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ORIGINAL ARTICLE

A conjugate of the lytic peptide Hecate and gallic acid: structure, activity against cervical cancer, and toxicity

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Abstract Conjugate compounds constitute a new class of molecules of important biological interest mainly for the treatment of diseases such as cancer. The N-terminus region of cationic peptides has been described as important for their biological activity. The aim of this study was to evaluate the lytic peptide Hecate (FALALKALKKALKK LKKALKKAL) and the effect of conjugating this macromolecule with gallic acid ($C_7H_6O_5$) in terms of structure, anti-cancer activity, and toxicity. An N-terminus GA-Hecate peptide conjugate was synthesized to provide information regarding the relationship between the amino-terminal region and its charge and the secondary structure and biological activity of the peptide; and the effects of gallic acid on these parameters. Peptide secondary structure was confirmed using circular dichroism (CD). The CD measurements showed that the peptide has a high incidence of α-helical structures in the presence of SDS and LPC, while GA-Hecate presented lower incidence of α-helical structures in the same chemical environment. An evaluation of the anti-cancer activity in HeLa cancer cells indicated that both peptides are active, but that coupling gallic acid at the N-terminus decreased the activity of the free peptide. GA-Hecate showed lower activity in non-tumor keratinocyte cells but higher hemolytic activity. Our findings suggest that the N-terminus of Hecate plays an important role in its activity against cervical cancer by affecting it secondary structure, toxicity, and hemolytic activity. This study highlights the importance of the N-terminus in antitumor activity and could provide an important tool for developing new anti-cancer drugs.

Keywords Hecate · Gallic acid · Bioconjugates · Cervical cancer · Hemolytic activity · Secondary structure

Introduction

Cancer has become a great concern in public health; it is caused by abnormal cells characterized by high metabolism and uncontrollable division. Cervical cancer is one of the most common cancer types in the world, mainly in less-developed regions (Ferlay et al. 2010). Human papiloma virus (HPV), poor genital hygiene, cultural factors, sexual behavior patterns, and smoking are some of the causal associations and etiological risk factors of cervical cancer (Kumar and Bhasker 2014). Although surgery and/or radiotherapy have been used to treat localized cancer, chemotherapy is still the treatment of choice for most cancers. Drugs are usually administered systemically and are therefore subject to variations in absorption, metabolism, and delivery to target tissues that can be patient-specific (Szakács et al. 2006). In addition, tumors could be located in regions of the body not easily accessible to drugs or could be protected by local environments due to increased tissue hydrostatic pressure or altered tumor vasculature (Szakács et al. 2006). Generally, the nature of conventional therapeutics, especially low molecular weight molecules, enables them to penetrate various body compartments and access numerous cell types and sub-cellular organelles (Vilar et al. 2012). However, cancer and non-tumor host

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cells share several common features (Szakács et al. 2006; Vilar et al. 2012) enabling these therapeutic molecules to interact non-specifically with non-tumor cells resulting in numerous side effects (Buolamwini 1999; Duncan 2006; Barrajón-Catalán et al. 2010). The harmful side effects of traditional chemotherapy have prompted an urgent need for novel selective anti-cancer drugs or therapeutic approaches (Szakács et al. 2006; Duncan 2006; Barrajón-Catalán et al. 2010; Buolamwini 1999; Pennarun et al. 2013). Peptides and bioconjugates that have demonstrated specific interaction with cancer cells and higher inhibition potential (Hansel et al. 2007a; Rosés et al. 2012; Pennarun et al. 2013; Yang et al. 2013) could constitute a new class of compounds for the development of novel cancer therapy drugs.

Small α-helical cationic antimicrobial peptides (cAMPs) have emerged as promising anti-cancer agents and a new strategy for cancer therapy (Gaspar et al. 2013). These peptides target, disrupt, and permeate cell membranes. Membrane disruption can occur through different mechanisms. Established mechanisms of action include the "barrel-stave" model, "toroidal pore" model (membrane lysis is achieved by pore formation), and the "carpet-like" model (induction of membrane disintegration and/or membrane micellization) (Castro et al. 2006). The interactions between the positive net charge of the peptides and cell membrane components are believed to be a key factor in the selective killing of cancer cells. The small α-helical cAMPs are more selective for the negatively charged membranes of cancer cells than for the membrane of normal cells. In addition, these molecules possess good penetration of tissues, broad spectrum of activity, act rapidly, demonstrate synergism with classic drugs and, in general, do not lead to the development of resistance. Hecate (Barr et al. 1995; Henk et al. 1995), an analog of melittin, is a 23 amino acids peptide (FALALKALKKALKKLKKALKK AL) with a high incidence of lysine (positive), leucine and alanine (nonpolar) (Rivero-Müller et al. 2007; Hansel et al. 2007b) that exhibits high anti-cancer activity (Hansel et al. 2007a; Gawronska et al. 2002; Leuschner et al. 2003). This peptide shares the main characteristics of other antimicrobial peptides, such as Ctx-Ha (Vicente et al. 2013) and Hylin-a1 (Crusca et al. 2011); it has a positive net charge, a high number of hydrophobic amino acids, and an amphipathic α-helix structure. The exact mode of action of this peptide on cancer cells has not been established. It is accepted that the plasmatic membrane is the main target, similar to mechanism of action proposed for other antimicrobial peptides (Fjell et al. 2011). A fusion of this peptide with the 81–95 amino acid fragment of chorionic gonadotropin-β was efficient in destroying LHRH-positive cells (Rivero-Müller et al. 2007). The proposed mechanism of action of this fusion conjugate is the destruction of cell membranes with minimal side effects. These studies show that using this peptide together with other molecules constitutes an interesting approach for the development of new active compounds.

Gallic acid ($C_7H_6O_5$) is a natural compound demonstrating anti-cancer activity against cervical, lung, colon, and leukemia cancer cells (Madlener et al. 2007; Bernhaus et al. 2009; You et al. 2010; You and Park 2010). Many studies (Sarjit et al. 2014; Korani et al. 2014; Kee et al. 2014; Sun et al. 2014) have demonstrated that modifying the molecular structure of gallic acid results in higher activity than the unmodified structure (Kitagawa et al. 2005; Cordova et al. 2011; Asnaashari et al. 2014). Alkyl gallates are powerful anti-viral agents used against several pathogens of clinical and veterinary importance (Hurtado et al. 2008); these compounds exhibit higher antioxidant properties (Aruoma et al. 1993) and antifungal activity against *Saccharomyces cerevisiae* and *Zygosaccharomyces* (Ko et al. 2001).

In this study, gallic acid (You and Park 2010; Ho et al. 2013) was coupled to the Hecate peptide to evaluate the effect of gallic acid on the secondary structure, anti-cancer properties, toxicity, and hemolytic activity of the linear peptide Hecate.

Materials and methods

Peptide synthesis

Peptide synthesis was performed manually by solid-phase peptide synthesis (SPPS) using the standard Fmoc (9-fluorenylmethyloxycarbonyl) protocol on a Rink-MBHA resin (Merrifield 1963) resulting in peptides in the amidated form. The experimental protocol used was the same as previously described (Howl 2005). Gallic acid was coupled to the N-terminus position of the Hecate peptide using standard coupling conditions. Gallic acid was coupled with DIC (*N*,*N*'-diisopropylcarbodiimide) in the presence of HOBt (*N*-hydroxybenzotriazole) at a 2-fold excess over the amino component in the resin, using 20 % dimethylformamide (DMF)/dichloromethane (DCM). A qualitative ninhydrin test was performed after 2 h to estimate the completeness of the coupling reaction; the recoupling procedure was performed when the ninhydrin test was positive. Cleavage from the resin and removal of the side chain protecting groups were performed simultaneously using TFA (trifluoroacetic acid), TIS (triisopropylsilane), and water (95:2.5:2.5, v:v:v, respectively). The crude peptides were precipitated with anhydrous ethyl ether, separated from soluble non-peptide material by centrifugation, extracted in 0.045 % TFA in water (solvent A) and lyophilized. Peptide purification, subsequent to dissolution in solvent A, was carried out with semi preparative HPLC (high performance liquid chromatography) using a reverse phase C_{18} column

(25 × 2.12 cm) with a 35–65 % linear gradient of solvent B (0.036 % TFA/acetonitrile) over 120 min. The flow rate was 5 mL/min and UV detection was carried out at 220 nm. Subsequent to purification, peptide homogeneity was evaluated by analytical HPLC, using a reverse phase column (25 × 0.46 cm with a 300 Å pore size) and a 5–95 % (v/v) linear gradient of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. The identity of the peptide was confirmed by Electrospray Mass Spectrometry and the final purity of the peptide was determined to be >95 %.

Cell culturing

Human cervix carcinoma cells (HeLa) and immortalized non-tumor human keratinocyte cells (HaCat) were routinely maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 % (v/v) heat inactivated FBS (fetal bovine serum), 1 × nonessential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin in plastic flasks (Corning) at 37 °C in a humidified 5 % CO_2 atmosphere.

MTT assay

HeLa and HaCat cells (5×10^3 cells/well) were seeded into a 96-well plate and incubated for 24 h prior to treatment. Next, supernatants were removed and substituted with 100 µL of DMEM supplemented with peptide concentrations ranging from 0.100 to 0.003125 mg/mL. Wells containing only cells and culture media were used as the positive control (100 % cell viability). The effects of the peptide on the cells were determined at 1, 24, and 48 h post addition of the peptide to culture media. The supernatants were removed, and a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100.0 µL) was added to each well and the plate was incubated for 30 min at 37 °C. Subsequent to incubation, the MTT crystals were solubilized with 100 µL DMSO (Dimethyl sulfoxide) and the absorbance was measured at 570 nm (Batista et al. 2014).

Cell morphology

Cell photography was conducted by optical microscopy using a Zeiss AxioVert A1 microscope. The cells were photographed subsequent to 0, 1, 24, and 48 h incubation with the compounds in a 96-well plate. Cell treatment was performed as for the MTT assay. Only the 24 h pictures are shown.

Hemolysis assay

Fresh human red blood cells (RBCs) were washed three times with 0.01 mol/L PBS (phosphate buffered saline) pH 7.4. A suspension of 1 % (v/v) erythrocytes was made with packed red blood cells resuspended in PBS. Synthetic peptides were dissolved in PBS at an initial concentration of 0.1 mg/mL and were then serially diluted in the same buffer to 0.003125 mg/mL. A 1 % Triton X-100 solution (v/v) and PBS were used as the positive (100 % lysis) and negative control (0 % lysis), respectively. Subsequent to 1 h incubation at 37 °C, the samples were centrifuged at 1000*g* for 5 min. One hundred microliter aliquots of the supernatant were transferred to 96-well microplates and the absorbance was determined at 540 nm. The assay was performed in triplicate.

Circular dichroism

Circular dichroism (CD) spectra were obtained between 190 and 260 nm with a JASCO J-715 CD spectrophotometer (Japan) under nitrogen flush with 1 mm path-length quartz cuvettes at room temperature. All CD spectra were acquired at 25 °C with a resolution of 0.2 nm. The peptide concentration used was 80 µmol/L. To investigate conformational changes caused by membrane environment, a titration was performed with 0–25 mmol/L sodium dodecyl sulfate (SDS) and 0–10 mmol/L lysophosphatidylcholine (LPC). CD spectra were typically recorded as an average of eight scans that were obtained in millidegrees and converted to molar ellipticity $[\theta]$ (deg cm^2/dmol). The helicity of the complete helix was calculated using the formula max $[\theta]_{222} = -40.000 \times [(1 - 2.5/n)] + (100 \times T)$, where n is the number of amino acid residues and T is the temperature in °C. The fractional helicity (percentage) of the peptide was calculated as $100 \times [\theta]_{222}/\max[\theta]_{222}$ (Jamasbi et al. 2014).

Statistics

Viability graphs are representative of a set of three independent assays. All statistical analyses were performed by one-way ANOVA with Tukey's post hoc test using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Hemolysis data is reported as the mean of a set of three independent assays.

Results and discussion

Several studies have shown that bioconjugates constitute a modern strategy for the synthesis of new compounds (Hudecz 2005; Lutz and Börner 2008; Mooney et al. 2009; Golubeva et al. 2011; Hirata and Nokihara 2014; Lebedyeva et al. 2014). Here, we synthesized and evaluated two molecules: (1) the anti-cancer peptide, Hecate (Hansel et al. 2001; Kumar et al. 2004); and (2) a new molecule,

gallic acid-Hecate peptide (GA-Hecate). The compounds were manually synthesized according to the standard Nα-Fmoc protecting group strategy using solid-phase peptide synthesis. Gallic acid was bound to the N-terminus of the peptide using standard reagents (DIC/HOBt) for amino acid coupling (Fig. 1). The compounds obtained were of a high level of purity (>95 %) and the identity of the peptide was confirmed by electrospray mass spectrometry (Table 1).

The activity of free gallic acid, the Hecate peptide, and conjugate GA-Hecate was evaluated against cervical cancer cells (HeLa), erythrocytes, and non-tumor cells (HaCat). Hecate peptide reduced HeLa cell viability by 50 % subsequent to 1 h of incubation (Fig. 2a) at concentrations of 0.1, 0.05, and 0.025 mg/mL. Subsequent to 24 and 48 h incubation, the peptide was still able to reduce cell viability by approximately 80 % using the same concentrations. Free gallic acid also demonstrated activity against tumor cells; its activity was similar to that of the free peptide at 0.1 mg/mL (inhibition of cell growth), but showed decreased activity at lower concentrations (Fig. 2b). Conjugated GA-Hecate decreased the peptide anti-cancer activity at a concentration of 0.025 mg/mL (Fig. 2c). These results show that the incorporation of gallic acid into the peptide does not lead to an additive effect; it is possible that other factors, which remain to be elucidated, are involved. Cancer cells overexpress phosphatidylserine and sialic acid that confer a negative charge to their membranes (Spector and Yorek 1985). The coupling of GA removes the positive charge of the amino-terminal group of the peptide, decreasing the net positive charge of the GA-Hecate conjugate. This difference could affect the electrostatic interaction between the cancer cell membrane and the peptides, decreasing its antitumor activity. HeLa cells have been used to determine the cytotoxicity of several molecules such as marine toxins and bismuth compounds (Pelin et al. 2014; Gao et al. 2015). HeLa cells have also been used to evaluate the antitumor potency of other cationic peptides (Slaninová

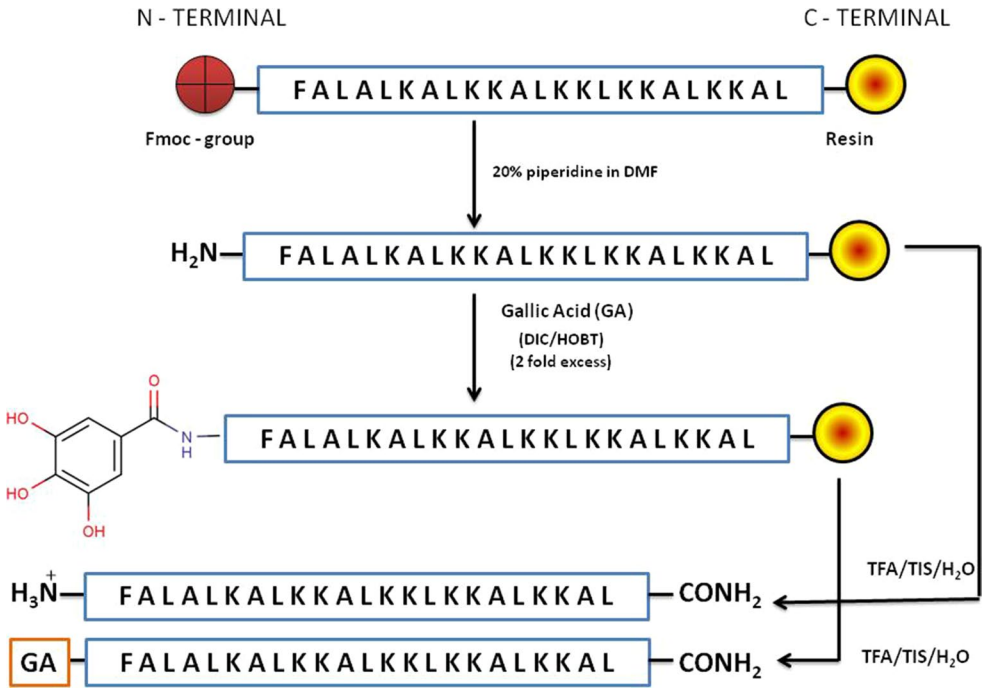

Fig. 1 Solid-phase peptide synthesis of the lytic peptide hecate and GA-Hecate

Table 1 Synthesis results and the physicochemical features of the synthetic peptides

Peptide	Yield (%)	Retention time[a] (min)	Mass (Da)		<μH[c]>	% Helicity		Net charge
			Observed[b]	Calculated		SDS	LPC	
Hecate	68.3	17.6	2535.6	2536.4	8.33	53	67	10
GA-Hecate	61.8	22.1	2688.0	2688.4	8.55	30	36	9

[a] Determined by RP-HPLC

[b] Determined by Mass Spectrometry

[c] Calculated using the MPEx program (Snider et al. 2009)

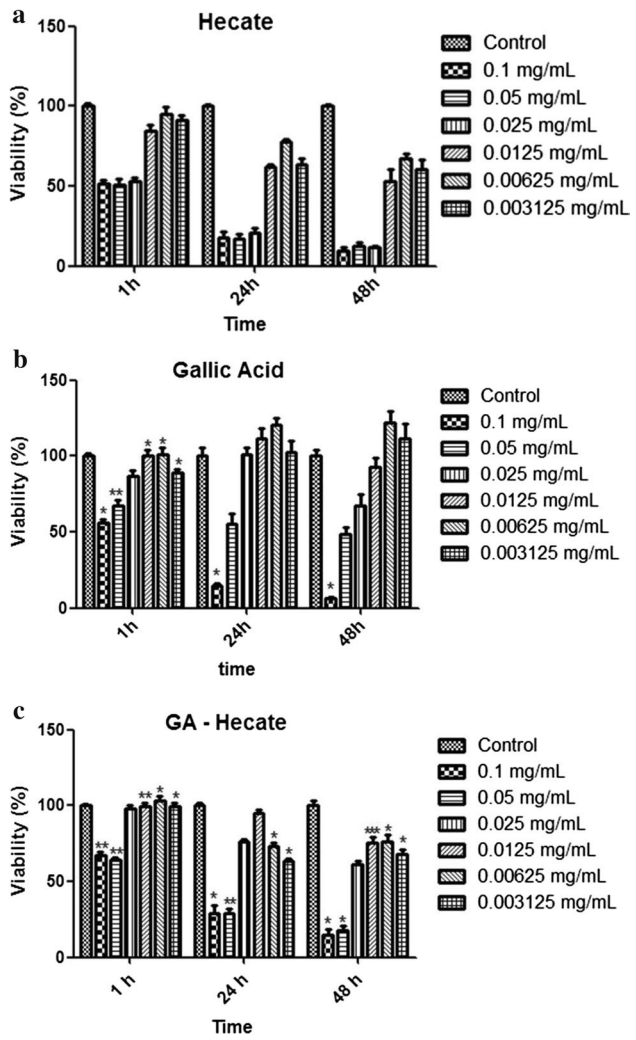

Fig. 2 Comparison of cervical cancer activity in HeLa cancer cells post treatment with different concentrations of Hecate (**a**), Gallic acid (**b**), and GA-Hecate (**c**) with 1, 24, and 48 h incubation. *$p > 0.05$ vs. Hecate **$p < 0.05$ vs. Hecate ***$p < 0.01$ vs. Hecate. The *bars* above the graphs designate the S.E

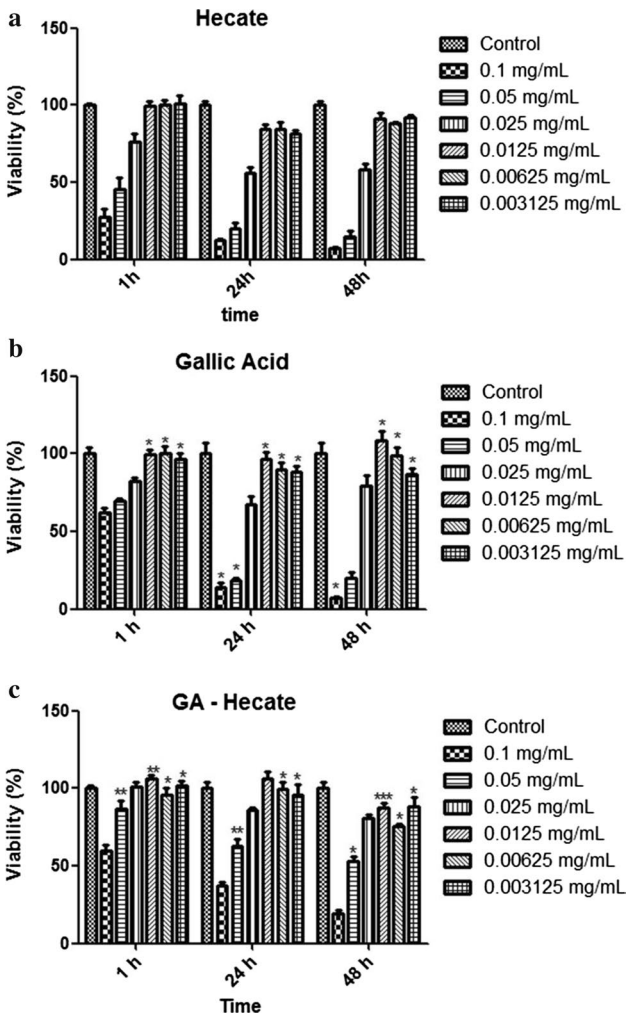

Fig. 3 Comparison of toxicity in non-tumor cells (HaCat) post treatment with the same concentrations used for antitumor activity. Hecate (**a**), Gallic acid (**b**), and GA-Hecate (**c**). *$p > 0.05$ vs. Hecate **$p < 0.05$ vs. Hecate ***$p < 0.01$ vs. Hecate. The *bars* above the graphs designate the S.E

et al. 2012) causing 50 % cell death at concentrations of 2.5–10 μmol/L. These values are similar to those obtained with Hecate in this study. Fluorescence assays have shown that the peptide lasioglossin III enters mammalian cells at higher levels only after reaching a toxic concentration, indicating that the mechanism of action of the tested peptides involves an initial permeabilization of the cell membrane (Slaninová et al. 2012).

To evaluate the toxicity of the peptide and conjugate, MTT assays and hemolytic tests were performed in non-tumor cells (HaCat). The cell viability assays (Fig. 3) show that Hecate has lower activity (toxicity) at 0.025 mg/mL against HaCat cells compared to HeLa cells. Similar results were obtained at concentrations of 0.05 and 0.1 mg/mL subsequent to 24 and 48 h incubation. The GA-Hecate conjugate resulted in less reduction in viability in non-tumor cells than in HeLa cells at 0.05 mg/mL. Thus, the toxicity of GA-Hecate is lower than the Hecate peptide. These results show that the addition of gallic acid to the N-terminal region of the peptide could decrease the specificity of the compound for cancer cells. Modifying the N-terminus of the peptide has been shown to affect the biological activity of other peptides (Cilli et al. 2007). Modifying the charge of the N-terminus of Hylin a1 changes its selectivity against gram negative bacteria (Crusca et al. 2011). These results are similar to those obtained for other antimicrobial peptides; camel and protegrin 1 inhibit the proliferation of HaCat keratinocytes at a concentration of 25 μg/mL, while other antimicrobial peptides have been shown to be cytotoxic at concentrations of 50 and 100 μg/mL (Baranska-Rybak et al. 2013). The hemolytic results

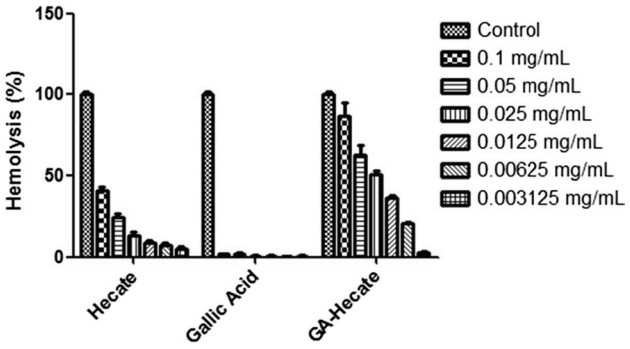

Fig. 4 Hemolytic activity of Hecate, Gallic acid, and GA-Hecate

show that gallic acid does not affect red blood cells (Fig. 4). In addition, the GA-Hecate conjugate promoted higher hemolysis than the Hecate peptide.

To further elucidate the mode of action of the Hecate and GA-Hecate compounds, the membrane morphology of the cells (HeLa and HaCat) was analyzed microscopically subsequent to incubation with different concentrations of the compounds. Cells exposed to these compounds demonstrated membrane damage post treatment, with membrane disruption occurring after 24 h (Fig. 5a). The results show that GA-Hecate causes less damage in both cell types than the peptide. Analysis of the images demonstrates cavity formation and loss of microvilli and membrane integrity (Fig. 5b). This rapid mode of action supports previous studies using lytic peptides in cancer cells indicating that these compounds cause cell necrosis (Bodek et al. 2005; Yates et al. 2011). Similar results were obtained using scanning electron microscopy of HeLa cells treated with the A12L/A20L (Ac-KWKSFLKTFKSLKKTVLHTLLKAISS-NH_2) peptide (Huang et al. 2011). Furthermore, although inhibition of cell growth was the same at 0.05 mg/mL, GA-Hecate resulted in increased damage to the membrane of HeLa cells compared to HaCat cells. These results suggest that at low concentrations, these compounds act via a mechanism other than necrosis. This is similar to the dual mode of action proposed for antimicrobial peptides (AMPs); at lower concentrations gomesin, tachyplesin, and linear-tachyplesin promote apoptosis. Conversely, higher concentrations of the AMPs result primarily in cell membrane disruption (Paredes-Gamero et al. 2012).

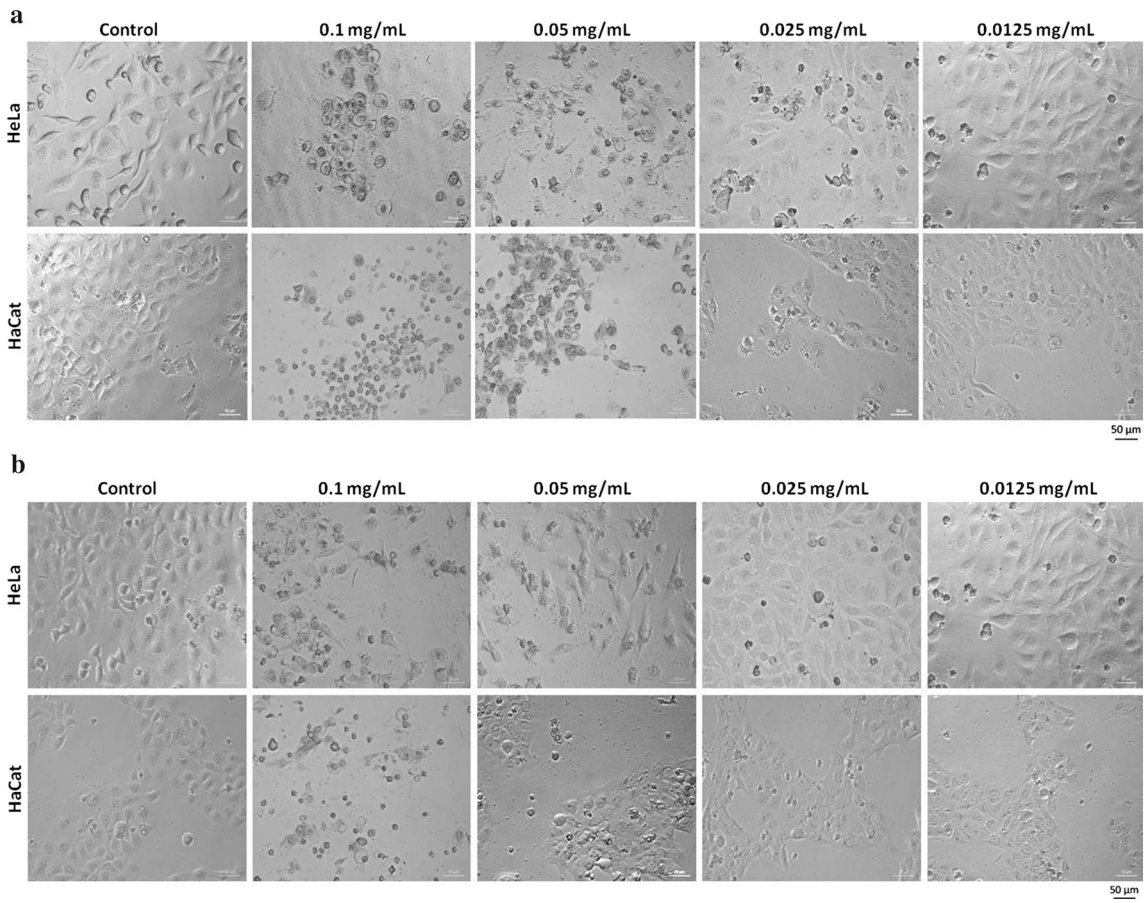

Fig. 5 Bioconjugate cytotoxicity images. HeLa and HaCat cells were exposed to the Hecate peptide (0–0.0125 mg/mL) (**a**) or GA-Hecate (0–0.0125 mg/mL) (**b**) and imaged using optic microscopy subsequent to 24 h incubation

To evaluate the relationship between the biological results, the structure and biophysical properties of the biomolecules, including hydrophobic moment (⟨μH⟩), net charge, hydrophobicity, and percentage of secondary structure elements were measured (Table 1). Biophysical parameters were obtained by computational analyses and CD spectroscopy was used to examine the secondary structure of these compounds in water and in presence of SDS and LPC micelles. SDS micelles mimic cancer cell membranes that possess a higher negative charge than non-tumor cells (Utsugi et al. 1991; Ran et al. 2002). LPC is chemically similar to phosphatidylcholine, a lipid present in the plasma membrane of certain organisms and human red blood cells. Our results show that gallic acid increased the hydrophobicity of the peptide (estimated by HPLC retention time; Table 1). Introduction of gallic acid at the N-terminus decreased the net charge, but did not affect the hydrophobic moment. Hydrophobic moment (<μH>) measures the probability that the secondary structure of the peptide is located at the interface between the

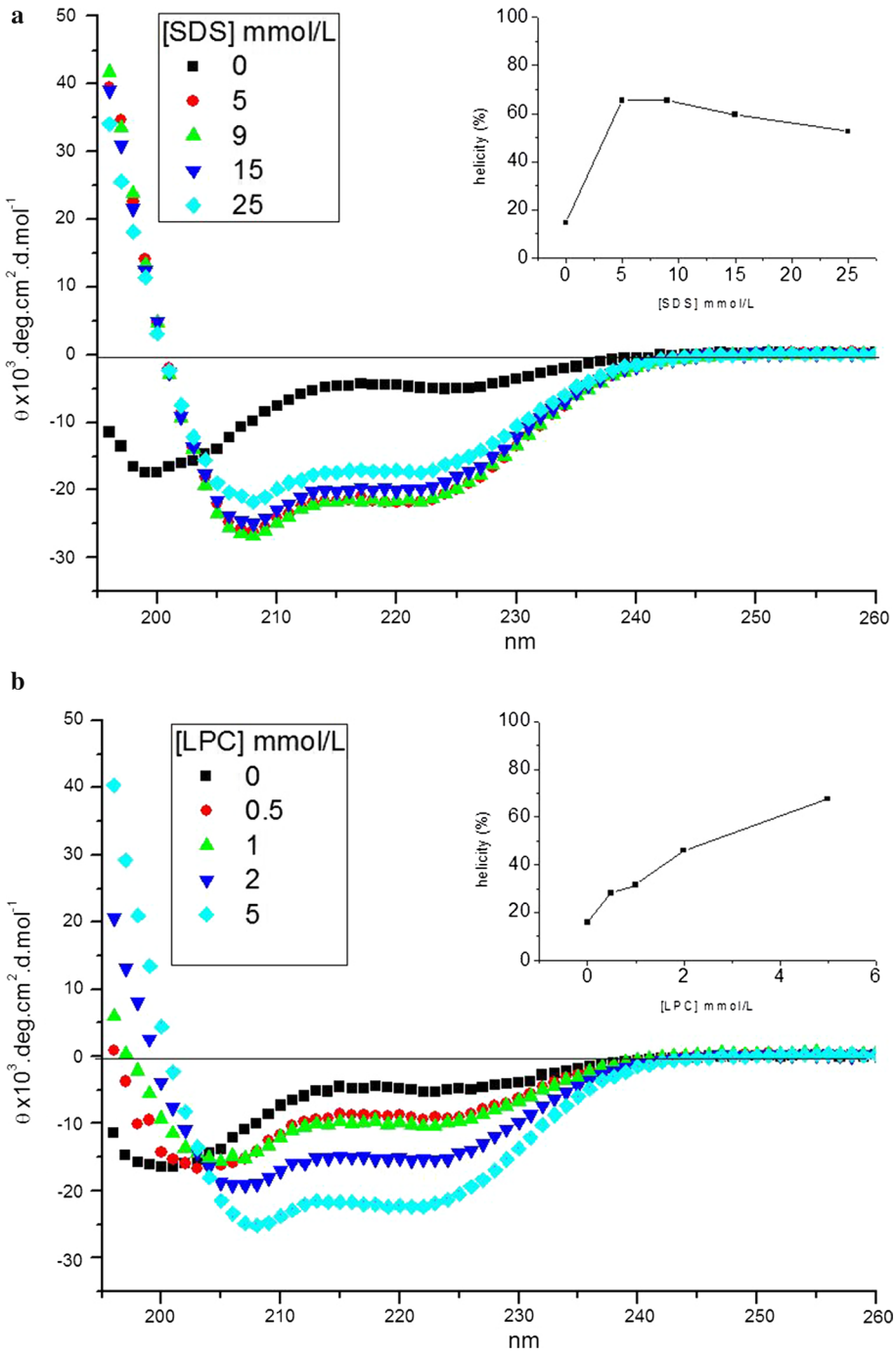

Fig. 6 CD spectra for titration of the compounds with SDS. Hecate (**a**) GA-Hecate (**b**). The *inset* panels of both spectra are designated as the percentage of α-helix versus concentration of surfactant

hydrophobic interior of a membrane and its polar surface (Eisenberg et al. 1984). Circular Dichroism Spectroscopy studies in water showed that the Hecate peptide and GA-Hecate adopted mainly a random coil conformation. A typical α-helix with double minima at 208 and 222 nm was induced in the peptide and GA-Hecate by the non-polar environment of LPC (zwitterionic) and SDS (negative) micelles (Figs. 6a, b, 7a, b). These results concur with previous findings reported for other antimicrobial peptides, such as the peptide Ctx-HA (Ceratotoxin-like Peptide from *Hypsiboas albopunctatus*) and Aurein 1.2 (Lorenzón et al. 2013). The addition of GA at the N-terminus position decreased the α-helix percentage of the peptide in these membrane mimetic environments. Hecate showed 53 and 67 % helical content in 10 mmol/L SDS and 5 mmol/L LPC, respectively, while the peptide conjugated with gallic acid showed 30 and 36 % in the SDS and LPC micelles, respectively.

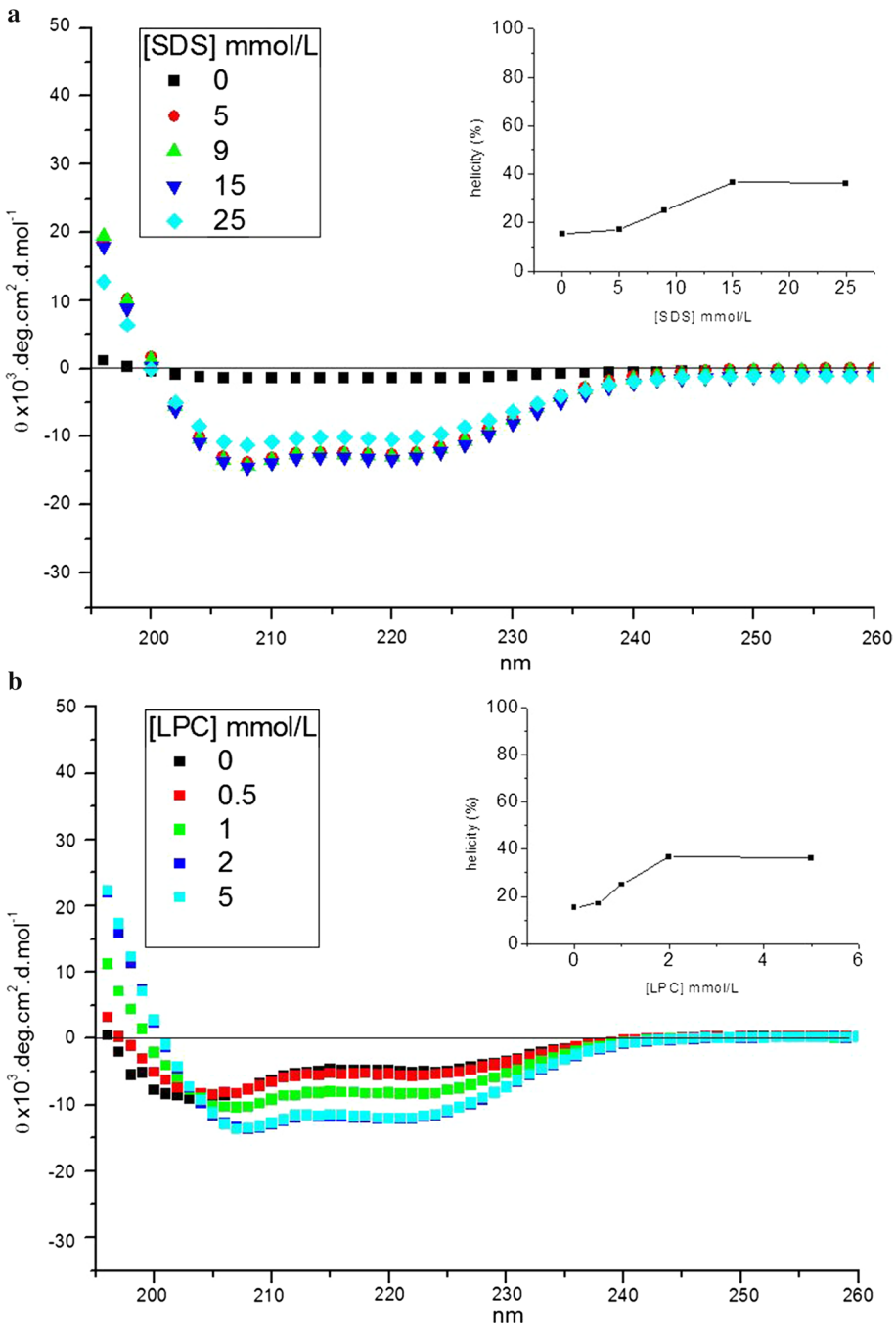

Fig. 7 CD spectra for titration of the compounds with LPC. Hecate (**a**) GA-Hecate (**b**). The *inset* panels of both spectra are designated as the percentage of α-helix versus concentration of surfactant

The high helicity, hydrophobicity, and amphipathic nature of cAMPs have been correlated with increased eukaryotic cell toxicity as measured by hemolytic activity (Cespedes et al. 2012). In this study, the addition of gallic acid at the N-terminus position of the peptide increased the hydrophobicity but decreased both the net positive charge and α-helix content in the presence of membrane mimetic environments. These modifications promoted changes in biological activity, decreasing anti-cancer activity and toxicity in non-tumor cells and increasing activity in human erythrocytes. The increase of hemolytic activity is in agreement with the findings of Dathe et al. (1997), which showed that the increase of hydrophobicity and hydrophobic moment substantially enhance the lysis of erythrocytes. In addition, previous reports have shown that the presence of positive charges, mainly at the N-terminus position (Vicente et al. 2013; Cilli et al. 2007) is important for increasing biological activity (Shin et al. 2001; Duval et al. 2009). In this case, the coupling of GA removes the positive charge of the amino-terminal group, decreasing the positive charge of the GA-Hecate conjugate. However, a direct correlation between the α-helix content and hemolytic activity was not found in our study; GA-Hecate showed lower α-helix percentage and higher hemolytic activity, indicating that there are others factors that affect the lysis of erythrocytes.

Conclusions

In this study, we demonstrate novel anti-cancer activity for the lytic peptide Hecate and that N-terminal modification could be used as a strategy for altering the specificity and toxicity of lytic peptides. Our results indicate that both Hecate peptide and GA-Hecate damage cancer cell membranes at high concentrations, reinforcing the hypothesis that these compounds act in the membrane. However, lower concentrations of the Hecate peptide did not cause substantial damage to cancer cells membranes, suggesting that at these concentrations the peptide acts in the membrane and also leads to apoptosis. Finally, our findings highlight the importance of the N-terminus charge for the anti-cancer activity of lytic peptides; a decrease in the positive charge of the peptide N-terminus reduces its antitumor activity. These results provide useful information for the design of conjugate peptides with selective targets properties.

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Conflict of interest The authors declare that they have no conflict of intrest.

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