

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

**NEW *IN VITRO* THERAPEUTIC APPROACHES OF ANTI-ACTIVIN A MIMETIC
PEPTIDE IN LUNG CELLS IN CANCER DISEASE**

Aluna: Natieli Saito

Orientadora: Profa. Dra. Vivian Alonso Goulart

Co-orientadora: Dra. Fernanda Van Petten de Vasconcelos Azevedo

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Aos trinta e um dias do mês de janeiro de dois mil e vinte e três, às 14:00 horas, reuniu-se via web conferência pela plataforma Cisco Webex, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e Resolução de nº 06/2020 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Professores Doutores: Vivian Alonso Goulart (Orientadora), Bruno Antonio Ferreira e Douglas Carvalho Caixeta. A participação dos dois últimos se deu por epístola. Iniciando os trabalhos o (a) presidente Dr (a). Vivian Alonso Goulart apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença dos participantes, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa. A seguir o senhor (a) presidente procedeu a leitura das epístolas enviadas pelos membros da banca. Em seguida os membros presentes, passaram a arguir o(a) candidato(a). Ultimada a leitura das epístolas e a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

(A) PROVADO.

Esta defesa de Dissertação de Mestrado é parte dos requisitos necessários à obtenção do título de Mestre. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



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DEDICATÓRIA

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APRESENTAÇÃO

O trabalho apresentado enfoca em uma doença que é muito comum na população e que seu tratamento não fornece grande expectativa de vida a seus pacientes. O câncer de pulmão possui um prognóstico ruim e tal associação é feita também quando há uma desregulação da presença de Activina A no órgão, além disso há falta de exploração de novas vias de tratamento, que podem ou não ser associadas a métodos já existentes, se faz essencial nas pesquisas.

A molécula de Activina A está presente em diversas funções no desenvolvimento da homeostase, como no desenvolvimento embrionário e regulação de diversas atividades metabólicas, contudo sua hiper expressão pode levar a via de desenvolvimento de doenças e do agravamento delas, assim como no câncer de pulmão, onde esta molécula está associada a desregulação celular que leva a proliferação das células pulmonares. Por isso, o presente trabalho foca no desenvolvimento de um peptídeo inibidor desta molécula (Activina A) que não é comumente explorada nesse tipo de doença, tornando esta pesquisa inovadora e uma ferramenta para a descoberta de novas vias de tratamento para o tumor pulmonar.

Esta dissertação será dividida em dois capítulos, o primeiro consta em uma fundamentação teórica sobre o tema e foca no tratamento da doença em questão, o segundo destrincha o tema proposto e demonstra todos os testes até então executados.

CAPÍTULO I – FUNDAMENTAÇÃO TEÓRICA

O câncer de pulmão é uma doença que atinge grande parte da população, sendo que em 2020 sua incidência no Brasil apresenta o terceiro tipo de câncer mais comum entre os homens e o quarto em mulheres. Atualmente é mundialmente identificado como o primeiro tipo de câncer com maior índice de mortalidade entre homens e o segundo entre mulheres (1). Este alto índice se justifica em grande parte devido ao tabagismo e a exposição excessiva ao mesmo e, atualmente, o uso de cigarros eletrônicos expõe ainda mais o organismo a diversos tipos de substâncias tóxicas e carcinogênicas que levam ao desenvolvimento de doenças pulmonares e cardiovasculares, além de que este aparato eletrônico aumenta a chance de experimentação ao cigarro convencional ou de sua dependência (2).

Essa doença pode ser diferenciada em quatro tipos histológicos mais comumente encontrados, sendo estes o espinocelular, adenocarcinoma, carcinoma de grandes células e carcinoma de pequenas células. Os diferentes tipos podem ser encontrados de forma heterogênea devido a localidade e hábitos locais. Comumente o câncer de pulmão é dividido em dois grandes grupos e são classificados de acordo com a sensibilidade de resposta à quimioterapia e radioterapia, sendo que adenocarcinoma, carcinoma de grandes células e espinocelular são considerados carcinoma de pulmão de células não-pequenas e possuem elevada sensibilidade aos tratamentos, e carcinoma de pulmão de pequenas células que não apresentam tanta resposta aos tratamentos (3).

A identificação precoce de câncer de pulmão é deficiente e isso faz com que os pacientes diagnosticados com esse tipo de tumor já estejam em fase avançada sintomática apresentando fadiga, anorexia, perda de peso, tosse (com sangue em sua maioria) e dores no peito. O diagnóstico comumente utilizado nessa doença é o uso de radiografia e biópsia, já que a imagem pode em certos casos indicar corpos estranhos no pulmão que não são tumor. Como o câncer de pulmão pode ser classificado em diferentes tipos, é necessária a realização de análise citológica após a biópsia, que possibilita definir qual a metodologia mais adequada de abordagem para o tratamento (4).

A terapia adequada para o câncer de pulmão varia de acordo com o tipo celular encontrado em diagnóstico, mas em sua maioria envolve altas doses de radiação ou tratamentos intensivos com quimioterápicos e compostos metálicos que agredem todo o organismo e geram desconforto ao paciente, além de não serem métodos resolutivos (5). Nenhum método atualmente utilizado foca em utilizar as vias hormonais locais para tratamento, somente utilizando métodos comumente utilizado em outros tumores.

A Activina A é uma molécula importante para o funcionamento pulmonar, e também está presente desde o desenvolvimento embrionário onde é indispensável, porém sua hiperexpressão e desregulação no pulmão pode levar a formação de danos celular relacionados a reparos teciduais, tanto no câncer de pulmão quanto em doenças inflamatórias locais (6, 7).

O funcionamento da Activina A acontece por meio de sua ligação com receptores extracelulares que após sofrerem fosforilação iniciam a cascata de sinalização interna gerando atividades tanto anti quanto pró-inflamatórias, sendo essencial para esta definição a quantidade da molécula encontrada externamente à célula (8-10). Por isso o desenvolvimento de algo que possa bloquear o funcionamento extremo da molécula pode se tornar uma via alternativa importante para combater doenças como o câncer de pulmão.

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CAPÍTULO II

Revista: Respiratory Research

New *in vitro* therapeutic approaches of anti-activin A mimetic peptide in lung cells in cancer disease

Resumo

O câncer de pulmão é uma doença severa que afeta grande porcentagem da população e os tratamentos atuais não fornecem uma longe expectativa de vida aos pacientes. Devido a estes fatores nós decidimos explorar outro caminho na doença do câncer e desenvolvemos um peptídeo antagonista de Activina A, uma molécula importante no tumor de pulmão, por meio do uso da técnica de phage display. Como experimentos iniciais foi feito experimentos *in silico* e *in vitro* com experimentos como citotoxicidade, proliferação, Wound Healing e outros que demonstraram a capacidade do peptídeo de agir nessa via. Os dados presente no estudo de proliferação em células de pulmão e da inibição de adesão de células de câncer de pulmão mostram a possibilidade de ação em inibidor específico associado a essa molécula, mostrando a capacidade de modificação da capacidade celular sobre migração, invasão e adesão, importantes características em células de câncer de pulmão.

Palavras-chave: câncer de pulmão; Activina A; peptídeo; células de pulmão.

Abstract

The lung cancer is a severe disease that affects a huge percentage of the population, and the current treatments doesn't offer long life expectancy to the patients. Because of this we decided to explore another path in cancer disease and develop a peptide antagonist of Activin A, a molecule that is important in lung tumor, using the phage display technique. As initial experiments we did *in silico* and *in vitro* assays like cell cytotoxicity, proliferation, Wound Healing, and others to demonstrate the capacity of this peptide to work in this way. The data present results in proliferation assay in lung cell culture and in inhibition adhesion in lung cancer cell,

that shown the possibility of action in specific receptor and to modify the cell capacity of migrate, invasion and adhesion, important characteristic in lung cancer cells.

Keywords: lung cancer; Activin A; peptide; lung cell.

Background

Activins are pleiotropic, dimeric cytokines from the TGF- β superfamily, initially described in 1986 for their ability for promoting follicle stimulating hormone (FSH) release (11). Activin A is the most important and well understood isoform in vertebrates, with homology as high as 95% between species. Structurally, the activin A molecule is comprised by two identical B-A subunits with functions associated with several biological process, such as, embryogenesis, cellular differentiation, neuroprotection, inflammation, ovarian and uterine development, myogenesis, bone formation, tissue repair, stem cells regulation, pluripotency, hematopoiesis, fibrosis development and progression and in cancer disease (6, 7, 12-14).

Activin A signaling is triggered by the molecule interaction with the extracellular portion of the receptor ActRIIA and ActRIIB. The initial interaction promotes conformational changes in the activin A molecule, allowing it to interact with the cell surface receptor ALK4. Those interactions promote the phosphorylation of the intracellular portion of the receptors, triggering a Smad2/3 signaling response which will result in the regulation of the gene expression. This process is self-regulated by the induction of the production of Smad7, which can inhibit the phosphorylation and signaling of Smad2/3, and by the production of follistatin, an activin A biological inhibitor (6, 8-10).

Being a pleiotropic molecule, activin A can have pro or anti-inflammatory effects, depending on the microenvironment. High levels of activin A have been observed on inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, sepsis or inflammatory bowel disease, and being responsible for influencing the release of other pro inflammatory factors such as TNF- α , IL-6 and IL-1 β , furthermore this molecule can be found in increase or decrease depending of the type of tumor, indicating a possible target in cancer treatment (8, 13-19).

In the lung, Activin A has been associated with the tissue repair mechanism, although high levels of Activin A are linked to the severity of pulmonary diseases as well as the development of lung adenocarcinoma. In pulmonary inflammatory diseases animal models, Activin A imbalance triggered pro-inflammatory and pro-fibrotic states, inducing the production of IL-6, IL-13, IL-1 β , TNF- α , TGF- β 1, endothelin, angiotensin, thrombin, extracellular matrix, connective tissue growth factor, as well as stimulating fibroblast proliferation, differentiation into myofibroblasts and the epithelial-mesenchymal transition (6, 8, 14, 15, 20-22).

The increase of Activin A is one of the marks in lung carcinoma when the cells develop insensitivity, undergo adaptation, and evade the regulatory system. Specifically on lung cancer, the overexpression of Activin A is correlated with the increase of cell proliferation, adhesion, and positive lymph node status, furthermore, the increase of the presence of this molecule is related with the poor prognosis about the patient. Currently the treatments for lung cancer consist in surgery, chemotherapy, radiotherapy and drug delivery in specific cases, the decision about the approach of the treatment is decided when the professional knows what kind of cells was involved in the carcinoma, but even with the treatments the life expectancy about the patient don't overcome 5 years and all the process is too aggressive. (23, 24).

Thinking in another way for treatment to lung, less aggressive and involving molecular pathway, the use of peptides as theranostic tools is mainly due to their structural diversity, small size, high binding specificity and easy and low-cost synthesis (25, 26). The screening process uses a target molecule and a random library of peptides, derived from phage clones that express binding peptides fused to their capsid proteins, usually pIII or pVIII, which can be selected as they form a strong bond with the molecule. After this binding, an elution process takes place to recover selected phage clones, and subsequent selection rounds ensure a close pool of particular clones. Genetic sequencing and bioinformatic analysis allow the prediction and synthesis of the selected peptides (25, 27, 28).

Our aim was to select peptides that can block the local activity of Activin A in the lungs, we hypothesized that the peptides can inhibited cell characteristics like proliferation and adhesion. For this study we use immortalized lung cells, Beas-2B

(normal bronchial epithelium) and A549 (human lung carcinoma), to beginning the characterization of the peptides, initially with the process to selected peptides in silico, followed for functionals studies in cell culture.

Materials and Methods

Phage Display

M13 phage library and *E. coli* ER2738 were part of the PhD-7mer phage display peptide library Kit (New England Biolabs Inc. - Ipswich, MA, USA). Recombinant human Activin A (Cat# 592006) and anti-Activin A antibody (Cat# 693603) were purchased from BioLegend (San Diego, CA, USA).

To select high-affinity binding peptides to Activin A, 100 μ L of recombinant human Activin A (10 μ g/mL) were immobilized in a high binding ELISA plate. Unbound or weakly bound Activin A molecules were removed by two wash steps with PBS 1X. The plate was incubated with 300 μ L/well of blocking solution (5% w/v BSA in PBS) for 1 hour at RT and 200 rpm agitation. After the removal of blocking solution, the well was washed six times with 200 μ L/well of PBS-T 0.05%, and 10 μ L of the phage library diluted in 90 μ L o PBS-T 0.05% were added to the Activin A coated well. Incubation was conducted for one hour at RT and 200 rpm agitation. Unbound phages were removed by washing the plate 10 times with PBS-T 0.05% (300 μ L/well). Phages that were specific bound to Activin A were competitively eluted for 1 hour with 100 μ L of anti-Activin A neutralizing antibody (0.1 μ g/ μ L), diluted in PBS 1X buffer at RT and 200 rpm agitation. Eluted phages were amplified in *E. coli* ER2738 strain, purified by PEG-8000/ NaCl precipitation, and used in the next round of selection. Three rounds of selection were performed for the enrichment of specific peptides. Titration was performed at the end and the beginning of each round to evaluate the rate of recovered phage clones.

Phage ELISA

After the screening, selected phages were submitted to an ELISA assay to verify binding affinity. High-binding 96 well plates (Greiner Bio-One) were coated and incubated overnight at 4 °C with 100 μ L of Activin A (10 μ g/mL). After blocking with PBS-BSA 5% (Sigma-Aldrich) at RT for 1 hour, wells were washed twice with PBS, and 1×10^{11} selected purified phages were added into each well, followed by

further incubation at 37°C for 1 hour. Wells were washed six times with PBS 1X. Anti-M13 antibody labeled with peroxidase (Amersham Biosciences) diluted at 1:5000 was added into each well followed by incubation for 1h at 4°C. Samples were revealed with TMB (BD Biosciences, San Jose, CA) following manufacturers' instructions. The reaction was maintained for 10 minutes and stopped with the addition of sulfuric acid 2M (10 µL/well). Each sample was assessed in triplicates and evaluated at a plate reader at 492 nm (TP Reader, Thermo Plate). Results were expressed by the median OD of the samples. Best performing samples (24 phages) had their DNA sequenced.

Phage DNA extraction and sequencing

Overnight cultures of *E. coli* ER2738 infected with the selected phages were prepared in a deep-well plate. After centrifugation to separate the bacterial cells, the supernatant was transferred to a deep-well plate, and 1/6 v/v of PEG/NaCl solution was added for phage precipitation. After precipitation and centrifugation, the phage pellet was resuspended in 100 µL of sodium-iodide buffer, to promote phage lysis. The lysate was submitted to absolute ethanol precipitation for 10 minutes and centrifugation at 10,000 rpm for another 10 minutes. Phage DNA was washed with 70% ethanol and resuspended in 15 µL of Milli-Q water. Before sequencing, phage DNA quality was assessed by 0.8% agarose gel electrophoresis and quantification was done in a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). Sequencing was performed by Myleus Facility (Belo Horizonte, Brazil), in a 3730 DNA Analyzer (Thermo Fisher Scientific). Following their instructions, 100 ng of phage DNA was mixed with 1 µL of M13 sequencing primer (10 µmol) (5'-OH CCC TCA TAG TTA GCG TAA CG-3', Biolabs) and Tris-EDTA buffer solution q.s. 7.5 µL.

In silico analysis

DNA sequences were translated using the online free software, ExPASy Translate (<http://web.expasy.org/translate/>). To predict and visualize tridimensional structures we used Pep-fold3 (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>) and Pymol (<https://pymol.org/2/>). Structural interaction and docking prediction between the selected peptides and the Activin A molecule as well as interaction and docking predictions between Activin A/follistatin

and Activin A/ActRII were assessed using the online software GalaxyPepDock (<http://galaxy.seoklab.org/>).

Peptide synthesis

After in vitro and in silico analysis, clones A7, B9 and E10 were selected, the first three clones that reached the higher reacted ELISA indices, to be synthesized. Synthesis was carried out by Genscript (GenScript, Piscataway, NJ), abiding the following structures.

Peptide	Structure
A7	ACPTPLAWLXXXXXXETVES[LYS(BIOTIN)]
B9	ACPRDLLWLXXXXXXETVES[LYS(BIOTIN)]
E10	ACPPTLHWLXXXXXXETVES[LYS(BIOTIN)]

Table 1 – Patent number: BR 10 2018 071971 8

Cell culture assays

The cells Beas-2B and A549 (ATCC CRL-9609 and CRM-CCL-185, respectively) were used for all cell culture assays. Cells were grown in high glucose DMEM medium (Gibco), supplemented with 10% fetal bovine serum and 1% gentamicin (Sigma-Aldrich) – complete medium - under standard culture conditions (37°C, 95% humidified air, and 5% CO₂). The medium was changed every three days. Assays were conducted when cells reached approximately 80% confluence.

Cytotoxicity assay

Approximately 1x10⁴ Beas-2B and A549 cells were plated in a 96 wells plate (Corning) with 100 µL high-glucose DMEM complete medium (Gibco) under standard culture conditions (37°C, 95% humidified air, and 5% CO₂). After 24 hours medium was removed and replaced with high-glucose DMEM complete medium with the treatments. Cells were treated with the synthetic peptides (A7, B9, and E10) at 1 µM, 10 µM, and 100 µM for 24 h. Groups were also treated with and without the synthetic peptide pmTGF-β1 (1 ng/mL), a peptide that mimics the effects of TGF-β1 (29). After the incubation period, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Calbiochem, Darmstadt, Germany) solution (5 mg/ml) was added to each well, and the culture was further incubated for 4 h at

37°C. A total of 100 µL of SDS/DMF solution was added to each well followed by 1 hour incubation. The absorbance was determined on a microplate reader at 570 nm. Relative cell cytotoxicity (%) was calculated using the formula: % Viability = [(treated cells)/(untreated cells)] x 100 and % Cytotoxicity = 100 – (% viability). Negative control cells were treated only with high-glucose DMEM medium. The assay was conducted in triplicates.

Proliferation assay

Approximately 1×10^5 cells were plated in 24 wells plate (Kasvi) with 500 µL high-glucose DMEM complete medium (Gibco) under standard culture conditions. After 24 hours the medium was removed and replaced with high-glucose DMEM complete medium and treated with the synthetic peptide E10 at 1µM, 10µM and 50µM, groups were also treated with and without the pmTGF-β1 (1ng/mL). After the incubations periods of 24, 48 and 72 hours, 50µL of the culture medium was removed and 50µL of MTT solution (5mg/mL) was added to each well, the culture was incubated for 4 hours at 37°C. The medium supernatant was removed and 100µL of DMSO (Dimethyl sulfoxide) was added each well. After this the supernatant was replaced for a different plate and the absorbance was determined at 570nm. Negative control cells were treated only with high-glucose DMEM medium.

In vitro migration assay

Approximately 1×10^4 cells were cultured in a 24 wells plate (Kasvi) with high-glucose DMEM complete medium (Gibco) until ~80% confluence was reached. The cell monolayer was scratched with a p200 pipet tip. Unattached cells and debris were washed away with PBS 1X. The cells were then treated with high-glucose DMEM complete medium supplemented with the synthetic peptides E10 at 1µM, 10µM, and 50µM for 24h. Groups were also treated with and without the synthetic peptide pmTGF-β1 (1 ng/mL). Images were gathered using an EVOS® FL Auto Imaging System (Thermo Fisher Scientific, Wilmington, DE). Cellular migration was measured through the occupied area using the software Image J. The assay was conducted in triplicates.

Cell adhesion assay

The approximately 6×10^4 A549 cells were incubated with the different concentrations of treatment with the peptide E10 in DMEM complete medium, with and without the pmTGF- β 1 for 30 minutes at 37°C. Negative control was only treated with DMEM complete medium. After the incubation period, 100 μ L of cells were placed in each well in a 96 wells plate (Corning) and incubated in the cell culture incubator for 2 hours. After this, the medium was removed and the plate was washed with PBS 1X and removed carefully, 100 μ L of new medium was added with 20 μ L of MTT reagent in each well. The plate was incubated 3 hours and was added 100 μ L of SDS 10% 0,01M HCl and read after 18 hours in the absorbance of 570nm, reader *Perkin-Elmer LS 55*.

Autophagy and MitoStatus

For both experiments, cells (Beas-2B and A549) were cultured in a 24-well plate (Kasvi) with approximately 1×10^5 cells per well, and the treatment with 10 and 50 μ M of E10 peptide in the presence and absence of pm-TGF- β 1 (1ng/mL), was made after 24 hours of growth. In the MitoStatus experiment, the cell culture supernatant after treatment was removed and put in smalls vats. The plate wells were washed with PBS 1X and after the trypsinization process, the trypsinized cells were placed in smaller cuvettes and centrifugated twice to wash the cells. After that, the cells were resuspended with the MitoStatus solution with *BD Pharmingen mitostatus TMRE* and incubated for 15 minutes at 37°C, for finish this resuspension was read in cytometer *C6-Accuri BD* with the FL2-A parameter.

In the autophagy experiment the cells Beas-2B and A549 plated was treated after 24 hours of growing and incubated for more 24 hours for treatment with the peptide E10. After the cells were incubated with acridine orange for 15 minutes in room temperature in the dark. After this period the cells were visualized in fluorescence microscope EVOS® FL Auto Imaging System (Thermo Fisher Scientific, Wilmington, DE) and photographed in different lights for visualization the coloration.

Statistical analyses

All tests were conducted using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, EUA). $P < 0.05$ was considered statistically significant.

Results

A7, B9 and E10 peptides phage display development and in silico analysis

A total of 46 clones were obtained after three rounds of selection, 24 clones were found to be more reactive through phage-ELISA assay (fig. 1.I and 1.II), and their DNA was sequenced (fig. 1.III). Ten unique peptides sequences were obtained. To evaluate the binding capabilities of the selected sequences, another phage-ELISA assay with the corresponding clones was carried out. Clones A7, B9, and E10 presented higher binding affinity amongst the selected phages.

Peptides A7, B9, and E10 were synthesized using a template of the pIII phage protein to maintain the same conformation found in the screening process. Three-dimensional structural evaluation and interaction modeling demonstrated that the peptides A7, B9, and E10 target the same region. Interestingly, that is the same region that interacts with the receptor ActRII and the Activin A natural inhibitor, follistatin. While the interactions of the peptides A7 and B9 with the activin A molecule were located mainly in the pIII protein portion of the peptides, the peptide E10 showed a more strong and specific interaction with the target molecule (fig. 1.IV and V).

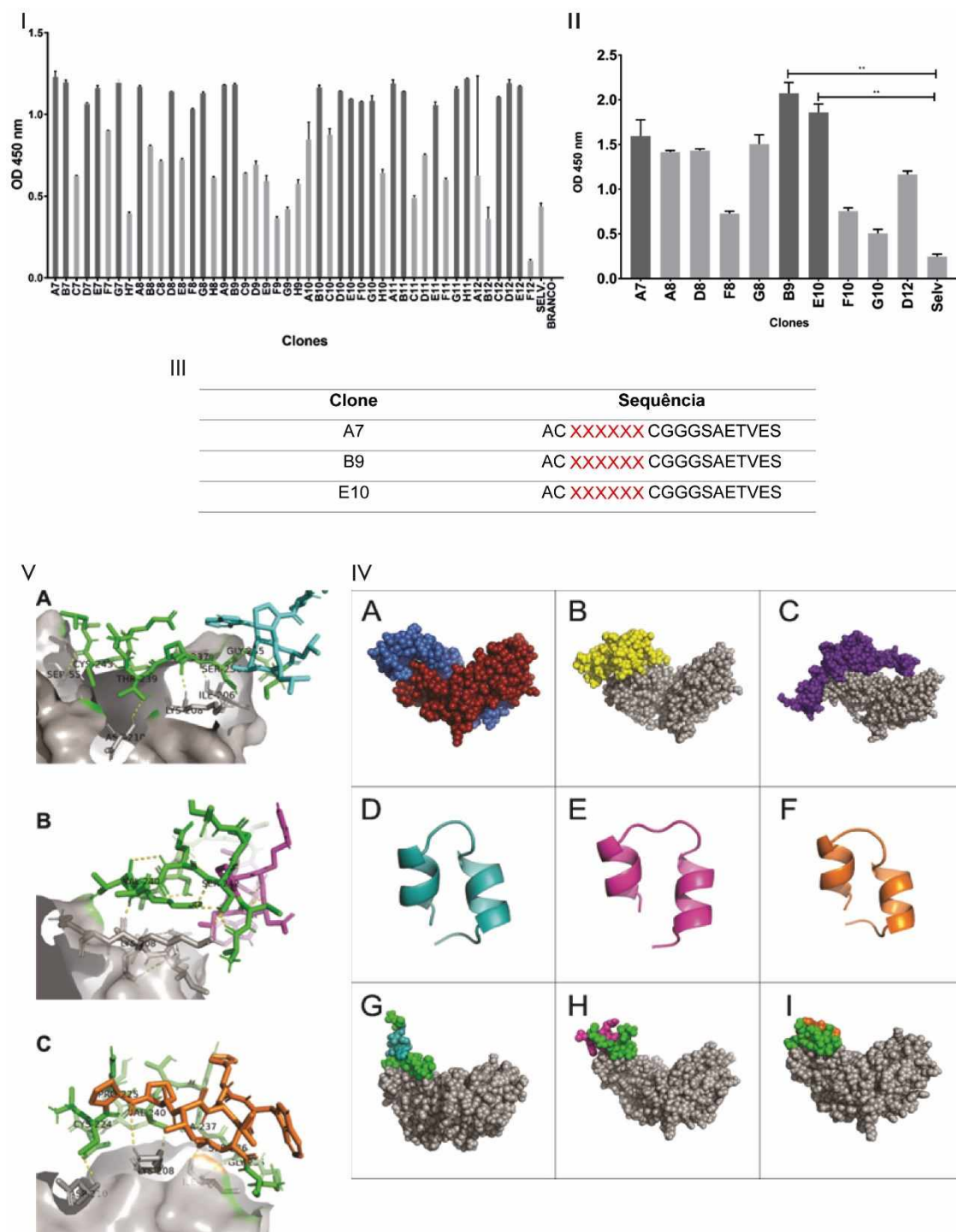


Figure 1 - I: Phage-ELISA evaluation of the reactivity of the 46 clones selected using the Activin A as a target. Darker columns highlight clones that showed ELISA index greater than one. II: ELISA assay demonstrating the reactivity of selected clones against Activin A. More reactive clones and with the sequences chosen for synthesis are highlighted. Clones B9 and E10 showed statistical significance against wild-type phage (Kruskal-Wallis and ANOVA $p < 0,05$). III: Sequences of peptides synthesized from selected clones. IV: Structural prediction models of the molecules used in this study. A) dimer of Activin A (PDB: 2ARV), B) interaction between Activin A (grey) and the ActRIIB receptor (yellow), C) interaction between Activin A (grey) and follistatin (purple), D) structural prediction of A7 peptide, E) structural prediction of peptide B9, F) structural prediction of peptide E10, G, H and I) prediction of the interaction of the Activin A molecule (grey) with the peptides A7, B9 and E10 respectively, in green the part common to the three peptides. V: Binding points between peptides A7, B9 and E10 with the Activin A molecule. A) Regions corresponding to the pIII protein sequence of the viral capsid, expressed together with the A7 peptide were able to bind to isoleucine (I206), to aspartic acid (D210) and to lysine (K208) from the ActRIIB receptor recognition site for the Activin A. B) the B9 peptide was capable of

covalently interacting only with lysine (K208) present at the recognition site of the Activin A receptor ActRIIB. C) Peptide E10 bound to isoleucine (I206), lysine (K208) and to aspartic acid (D210) present in the recognition site of the Activin A receptor ActRIIB. In the case of the E10 peptide, one of the specific amino acids of its sequence, proline, was responsible for the call.

E10 peptide decrease and cells proliferation

A549 and Beas-2B cells were treated with 1, 10, and 50 μM of the peptides A7, B9, and E10, with and without the addition of pmTGF- β (1 ng/mL) and monitored for 24 hours. After 24 hours, all peptides were able to demonstrate a significant reduction in proliferation, after comparing control and cells treated with different peptides, in both cells. In figures 2.A and 2.C it can be observed that the treatments did not present cytotoxicity, when compared with the control without treatment.

In the figures 2.B and 2.D, it can be seen that after treatment, none of the cells showed a proliferative effect at different doses and times and there was no significant difference between control and timing. Meanwhile, in A549 cells it can be noted that the E10 peptide at 48 and 72 hours of treatment when compared with the control at the concentration of 10 and 50 μM and pmTGF- β (1ng/mL) at 48 hours and the same peptide at a concentration of 50 μM with and without pmTGF- β showed a reduction in cell proliferation.

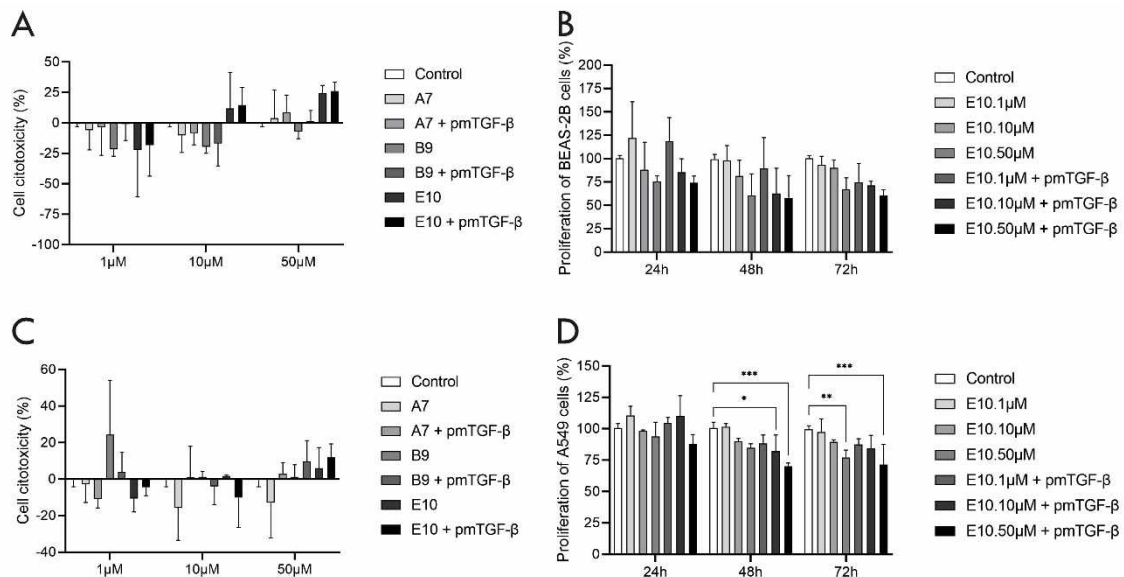


Figure 2 – In A and C have the analysis for cell cytotoxicity with the cells Beas-2B and A549, respectively, in different concentrations of 1, 10 and 50 μM in the three types of peptides A7, B9 and E10 with and without the addition of pmTGF- β (1ng/mL). The positives values correspond a percentage of cytotoxicity and the negative values correspond the opposite of cytotoxicity, in other words, the grow cell even with the treatment. In B and D have the cell proliferation assay

represents in percentage of viability with Beas-2B and A549, respectively. Measured in three different hours, 24, 48 and 72 of treatment in analysis of two-way ANOVA with Dunnett and $p < 0,001$, demonstrated statistical variance highlighted with asterisks.

E10 peptide decrease the tumoral cells (A549) migration and normal cells (Beas-2B) in different concentration

In Beas-2B cells, wound closure was observed even with the use of the peptide (figure 3.A and 3.B), in 24 hours at a concentration of 50 μM with pmTGF- β (1ng/mL) showed a significant difference when compared to the control, the E10 peptide was able to inhibit the migration of Beas-2B.

Image observations captured at 0, 6 and 24 hours demonstrated that the peptide E10 was able to inhibit cellular migration on A549 cells in the presence or absence of pmTGF- β 1 (figure 3.C and 3.D). That wasn't notice difference between the different concentrations of this peptide in any hour observed. When comparing the percentage of scratch wound coverage of the cells, Beas-2B showed faster migration when compared to A549 at a 24 hour. The Beas-2B showed in all treatments reached the mark of 80% coverage while in A549 the cells reached a maximum of 60% coverage.

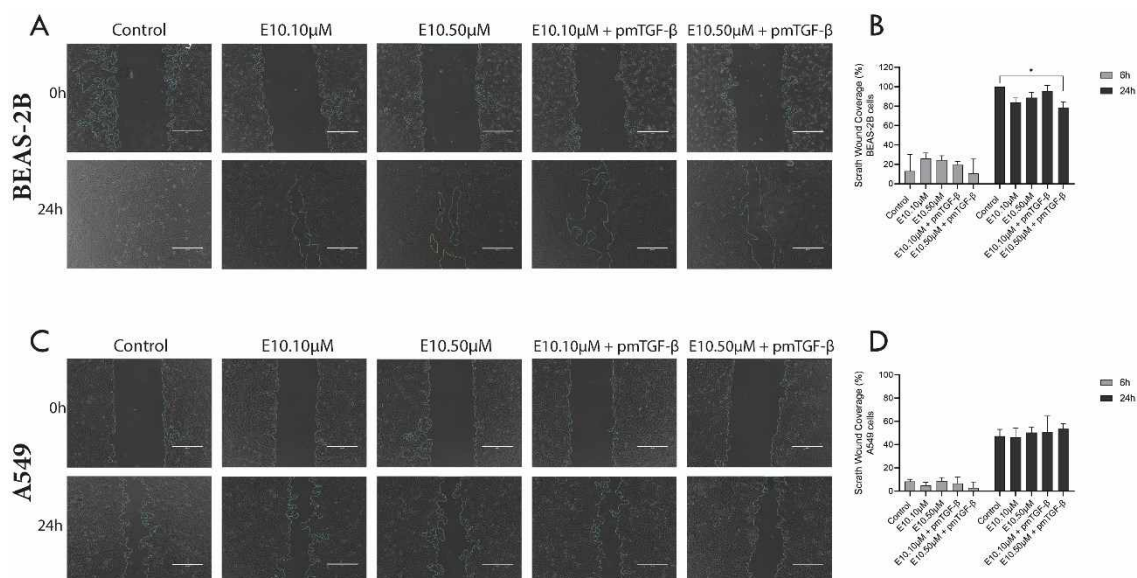


Figure 3 – A and C represents the photos make during the wound healing assay in two different hours 0 and 24, the risk is delimited by a colored trace to facilitated visualization. In B and D we have the statistical analysis derived from the area analysis in the photos, it's made two-way ANOVA with Dunnett test and $P < 0,001$ and the statistical variance is highlighted with asterisks.

E10 peptide inhibited the adhesion in the A549 cells

The A549 cell line was observed before the treatment with the E10 peptide, and we discover the potential of inhibition of the peptide in the 10 and 50 μM concentration (figure 4), that inhibits above 15% of the cells to join the surface after treatment and even with the presence of pmTGF- β 1, the peptide in the 10 μM concentration can inhibits approximately 35% of adhesion in the A549 cells.

Comparing with the control the peptides have a significant difference and demonstrate a elevate difference statistics. Any difference was observed between the control and the treatment with E10 in the concentration of 50 μM and with the pmTGF- β 1.

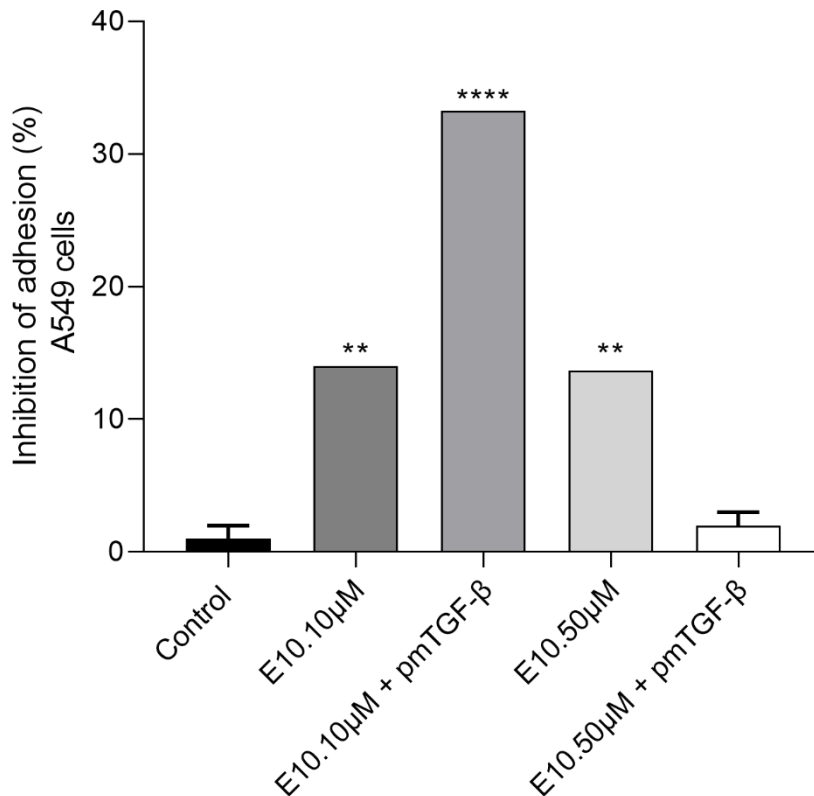


Figure 4 – Statistical analysis from inhibition adhesion in A549 cell with E10 peptide with and without the addition of pmTGF- β in ANOVA test with Dunnett and $p < 0,001$. The statistical variance is highlighted with asterisks.

MitoStatus and Autophagy

With the *BD Pharmingen mitostatus TMRE* reagent, a fluorescent dye that is sequestered by activated mitochondria we can read the live cells in the cytometer and measure the number of cells that is viable or not. In this assay, live cells acquire the fluorescent dye contrary to cells that suffer death processes.

In both cells (Beas-2B and A549) we use as positive control for death cells treated with hydrogen peroxide concentrated, to cause purposely depolarization of the mitochondria, which can't be colored with the reagent. In this experiment, we measure the number of living cells, and comparatively, in any cells, we note difference between the treatments, but comparatively the positive control in all the other treatments has a lot of live cells, as indicated in the figure 5.A and 5.C with the high peaks. It demonstrates that the treatments with the E10 peptide, and with the pmTGF- β 1 don't induce the death cell in mitochondrial path, because the major of the cells stay with the polarized mitochondria.

In the autophagy test using acridine orange, we present 3 images (figure 5.B and 5.D). The first is the GFP marks the cytoplasm in green, the second is read FITC in red marking the acid components, and finally, we present the "merge" which is the mixture between the two lenses and the overlay has an orange color which represents the autophagic action of the treatment, which may be due to the action of autophagosomes. In the A549 cells can be notice the difference color when comparing the control with the treatments specially in the addition of pmTGF- β , this can't be note in the Beas-2B cells.

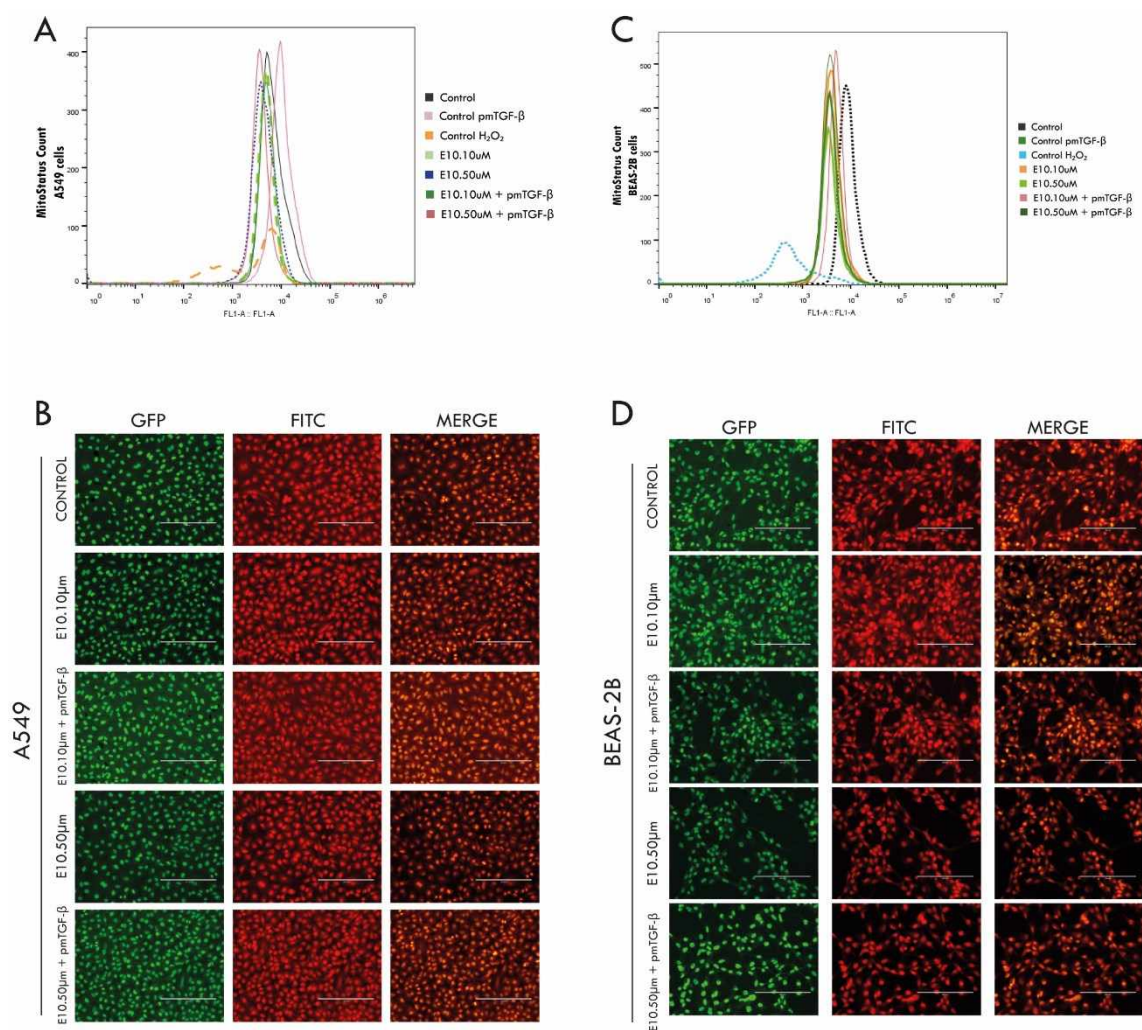


Figure 5 – A and C represents the TMMitoStatus assay in different colors representing the different treatments, above the image have the parameter utilized in the analysis, that is FL1-A:FL1-A utilizing the flow cytometer . B and D have the photos made in autophagy assay and have three different lens, the GFP (green), FITC (red) and MERGE (mix with green and red), the last lens shows the orange color that marks the autophagosomes and can be compared with the control image.

Discussion

The pleiotropic characteristic of Activin A and the relation of this molecule with different functions like tissue repair, pluripotency and progression in cancer disease make this hormone an interesting target in the research for a new way to fight a strong disease such as lung cancer (6, 7, 11-14). The fact of lung cancer is a disease that is the most commonly diagnosed and the primary cause of death in men and the fourth mostly commonly and the second cause of cancer in women (30) makes the lung cancer cells an interesting target for study.

Currently the treatments for lung cancer are equal for the most part of cancer disease and depends of the development stage, furthermore, lung cancer has a differential because it can be divided in various type of cells and this delineated the specific treatment (31, 32), but it does not define the success of the processing or the cure and the most part of the procedures are invasives and aggressive.

In this study, we researched for a new molecule that could become a new treatment for lung cancer and, for that, we developed an Activin A antagonist through a phage display assay, which enabled the discovery based on the role of this molecule (20-22). We were able to screen 24 phage clones, which display 10 unique peptide sequences. Among them, three sequences (A7, B9 and E10) showed high affinity for the target region of the Activin A molecule. A region already described as crucial for the interaction between Activin A and the ActRIIB receptor or the follistatin molecule (33, 34). As demonstrated by Mason et. All the amino acids isoleucine (I30 and I100), aspartic acid (D104), lysine (K102), proline (P32 and P88), arginine (R87), alanine (A31) and phenylalanine (F17) in the structure of Activin A molecule is part of the ActRIIB receptor recognition site.

Considering this region, A7 was able to covalently bound to the lysine, aspartic acid, and isoleucine. The A7 sequence was present in 43,48% of the selected phage clones, suggesting that the final A7 structural conformation played an essential role in the interaction and the selection process. In the case of the B9 peptide, predictions pointed to interaction only with the lysine, deeming this interaction weak for our purposes. For E10 peptide, we observed a more complex and strongest bound, as the E10 interacts with the lysine (K102), aspartic acid (D104) and isoleucine (I100) in the ActRIIB receptor region of the Activin A. In this case, one specific and four non-specific amino acids of the E10 sequence were part of the complex. Showing high specificity and demonstrating a high probability of activin A function inhibition. Therefore, the experiments were carried out with E10 peptide.

As described, the overexpression of Activin A in lung cancer represents a poor prognosis for the patient, and even subject to treatment, life expectancy does not exceed 5 years (23, 24). Because of this, our work focused on discovering another way to minimize the damage caused by cancer and increase patient

survival. The study began with two lung cells, Beas-2B and A549, to explore the ability of these peptides to act in different pathways of tumor growth and cell death.

Initial assay to discover the cytotoxic potential of this peptides is using the MTT reagent and shows the capacity of E10 peptide to be a possible way against tumor cell. For begging we use only one type of lung cancer cell to start the experiments, and as shown in silico the E10 have a potential to binding strongly in the receptors at cells and modified the capacity of grow. On the figure 2 can be notice the statistical difference between the proliferation in A549 cell in 48 and 72 hours, that shows the reduce of grow in this cell using the peptide E10 with and without the inductor pmTGF- β (29, 35), that represents a potential in a differential path in the tumor disease (36, 37).

According to the results in previous tests the wound healing demonstrated a capacity of Beas-2B cells to grow and migrate to the scratch allowed the cells to invade the risk, informing that peptide don't interview in this way in Beas-2B cells, only with the maximum treatment with addition to pmTGF- β that shown a short opening even after 24 hours. In A549 cell can be notice that haven't difference between the control and the treatments, but the maximum closure is about 60%, that demonstrated the reduction of capable to invade and migrate the scratch (38).

The adhesion of the cells has an important role in cancer disease, because is related to grow, migrate and invasion (39). The results shown a reduction in A549 cell capable to maintaining this characteristic, the figure 4 exhibits the inhibition of adhesion in A549 cell and relate the results to the other statistics, that shown a reduction of the capable of this cell to stay with the high tumor capacity (40).

Another path in cancer disease is the regulation of the cell cycle and the death pathways of these cells, allowing inhibition of tumor growth, proliferation, adhesion and migration, important pathways for tumor establishment and progression. We verified how E10 peptide can act on one of the death pathways, for that, TMMitoStauts Red was used to analyze the mitochondrial viability under the different treatments and we observed that the peptide does not affect this pathway in cell viability (41-44). And in the autophagy pathway we can observe the formation of orange color in the fusion lenses at some concentrations, but it is not possible to

quantitatively measure the formation of possible autophagosomes (45, 46). As shown, we can observe the action of the E10 peptide in the lung cells at migration and proliferation, to go deeper in the results we need to make other experiments that corroborate the results already found and that enable a better analysis of the cell death pathway, such as the use of cell markers and cytokines ELISA.

Conclusion

In this study, we can observe the action of 3 different peptides developed to act as Activin A antagonists in lung cancer disease. These peptides have a specific receptor, and in the in-silico analysis the E10 peptide demonstrated a greater binding capacity to its receptor, presenting possible greater effects and stability, therefore, our study focuses more on this molecule, which presented excellent results against a proliferation, migration, adhesion inhibition and autophagy assay.

Being a possible path for the treatment of lung cancer, the peptide needs to be better studied. One of the suggestions is to verify its activity in the epithelial-mesenchymal transition assay, analysis of cell death by annexinV-PI, as well as analysis of inhibition against different substrates in order to verify its possible action in the organism, studies including cytokines and other types of pulmonary cells. The ability of this peptide and the others to act in addition to other drugs and under different conditions needs to be further explored.

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Declarations

1. Ethics approval and consent to participate.

Not applicable.

2. Consent for publication.

Not applicable.

3. Availability of data and materials.

All data generated or analyzed during this study are included in this published article.

4. Competing interests.

The authors declare that they have no competing interests.

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6. Author contributions.

The authors Natieli Saito and Victor Alexandre Félix Bastos did a collaborative work, contributing equally to the study and therefore share the first authorship. The last author, Thúlio, is the teacher responsible for the project. The authors Fernanda and Vivian contributed in the development of the research and in the writing of the article. The other authors contributed to the execution of tests during the study. All authors reviewed the manuscript.

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