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# Effects on Cell Viability and on Apoptosis in Tumoral (MCF-7) and in Normal (MCF10A) Epithelial Breast Cells after Human Chorionic Gonadotropin and Derivated-Angiotensin Peptides Treatments

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## ABSTRACT

Angiotensin-(1 - 7) [Ang-(1 - 7)] is an endogenous heptapeptide hormone of the renin-angiotensin system that has anti-proliferative properties. The aim of this work was to evaluate the anti-proliferative and pro-apoptotic properties of Ang-(1 - 7) and of Ang-(1 - 7)-substituents 9-fluorenylmethyloxycarbonyl (Fmoc) e Ang II-derivatives containing the TOAC (2,2,6,6-tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid) in normal (MCF10A) and in tumoral (MCF7) epithelial mammary cell lines. Both cell lines received an hCG and angiotensin peptides 24-hour treatment, in combination or alone followed by cell viability, apoptosis and cell cycle assays performed by flow cytometer (GUAVA). After hCG, Ang-(1 - 7), hCG + Ang-(1 - 7) and hCG + Ang-(1 - 7)-Fmoc treatments, MCF7 displayed cell viability decrease and mid-apoptosis increase. We also observed cell viability decrease in MCF10A after Ang-(1 - 7), Ang-(1 - 7) Fmoc and hCG + AngII Toac treatments. These cells had an increase in late apoptosis and necrosis after AngII Toac, hCG + Ang-(1 - 7) and hCG + Ang-(1 - 7)-Fmoc treatments. Regarding the cell cycle analysis, we did not observed any changes in cell cycle phases. In summary, cell viability was decreased and apoptosis (initial, mid and late) was increased after hCG and/or Ang-(1 - 7) peptides treatments. These results point out hCG and Ang-(1 - 7) as effective compounds to inhibit cell proliferation, since they decrease cell viability and increase apoptosis in both normal and in tumoral breast cells, being the effect more pronounced in the tumoral cell line. Our results support the idea of investigating more closely the putative use of these compounds as novel therapeutic agents for breast cancer.

**Keywords:** Angiotensin II; Angiotensin 1 - 7; Angiotensin II Type 1 Receptor (AT1R); Breast Cancer; Apoptosis; Human Chorionic Gonadotropin.

## 1. Introduction

Breast cancer is the most common cancer among American women, except for skin cancers. About one in eight (12%) women in the US will develop invasive breast cancer throughout her lifetime. The American Cancer Society's estimates that 226,870 new cases of breast cancer will be diagnosed in 2012 in the United States; about 63,300 new cases of carcinoma *in situ* (CIS) will be diagnosed and about 39,510 women will die from breast cancer [1].

The renin angiotensin system (RAS) is a hormone widely known for regulating blood pressure. The proteolytic cascade of the RAS begins with the release of renin (REN), an aspartyl protease which cleaves angiotensinogen (AGT) produces Angiotensin I (Ang I), which is hydrolyzed by angiotensin converting enzyme (ACE) and releases Angiotensin II (Ang II); this octapeptide ex-

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erts its functions through its specific membrane receptors, Angiotensin II receptor Type 1 and Type 2 (AGTR1 and AGTR2, respectively) [2]. Most of the physiological effects are attributed to the signaling pathways activated by the AGTR1. However, other components of the renin-angiotensin system (RAS) have been described as having mitogenic and angiogenic activities [3,4]. Since angiogenesis and proliferative processes are related to the development, progression and metastasis of cancer, we believe that there might be an association between these other RAS components with cancer [5]. Recent studies have shown that at a local tissue level, the components of the RAS influence tumor growth by changing its microenvironment [6,7]. Angiotensin-(1 - 7) [Ang-(1 - 7)] is an endogenous peptide hormone that functions as a vasodilator [8] with antihypertensive [9], antiproliferative [10-12] and antiangiogenic properties [13]. The formation of Ang-(1 - 7) from Ang I requires the action of three tissue endopeptidases: prolyl endopeptidase, neutral endopeptidase and thimet oligopeptidase [14-16]. Ang-(1 - 7) may also be synthesized from Ang II by the action of the ACE2 (Angiotensin converting enzyme 2) [17,18] or from Angiotensin-(1 - 9) [19]. Ang-(1 - 7) may be hydrolyzed by ACE forming Ang-(1 - 5) and Angiotensin-(1 - 3) [20,21]. The existence of a receptor for Ang-(1 - 7) is controversial. Most studies have shown that this Ang-(1 - 7) exerts its physiologic effects probably through activation of a unique G protein-coupled Ang-(1 - 7) [AT (1 - 7)] receptor encoded by the mas oncogene (mas1) [22].

The aim of this work was to evaluate the anti-proliferative and pro-apoptotic properties of Ang-(1 - 7), of Ang (1 - 7)-FMOC and of Ang II-TOAC analogues in normal (MCF10A) and in tumoral (MCF7) epithelial mammary cells. These cell lines were also treated with Human Chorionic Gonadotropin (hCG), a hormone that elicits life-long refractoriness to carcinogenesis by differentiation of the breast epithelium in order to assess any possible synergistic effect of these compounds.

## 2. Materials and Methods

### 2.1. Peptides

Ang II-TOAC, an Ang II analogue containing the TOAC (2,2,6,6-tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid) spin label synthesized by solid phase methodology [23] and Ang-(1 - 7)-FMOC, an Ang-(1 - 7) analogue containing the FMOC (9-fluorenylmethyloxycarbonyl) group was used for protection of the amine function [24]. These peptides were supplied by dr. C.R. Nakaie (department of Biophysics/UNIFESP-BRAZIL). hCG was obtained from Ovidrel®.

### 2.2. Cell Culture and Treatments

MCF7 cells (HTB-22, ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM), 4.5 g/l of glucose, supplemented with 5% fetal calf serum, 100 U/mL of penicillin (PAA), and 100 mg/mL of streptomycin (PAA). MCF10A cells (CRL-10317, ATCC) were cultured in DMEM/F-12 medium (PAA, Carlsbad, CA) supplemented with 10 mg/mL of human insulin (Sigma, St. Louis, MO), 20 ng/mL of epidermal growth factor (Sigma, St. Louis, MO), 0.5 mg/mL of hydrocortisone (Sigma, St. Louis, MO), 5% horse serum (Invitrogen), 100 U/mL of penicillin (PAA) and 100 mg/mL of streptomycin (PAA). All the cells lines employed in this work were cultured at 37˚C in a humidified atmosphere and 5% $CO_2$. The following treatments were performed on these cells: control (1); hCG (2); Ang II (3); Ang-(1 - 7) (4); Ang-(1 - 7)-FMOC (5); Ang II-TOAC (6); hCG + Ang II (7); hCG + Ang-(1 - 7) (8); hCG + Ang-(1 - 7)-FMOC (9); hCG + Ang II-TOAC (10).

### 2.3. Flow Cytometer (GUAVA™) Assays

MCF7 and MCF10A cells were seeded into 24-well plates with DMEM supplemented with 10% FBS in the absence (control) or presence of hCG. The cells were treated with 20 μM of peptide, the most active concentration value [25,26], and Angiotensin-deriveted peptides [Ang-(1 - 7)-Fmoc and Ang II-Toac] in the concentration of $10^{-6}$ M. After 24 hours of treatment, it was possible to determine viability index, and percentage of apoptosis, and cell cycle by means of biochemical assays in flow cytometer (Guava EasyCyteTM-Millipore) using kits ViaCount® Guava, Guava Nexin® and cell cycle assay®, respectively.

### 2.4. Statistical Analysis

The results for cell proliferation, apoptosis, and cell cycle assays were analyzed by descriptive statistics (means and standard deviation) and inferential statistics through the Student's *t*-test with significance level of 5% ($p < 0.05$).

## 3. Results

### 3.1. Cell Viability Assays

MCF-7 cells showed cell viability decrease while the mid-apoptosis increased after Ang-(1 - 7), Ang-(1 - 7)-FMOC and hCG + Ang II-TOAC treatments (**Figure 1(a)**). On the other hand MCF10A cells showed cell viability decrease, while the mid-apoptosis increased after Ang-(1 - 7) and hCG + Ang-(1 - 7)-FMOC treatments (**Figure 1(b)**).

### 3.2. Apoptosis Assays

Late apoptosis and dead cells ratio increase in MCF-7 cells after hCG, Ang-(1 - 7), and hCG + Ang-(1 - 7)-FMOC treatments (**Figure 2(a)**). Late apoptosis or

 

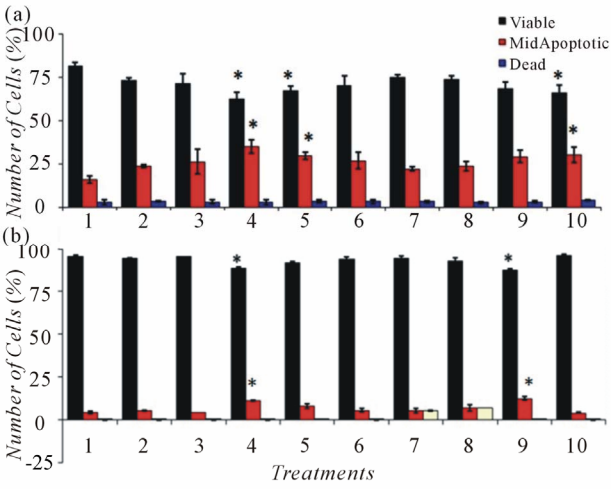


**Figure 1. Cell viability analysis after peptides, analogues and hCG treatments in MCF-7 (a) or MCF10A (b) cells. control 1); hCG 2); Ang II 3); Ang-(1 - 7) 4); Ang-(1 - 7)-FMOC 5); Ang II-TOAC 6); hCG + Ang II 7); hCG + Ang-(1 - 7) 8); hCG + Ang-(1 - 7)-FMOC 9); hCG + Ang II-TOAC (10). *p < 0.05 (compared to controls).**

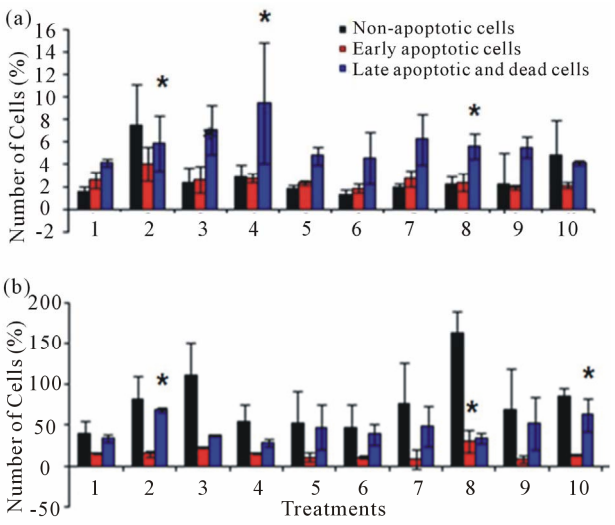


**Figure 2. Apoptosis analysis after peptides, analogues and hCG treatments in MCF-7 (a) and in MCF10A (b) cells. control 1); hCG 2); Ang II 3); Ang-(1 - 7) 4); Ang-(1 - 7)-FMOC 5); Ang II-TOAC 6); hCG + Ang II 7); hCG + Ang-(1 - 7) 8); hCG + Ang-(1 - 7)-FMOC 9); hCG + Ang II-TOAC 10). *p < 0.05 (compared to controls).**

dead cells increase after hCG treatment while early apoptosis in creases in MCF10A cells after hCG + Ang-(1 - 7)-FMOC treatment (**Figure 2(b)**).

### 3.3. Cell Cycle Assays

MCF7 and MCF10A cells did not evidenced alterations for G0/G1 or S cell cycle after treatments. Except for hCG that increases G2/M in MCF-7 (**Figure 3(a)**) and

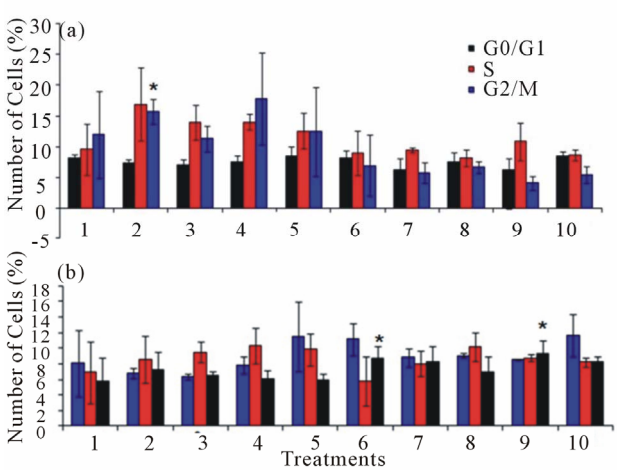


**Figure 3. Cell cycle analysis after peptides, analogues and hCG treatments in MCF-7 (a) and in MCF10A (b) cells. control 1); hCG 2); Ang II 3); Ang-(1 - 7) 4); Ang-(1 - 7)-FMOC 5); Ang II-TOAC 6); hCG + Ang II 7); hCG + Ang-(1 - 7) 8); hCG + Ang-(1 - 7)-FMOC 9); hCG + Ang II-TOAC 10). *p < 0.05 (compared to controls).**

for Ang II-TOAC and hCG + Ang-(1 - 7)-FMOC that increase G2/M in MCF-10A (**Figure 3(b)**).

## 4. Discussion

Ang II stimulates proliferation of AT1R-positive breast cancer cells through PI3-kinase/Akt pathway activation [27] and Ang-(1 - 7) has a clinical and pre-clinical activity in vascular sarcomas by reducing HIF-1alpha and PlGF genes expression [28]. In the present study, cell viability was decreased and mid-apoptosis was increased in both tumoral and in normal cells (MCF-7 and MCF10A) after Ang-(1 - 7) and analogs containing TOAC or FMOC treatments. Also, we observed that Ang II containing the TOAC substituent became an anti-proliferative hormone. Late apoptosis and cell death were increased after hCG, Ang-(1 - 7), and Ang-(1 - 7)-FMOC trreatments in MCF-7 cells. Early apoptosis was increased after stimulation with Ang-(1 - 7) and the effects of the peptides containing an FMOC group were powered by hCG in MCF10A cells.

Furthermore, cell cycle changes were not observed after peptide treatments, only hCG increased the stationary phases of the cell cycle (G2/M) in MCF-7 cells. Moreover in MCF10A cells, only the peptides containing the groups FMOC and TOAC triggered this same type of change. Therefore it is possible to say that the TOAC and FMOC substituent groups potentiate the anti-proliferative or pro-apoptotic effect in both cell types. Moreover we could also observe that Ang II becomes anti-proliferative in these mammary cells when the TOAC group is added to the hormone molecule and finally that hCG enhances the effects of the peptides.

 

In summary, cell viability was decreased and apoptosis (initial, mid and late) was increased after hCG and/or Ang-(1 - 7) peptides treatments. These results point out hCG and Ang-(1 - 7) as effective compounds to inhibit cell proliferation, since they decrease cell viability and increase apoptosis in both normal and tumoral breast cells, being the effect more pronounced in the tumoral cell line. Our results support the idea of investigating more closely the putative use of these compounds as novel therapeutic agents for breast cancer.

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