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# The Design and Anticancer Activity of a Citropin1.1 Hybrid Peptide with Selective Activity Against Highly Invasive Metastatic Cell Lines

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ABSTRACT

Citropin 1.1 is an amphipathic alpha-helical cationic peptide that exhibits potent anticancer activity *in vitro*. Citropin 1.1 was found to be active against 60 cancer cell lines, and this activity was mainly attributed to its ability to bind and lyse membranes of cancer cells. One of the major drawbacks of developing Citropin 1.1 as an anticancer agent is its lack of apparent selectivity toward cancer cells and its ability to cause significant lysis of normal human erythrocytes and mammalian cells at high concentrations. This low selectivity index places severe restraints on the development of Citropin 1.1 as a novel anticancer agent. In this study, we have designed a Citropin 1.1 analog named Citropin A that retained the biological activity of the parent peptide. Citropin A was fused to an anionic fragment in order to neutralize the positive charge carried on the parent peptide rendering it inactive. The resultant hybrid peptide named Citropin-MMP was designed to contain a Matrix metalloproteinase (MMP) cleavable consensus sequence that would be cleaved to release the active Citropin A once it encounters highly metastatic MMP producing cancer cells. Citropin-MMP was found to be completely inactive against non-MMP producing cancer cells and normal mammalian cells. However, when Citropin-MMP was administered to MMP producing cells, its antiproliferative activity was regained, and the peptide displayed exclusive activity against MMP producing cancer cell lines. The data of our study indicate that this enzyme-based cleavage strategy could prove to be successful for the development of Citropin-MMP as a novel therapeutic agent for the purpose of inhibiting the proliferation and invasion of highly metastatic invasive cancer cells.

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## INTRODUCTION

Cancer metastasis is responsible for 90% of cancer-related deaths (Weber, 2008). Cancer cells acquire the ability to invade other tissue by over-expressing certain enzyme proteases, specifically Matrix metalloproteinases (MMPs) 2 and 9. These enzymes play a major role in the degradation and remodeling of the extra-cellular matrix (ECM), which aids in enhancing tumor invasiveness by creating passages within the ECM that allows the tumor cells to migrate and invade surrounding tissues (Cairns *et al.*, 2003; Gattoni-Celli and Pozzatti, 1992). Different strategies have been implemented to efficiently

target the problem of metastatic cancers, but the use of chemotherapeutic agents remains the main choice offered for patients within the clinical setting (Kalkanis *et al.*, 2010). Standard chemotherapy treatment for cancer patients could prove to be very distressful, as the treatment usually includes multiple treatment regimens that can induce serious side effects and have tremendous physical and social consequences on the patients (Dimeo *et al.*, 1999). In addition to the high toxicity profile of such agents, cancer cells can rapidly develop resistance against chemotherapy through the amplified expression of multidrug resistance proteins that maintain a low-level concentration of these drugs intracellularly (Fletcher *et al.*, 2010).

Anticancer peptides (ACPs) are a group of biologically active peptides that have caught the attention of the scientific community in the recent decade. ACPs have been able to demonstrate potent antiproliferative activities against a wide range of malignant cell lines *in vitro* with different modes of action (Sookraj *et al.*, 2010; Almaaytah *et al.*, 2012; Gaspar *et al.*, 2012). ACPs are usually composed 49 of peptides with less than 50 amino acids and display a hydrophobic nature (Gabernet *et al.*, 2016). These peptides are positively charged, a factor that is crucial in their mode of action and fold in alpha-helical structures once in contact with hydrophilic solvents. (Schweizer, 2009). The positive charge carried on most ACPs is crucial to their effective mode of action as it is responsible for the initial binding of the peptides to the malignant cells. (Riedl *et al.*, 2011) . During carcinogenesis, cancer cells gain an additional overall negative charge as a result of the structural abnormalities that are accompanied with the process of malignancy that leads to an increased expression of negatively charged phosphatidyl serine's and O-glycosylated mucins on the surface of cancer cells and as a result facilitating the binding of ACPs to these membranes (Utsugi *et al.*, 1991; Yoon *et al.*, 1996). These macromolecules Could be the major factor behind ACPs' apparent selective activity that is principally mediated by electrostatic interactions. Among the anticancer peptides, Citropin 1.1 is a potent anticancer peptide that was isolated from the skin secretions of the blue mountain tree frog *Litoria citropa* (Wegener *et al.*, 1999) . When assessed for its anticancer activity, Citropin 1.1 was found to be cytotoxic against 60 cancer cell lines within the micromolar concentration range according to the national cancer institute regimen (Doyle *et al.*, 2003) . The activity of Citropin 1.1 is attributed to its cationic nature, helicity, and its ability to interact with membranes in a detergent-like manner (Chen and Mark, 2011). Although Citropin 1.1 displayed potent anticancer activity with substantial selectivity against neoplastic cells, Citropin 1.1 also killed normal cells at higher concentrations (Dawson *et al.*, 2010) , a feature that places severe restraints on the development of Citropin 1.1 as a novel anticancer agent. In this study, we designed a Citropin 1.1 analog that would retain the biological activity of the parent peptide; this peptide was linked to an anionic peptide fragment via a linker containing an MMP-2/MMP-9 cleavable consensus cleavage site (GPLGIAGQ) in order to mask the cytolytic activity of Citropin1.1 (Kridel *et al.*, 2002). This anionic fragment displays a net negative charge of -1, which neutralizes the positive charge on Citropin 1.1. As charge is considered one of the major structural parameters controlling ACPs activity, neutralizing the positive charge will render the peptide inactive. Additionally, this fragment allows the conversion of the inactive fusion peptide to its active free form, Citropin A, once it encounters MMP expressing cancer cells. This will allow the Citropin A to exert its anticancer activity exclusively in metastatic cancer cells expressing significant levels of the enzymes MMPs. This hybrid peptide along with Citropin 1.1 and Citropin A was tested against different cancer cell lines with variable MMPs expression levels, and our results have shown that the hybrid peptide displays enhanced selectivity towards MMP expressing cancer cells with no effects against non-neoplastic mammalian cells or non-MMP expressing cancer cells.

## MATERIALS AND METHODS

### Peptide Synthesis

Citropin 1.1, Citropin A, and the hybrid peptide Citropin-MMP were custom synthesized commercially by Genscript (USA). Fmoc chemistry was utilized to synthesize the peptides reaching a purity of > 95%. The purification method involved continuous rounds of reverse-phase liquid chromatography. The mass of the peptides was confirmed using MALDI-TOF mass spectrometry.

### Cell Culture

The following cell lines (PC3 human androgen-independent prostate cancer cell line, LNCaP human androgen-dependant prostate adenocarcinoma cell line, DU-145 human hormonal-independent carcinoma cell line, MCF-7 Human breast carcinoma and Vero African green monkey kidney cell line), purchased from the American Type Culture Collection were employed in the study. The cell lines were cultured in either RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1%

L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 mg/ml streptomycin. HUVECs (human *umbilical vein endothelial cells)* were cultured in DMEM medium supplemented with 10% FBS, 1% L-glutamine, 10 ng/ml epidermal growth factor (EGF) and 50 U/ml penicillin and 50 mg/ml Streptomycin. HUVECs were used within 10 passages. The cells were seeded into 150 $cm^2$ culture flasks. Cells were cultured as monolayers in a humidified environment of 5% CO2 95% air at 37ºC.

**Detection of MMP by (RT)-PCR analysis**

Poly A mRNAs were extracted from the cultured cells using the Dynabeads mRNA® DIRECT™ kit technology (Invitrogen, USA). RT-PCR was employed for obtaining the cDNA library and by employing the Advantage RT-PCR Kit (Clontech, USA). As described by the manufacturer, 1 $\mu$g of each purified mRNA was reverse transcribed into their correspondent cDNA using random hexamer primers. The resultant cDNA library was interrogated by using three sets of gene-specific primers MMP-2, MMP-9 and $\beta$-actin: MMP-2F (5′-tctcctgacattgaccttggc-3′)/MMP2-R (5′-caaggtgctggctgagtagatc-3′) for human MMP-2 genes; MMP-9F (5′-ttgacagcgacaagaagtgg-3′)/MMP-9R (5′-gccattcacgtcgtccttat-3′) for human MMP-9 genes; $\beta$-actin-F (5′-GCATCACACCTTCTACAATGAGC-3′)/$\beta$-actinR (5′-GCTCATAGCTCTTCTCCAGGG-3′) for the $\beta$-actin gene which was used as an internal control. The annealing temperature for the primer pairs was as follows: MMP-2F/MMP-2R, 55°C; MMP-9F/MMP-9R, 65°C; $\beta$-actinF/ $\beta$-actinR, 58°C.After PCR amplification, the products were analyzed using 1% agarose gel electrophoresis. The predicted PCR product size for MMP-2, MMP-9, and $\beta$-actin are 225bp, 554 bp, and 480 bp, respectively.

**Gelatin Zymography**

The cancer cell lines cell lines were grown onto 10 cm tissue culture plates and cultured routinely at 37 °C and under a $CO_2$ concentration of 5%. After reaching the satisfactory cell mass and the confluence of 70%, the plates were replenished by 2 ml media devoid of serum. The cells were incubated for 72 hours and then centrifuged and ultra-concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore, USA). A standard SDS-PAGE was performed in order to proceed with the gelatin zymography by the addition of 0.1% gelatin to the gel cast. Each sample was prepared for the experiment by employing a nonreducing loading buffer. After the completion of the electrophoresis, gelatinases were renatured by employing 2.5% Triton X-100. Gels were then incubated at 37°C for 24 h in an incubation buffer [50 mm Tris-HCl, pH 7.5, 200 mm NaCl, and 5 mm $CaCl_2$ and 5 mM $ZnCl_2$], and then were stained with 0.25% Coomassie Blue R 250 and destained later.

**Cell Proliferation Assay**

MTT assay was employed for the determination of the cytotoxicity of the peptides against all cells employed in this study. 5000 cells per well were seeded into 96-well culture plates and loaded with different concentrations of the designed peptides. Following a 24-hour incubation period, 20 $\mu$l of the MTT solution was added to each well and further incubated for four hours. Following the formation of the formazan crystals, DMSO was added to dissolve these crystals and produce the purple color, which can be measured spectrophotometrically using an ELISA Microplate Reader. GraphPad Prism software was used for statistical analyses.

**Lactate dehydrogenase (LDH) release assay**

Lactate dehydrogenase assay is a powerful tool to measure cell damage and toxicity as more LDH is released in response to cell death. LDH assay was performed according to the manufacturer's instructions (LDH Cytotoxicity Assay Kit, Cayman, USA). Briefly, 5000 cells of each cell line employed in this study were seeded into a separate well of a 96-well plate and incubated at 37 °C for 24 hours. Different peptide concentrations were added to each well as with the MTT assay. After the incubation period, the plates were centrifuged 400 x g for 5 minutes, and the supernatants collected and mixed with the manufacturer's reaction solution.

Briefly, each cell line used in this experiment (PC-3, LNCaP, and DU 145) was seeded on a 96-well plate at a density of 5000 cells/well for 24 h at 37 °C in the absence or in the presence of the peptides at different concentrations. Following the incubation period, the cells were at centrifuged at 400 x g for 5 minutes, and 100 ul each of the supernatants and the reaction solution were mixed in a 96-well plate, and absorbance values were recorded at 492 nm with a 96-well microplate reader. 2% Triton-X100 was used as a positive control (Santa Cruz, USA) and the employed for the relative absorbance measurement of the peptide treated cell lines

**Erythrocyte hemolysis assay**

Hemolytic activities of the peptides were assessed by incubating a 4% suspension of human erythrocytes (RBCs) with each of the peptides, in turn, for periods of 60 min. RBCs were repeatedly washed with a solution of 0.9% NaCl and immediately centrifuged. The peptide treated RBCs were later incubated for sixty minutes at 37º C. Following the incubation period, the RBCs were centrifuged at 900 x g

for 5 min, and the supernatant collected for analysis with 200 $\mu$l transferred a triplicate into a 96 well plate and its absorbance measured at 570 nm. Triton X-100 treated RBCs were used as the positive control, while a non-peptide solution of NaCl treated RBCs was used as the negative control.

## RESULTS AND DISCUSSION

### Design of the Citropin 1.1-MMP Hybrid peptide

Although Citropin 1.1 has been found to display moderate selective activity against neoplastic cell lines when compared with mammalian cells, it also displayed significant effects on the viability of mammalian cells and human erythrocytes at higher concentrations. To amplify the selectivity of Citropin 1.1 towards neoplastic cells, we have designed a hybrid peptide to act as a pro-form of Citropin 1.1 that would be initially inactive against both neoplastic and mammalian cells. This hybrid peptide is composed of the modified active form of the peptide Citropin 1.1 named Citropin A, which displays a net positive charge of +1 fused to an anionic fragment (IAGQGGD). This anionic fragment would neutralize the positive charge on the parent peptide Table 1. In addition to that, the hybrid peptide also contains an MMP-2/MMP-9 cleavable consensus cleavage site (GPLGIAGQ) located at the C-terminal end of Citropin A Table 1. This hybrid peptide will be cleaved once in contact with cells expressing significant amounts of the enzymes MMPs, releasing the active Citropin A to exert its effect exclusively against highly invasive MMP producing cell lines Figure 1.

### RT-PCR Analysis

RT PCR was used to investigate MMP-2 and MMP-9 mRNA expression in the five cancer cell lines used in this study (LNCaP; DU-145; PC3; MCF-7). As shown in Figure 2A, MCF-7 cells did not express both MMP-2 and MMP-9, while LNCAP cells strongly expressed MMP-2, but MMP-9 was weakly expressed. PC-3 and DU-145 cells strongly expressed both MMP-2 and MMP-9.

### Gelatin Zymography

The gelatin zymography results of the various neoplastic cell lines are shown in Figure 2B. As shown in the figure, no clear bands were shown in the conditioned media from MCF-7 cell lines, which clearly indicates that the cell lines are not expressing any MMPs. The conditioned media from the PC-3 and DU-145 showed the strongest expression of both MMP-2 and MMP-9, while the conditioned media from the LNCaP cell lines showed that these cell lines are only expressing MMP-2 but not MMP-9. The data generated from the zymography experiments confirm the RT-PCR results in regard to MMP-2 and MMP-9 mRNA expression, which should correlate with the behavior of the hybrid peptide against these cell lines according to our hypothesis.

### Cell Cytotoxicity and LDH release assays

When assessed for their activity against neoplastic cell lines. Both Citropin 1.1 and its modified analog Citropin A managed to inhibit the proliferation of all neoplastic cell lines in a dose-dependent fashion. In Figure 3A and 3B Citropin 1.1 and Citropin A displayed potent activity with an average $IC_{50}$ of 32.5 $\mu$M and 42.9 $\mu$M respectively, against all five cancer cell lines tested (Table 2). When the two peptides where assessed for their antiproliferative activity against mammalian cells, both Citropin 1.1 and Citropin A killed human HUVEC cell lines Vero cell lines (Figure 4A, Figure 4 B) with an average $IC_{50}$ values of 61.9 and 64.9 respectively (Table 2) which clearly indicates that the two peptides affect the viability of normal cells at higher concentrations. The impact of the hybrid peptide Citropin-MMP on neoplastic cell viability and proliferation was also assessed by the MTT assay. Citropin-MMP displayed potent, selective activity against the MMP expressing cell lines (PC3, DU-145) in a similar manner to Citropin 1.1 (Avg $IC_{50}$ of 47.4), Citropin-MMP displayed moderate activity against LNCAP cell line which was found to produce only MMP-2 and didn't display any activity against the non MMP producing cell line MCF-7 (Figure 3C) and normal cells (HUVEC, Vero) Figure 4C which clearly display that the hybrid peptide is only targeting MMP producing cells in a selective manner. These results confirm our hypothesis that the hybrid peptide is being cleaved by the MMPs releasing Citropin A, which is exerting its activity within the MMP producing cells microenvironment. The peptide's ability to disrupt cellular membrane integrity was also assessed using the LDH cytotoxicity assay, which is a good indicator of the peptide's lytic ability and their ability to cause membrane damage as cells with damaged membranes release LDH into the extracellular environment. Citropin 1.1 and Citropin A increased LDH release from all five cancer cell lines tested in a dose-dependent fashion displaying a correlation with the decrease in the viability of cancer cell lines in the previous experiments (Figure 5A, 5B). Citropin 1.1 and Citropin A displayed potent activity with an average $IC_{50}$ of 50.4 $\mu$M and 59.0 $\mu$M, respectively, against all five cancer cell lines tested (Table 2 ). When tested against mammalian cells, both peptides also displayed potent activities against Vero and HUVEC cell lines at high concentrations Figure 6A and 6B, clearly confirms the weak selectivity of the peptides against neoplastic cells as membrane damage was

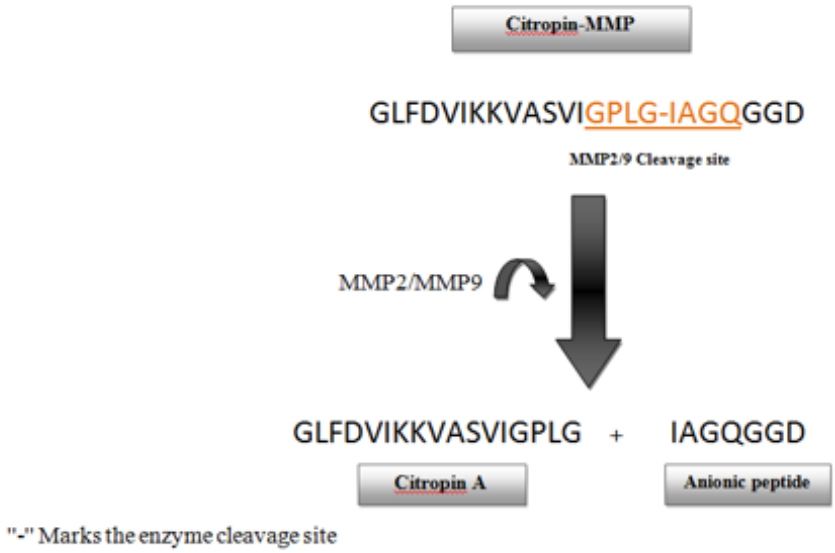


**Figure 1: Schematic illustration of Citropin-MMP cleavage by Matrix metalloproteinases 2 & 9. The cleavage of the peptide leads to the formation of the active peptide Citropin A and an inactive anionic peptide. The cleavage site is underlined**

**Table 1: Peptide Sequences**

| Peptide name | Peptide sequence |
|---|---|
| Citropin 1.1 | GLFDVIKKVASVIGGL |
| Citropin A | GLFDVIKKVASVIGPLG |
| Citropin-MMP | GLFDVIKKVASVIGPLGIAGQGGD |

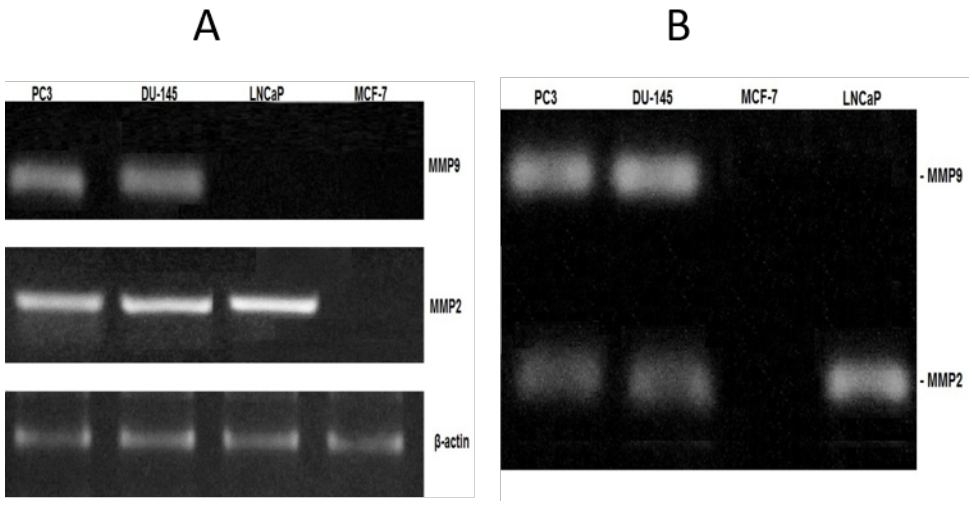


**Figure 2: MMP-2 and MMP-9 mRNA expression within the cell lines employed in the study. (A) RT-PCR analysis of Each mRNA extracted from the cell lines employed in the study and employing MMP-2 and MMP-9 gene-specific primers, $\beta$-Actin gene represents the control (B) Gelatin zymography. SDS gel embedded with gelatine as employed for the zymography assay. Coomassie brilliant blue was used as the staining reagent. The white bands in the dark backgrounds indicate the presence of gelatinases (MMP-2 and MMP-9)**

reported in both normal and neoplastic cells. The $IC_{50}$ values of the peptides in the LDH release assays were closely correlated with the antiproliferative studies (Table 3).

The hybrid peptide Citropin-MMP displayed selective membrane disruptive activity against neoplastic cells in a potent and dose-dependent manner (Figure 5A, 5B). The maximal membrane damage and lytic activity of Citropin-MMP were observed against the PC3 cell line with an $IC_{50}$ of (67.6 mM). In addition to that, Citropin-MMP didn't display any activity against the MCF-7 cell line and the normal mammalian cells (Figure 5C, Figure 6C). The data from the cytotoxicity and LDH release experiments confirm our hypothesis that Citropin-MMP is being cleaved by both MMP-9 and MMP-2 to release the active form of the peptide Citropin A which is killing cancer cells in correlation with the degree of MMP expression.

**Hemolytic activity**

To examine the toxicity of the peptides against mammalian erythrocytes, we tested the peptide at several concentrations against human erythrocytes. At concentrations equal to their $IC_{50}$ values, which are needed to inhibit the proliferation of the neoplastic cells and specifically at a concentration of 50 $\mu$M, both peptides Citropin 1.1 and Citropin A caused significant hemolysis of 33.7% and 39.5% respectively when incubated with human erythrocytes for 60 min. When the concentration was raised to 80 $\mu$M, percentage hemolysis values determined for Citropin 1.1 and Citropin A were 79.5 % and 85.8 %, respectively (Table 4). These results clearly indicate that both peptides suffer from the lack of selectivity between mammalian cells and neoplastic cells as the two of these peptides produced significant hemolysis when incubated with human erythrocytes. (Jo *et al.*, 2012). When the hybrid peptide Citropin-MMP was assessed for its hemolytic activity, no strong hemolysis was observed at a concentration of 60 $\mu$M, which displayed significant cytotoxic effects against neoplastic cells (4.8 % hemolysis). Even when the concentration was raised to 90 $\mu$M which is approximately 1.5 times higher than the average $IC_{50}$ value of the peptide against neoplastic cells, little hemolysis was observed and it never exceeded 5% which clearly indicates that the peptide is only active against MMP producing cells with no or little activity against mammalian cell that is no expressing any MMPs. All the data representing the hemolytic activity of the peptides are summarized in Table 4.

Over the last twenty years, several ACPs with significant anticancer activity have been identified (Jo *et al.*, 2012; Chuang *et al.*, 2009; Baker *et al.*, 1993; Chernysh *et al.*, 2002). Although ACPs have proven to be efficient in killing cancer cells in vitro, several obstacles hinder the development of ACPs as successful therapeutics. One of the major obstacles facing the development of most ACPs is the issue of target selectivity as several members of this group of peptides interact with host cellular membranes of a variety of tissues inducing severe toxicity, which would limit the application of these peptides for the treatment of cancer. Among the ACPs, Citropin 1.1 received great interest as a potential candidate for drug development as an anti-cancer agent since it was found to display cytotoxic activity against 60 different cancer cell lines. The activity of Citropin 1.1 is attributed mainly to its ability to interact with the negatively charged membranes of target cells via electrostatic interactions and the formation of peptide aggregates on the surface of membranes, this aggregation of peptides on membrane surfaces leads to the insertion of peptides through the lipid membranes and the formation of transient pores which places severe physical strain on the membrane leading to cell lysis and burst (Doyle *et al.*, 2003). Although Citropin 1.1 displayed substantial selectivity toward cancer cells, it also affected the viability of normal mammalian cells such as human erythrocytes, kidney epithelial cells, and human umbilical vein cells Figure 5 A. To avoid this obstacle of having a peptide with high activity and low selectivity against cancer cells, a hybrid peptide was designed based on a Matrix metalloproteinases activation strategy that would mask the deleterious effects of Citropin 1.1 and would only activate this hybrid peptide once it encounters an MMP producing cell. This hybrid peptide was designed by joining a modified form of Citropin 1.1 named Citropin A to an anionic fragment that would shield the positive charge on the parent peptide. We reasoned that this neutral hybrid peptide would lack the ability to interact with the host cellular membranes as it lacks the positive charge needed for the initial step of peptide binding to membranes through electrostatic interactions. In addition to the previously mentioned feature, this hybrid peptide also contains an MMP-2/MMP-9 cleavable consensus sequence that would serve as a targeting function. The hybrid peptide would be cleaved once it comes in contact with an MMP producing cell to release the active Citropin A, which displayed significant activity in killing cancer cells. This strategy would allow the peptide to be active only against highly invasive metastatic cell lines.

In agreement with our hypothesis, the hybrid peptide Citropin-MMP exhibited MMP specific activity.

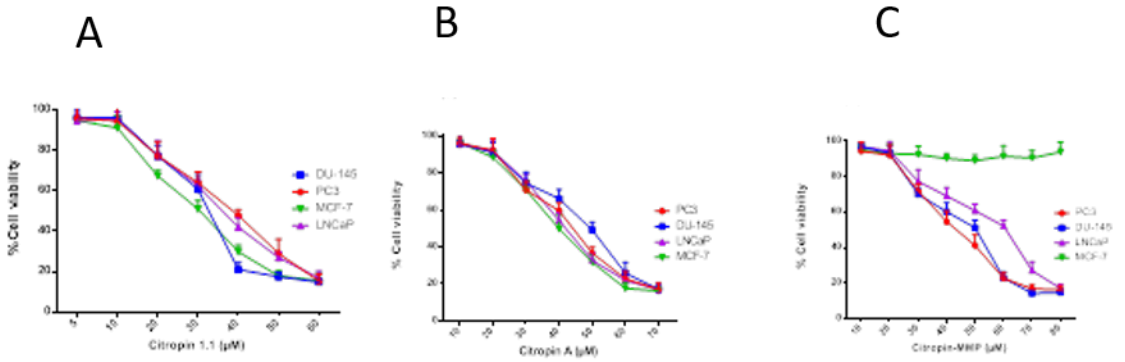


**Figure 3: The MTT antiproliferative assay results for (A) Citropin 1.1, (B) Citropin A, and (C)Citropin-MMP against five different cancer cell lines PC3, DU145, LNCaP, MCF7, andCaco-2 cells lines. Error bars represent standard error from mean cell proliferation, as determined by repeated experiments**

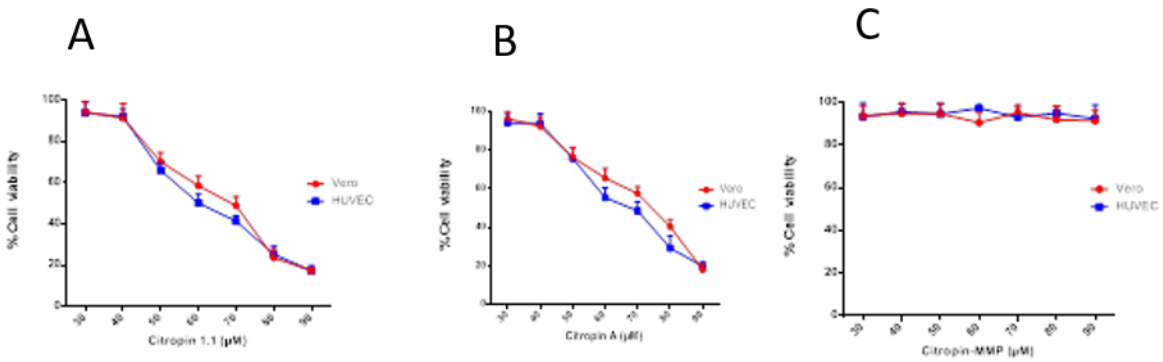


**Figure 4: The MTT antiproliferative assay results for (A) Citropin 1.1, (B) Citropin A and (C) Citropin-MMP against two different noncancerous mammalian cell lines, Vero and HUVEC cell lines. Error bars represent standard error from mean cell proliferation, as determined by repeated experiments**

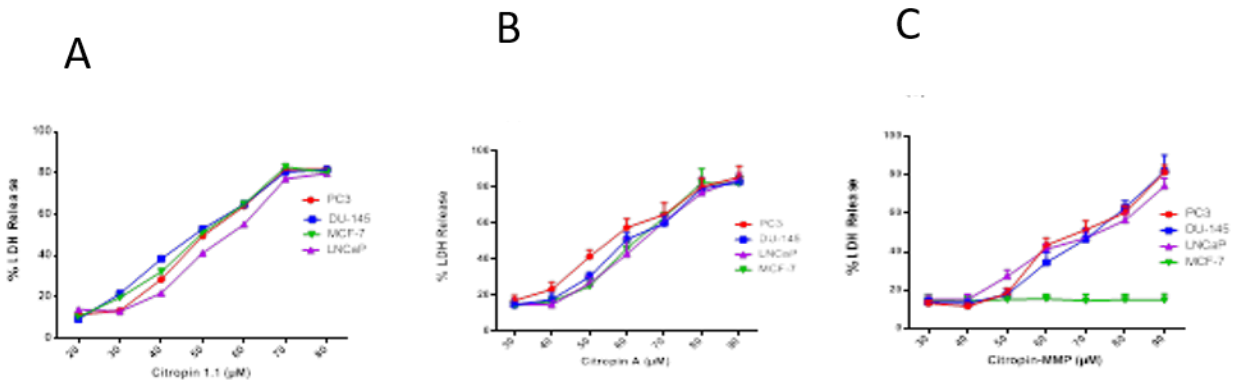


**Figure 5: Effect of (A) Citropin 1.1, (B) Citropin A and (C) Citropin-MMP on cytotoxicity measured through the LDH assay against four different cancer cell lines PC3, DU145, LNCaP, MCF-7cells lines. Errorbars represent standard deviation from mean cell proliferation, as determinedby repeated experiments**

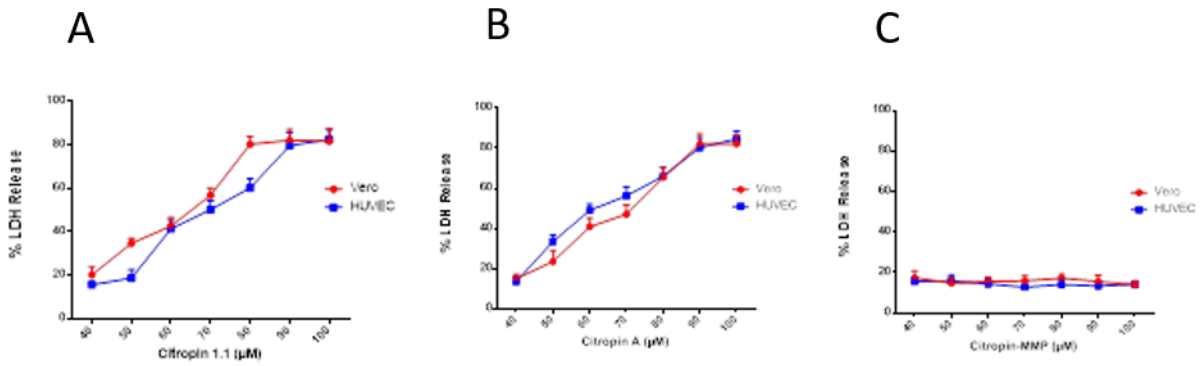


**Figure 6: Effect of (A) Citropin 1.1, (B) Citropin A and (C) Citropin-MMP on cytotoxicity measured through the LDH assay against two different noncancerous cell lines, Vero and HUVEC celllines. Error bars represent standard deviation from mean cell proliferation, as determined by repeated experiments**

**Table 2: Cytotoxicity effects ($IC_{50}$ value) using MTT assay of Citropin 1.1, Citropin A and Citropin-MMP on five tested cancer cell lines, and two non-cancerous mammalian cells**

| Cell line | Citropin 1.1 | Citropin A | Citropin-MMP |
|---|---|---|---|
| PC-3 | 36.2 | 42.4 | 42.6 |
| DU-145 | 31.2 | 46.7 | 44.9 |
| LNCaP | 34.9 | 41.5 | 55.1 |
| MCF-7 | 28.4 | 39.3 | n.d |
| Vero | 63.0 | 68.9 | n.d |
| HUVEC | 60.9 | 60.9 | n.d |

n.d.: not determined

**Table 3: Cytotoxicity effects ($IC_{50}$ value) using LDH assay of Citropin 1.1, Citropin A and Citropin-MMP on five tested cancer cell lines, and two non-cancerous mammalian cells**

| Cell line | Citropin 1.1 | Citropin A | Citropin-MMP |
|---|---|---|---|
| PC-3 | 50.0 | 54.98 | 67.6 |
| DU-145 | 48.9 | 59.78 | 69.4 |
| LNCaP | 54.3 | 62.0 | 69.3 |
| MCF-7 | 48.3 | 60.9 | n.d |
| Vero | 61.46 | 67.2 | n.d |
| HUVEC | 68.35 | 62.9 | n.d |

n.d.: not determined

**Table 4: Hemolytic effects of Citropin 1.1, Citropin A, and Citropin-MMP on human erythrocytes after 60 min incubation**

| Peptide | 90 $\mu$M | 80 $\mu$M | 70 $\mu$M | 60 $\mu$M | 50 $\mu$M | 40 $\mu$M | 30 $\mu$M | 20 $\mu$M |
|---|---|---|---|---|---|---|---|---|
| | | | | % hemolysis after 60 min | | | | |
| Citropin 1.1 | 86.3 | 79.5 | 55.1 | 39.8 | 33.7 | 22.9 | 19.8 | 7.2 |
| Citropin A | 88.6 | 85.8 | 66.0 | 50.1 | 39.5 | 26.2 | 17.8 | 13.1 |
| Citropin-MMP | 9.6 | 8.8 | 6.3 | 4.8 | 3.8 | 3.7 | 1.9 | 1.9 |

Neoplastic cells that expressed significant amounts of MMPs (PC3, DU-145, and LNCaP) were sensitive to Citropin-MMP Figure 4 A, 6A, whereas the deficient MMP neoplastic cells such as MCF-7 and Caco-2 were resistant to the activity of the peptide. Hemolytic studies against human erythrocytes confirmed the previous results as the erythrocytes were resistant to hemolysis by Citropin-MMP when compared to the parent peptides, which achieved significant t hemolysis at high concentrations. These results confirm that Citropin-MMP activity is enzyme-dependent, and the selectivity of Citropin A was enhanced significantly due to the structural changes applied to the parent peptide.

## CONCLUSION

Since highly invasive tumors secrete large amounts of matrix metalloproteinases and are responsible for the process of metastasis, we reason that Citropin-MMP would act exclusively upon invasive tumors without producing any deleterious side effects on the host cells. As metastatic tumors are still incurable, this strategy could prove to be valuable in the development of Citropin 1.1 as a novel anti-cancer agent and prove to be successful for the purpose of inhibiting the invasion of malignant cells.

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