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Evaluation Of The Cytotoxyc Effects Of LMDF5-7 On A Human T-Cell Leukemia Cell Line

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DEDICATION

This thesis is dedicated to the people who have been on this journey with me, which either left something good or bad, but make me the person I am today. More importantly, this thesis is dedicated to my family, especially my parents. They have always supported me during my time in graduate school, and they know how difficult the situations have been. My parents gave me the opportunity to want more in life, gave me the best advice, shared their knowledge, and never gave up on my dreams. Also, to my sisters, who are examples of how women can stand up for their ideals, be hardworking, and be independent.

EVALUATION OF THE CYTOTOXYC EFFECTS OF LMDF5-7 ON A HUMAN T-CELL LEUKEMIA CELL LINE

by

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THESIS

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ABSTRACT

According to World Health Organization (WHO), cancer is the second leading cause of death worldwide, with 9.6 million deaths in 2018[1]. In the United States, an estimated 434,982 people were living with cancer during 2017[2]. Leukemia is the most common blood cancer in children from birth to 14 years old[3]. In particular, Hispanic children in the United States experience a higher incidence rate of leukemia[4]. Therefore, the discovery of novel cancer therapies is necessary to help those affected survive the disease. A new compound, LMDF5-7 was investigated and found to have anticancer properties that can help to treat cancer. To determine how this compound kills cancer cells, we evaluated its potential to activate cell death pathways by searching for mitochondria depolarization, reactive oxygen species (ROS) production, caspase-3 activation, and cell cycle proliferation. In this work, we evaluated the cytotoxic effects of LMDF5-7 in various cancer cell lines and demonstrated potent cytotoxicity against cancer cells, specifically leukemia cells. LMDF5-7 is a novel compound that targets cancer cells and is less sensitive to non-cancerous cells. This compound induces cell death, causes accumulation of ROS, and leads to the loss of mitochondrial membrane integrity. Additionally, this compound disrupted cell cycle progression that caused DNA fragmentation and induced apoptosis in a T-lymphoblast leukemia cell line.

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INTRODUCTION

One out of six deaths is due to cancer worldwide, making it the second leading cause of death in the world[1]. Cancer is the rapid production of abnormal cells that grow beyond their usual limits. Cancer cells remove normal cell division restrictions, create uncontrollable growth and abnormal functions. There are more than 277 different types of cancer, and there are many different stages that are indicated by gene mutations[5]. Cancer cells can invade adjacent parts of the body and spread to other organs, known as metastasis[6]. The malfunctions that cause the uncontrollable growth can develop into a lump, mass, or tumor, but some cancers involve the blood or blood-forming organs where they do not form tumors.[7]

Several risk factors may increase your chances of getting cancer. The chances of being diagnosed with cancer depend on age, family history, lifestyle, health conditions, and habits such as smoking and drinking[8]. Also, socioeconomic status can have an impact on treatment and survival rates. Wealthy countries have access to information about the disease, a better health system, quality treatment, and survivorship care, leading to improved survival rates of some cancer types due to early detection. However, the most affected and least prepared are low-income and middle-income countries that do not have access to quality diagnosis and treatment.[9] In the United States, there were an estimated 1,806,590 cancer cases and 606,520 deaths in 2020[10]. Cancer will affect a larger group of the United States population by 2021. It is expected to reach almost 1.9 million new cases, and approximately 608,570 Americans are expected to die of cancer, especially since COVID-19 increases the risk[11].

It is estimated that every 3 minutes, one person in the US is diagnosed with blood-related cancer[12]. Blood cancer is known as leukemia, and it involves the bone marrow and the lymphatic

system. Several types of leukemia exist. The most common and fast-growing ones are Acute Lymphocytic Leukemia (ALL) and Acute Myeloid Leukemia (AML)[13]. Leukemia develops when the DNA in blood cells, known as leukocytes, change and mutate, leading to uncontrollable proliferation[14]. Normally, white blood cells are only about 1% of your blood and responsible for protecting us against illness and disease[15]. The white blood cells are potent infection fighters in our bodies, and they grow and divide as needed. The bone marrow of people with leukemia produces an excessive amount of abnormal white blood cells, surpassing red blood cells and cause cancer [16].

Chemotherapy, surgery, and radiotherapy are the most common types of cancer treatments available nowadays[17]. Several problems exist with the proper selection of cancer treatments. These include unavailability of effective biomarkers, metastasis, among others, but especially drug resistance[18]. Resistant cancer cells develop certain unique mechanisms by which they can escape harmful agents.[19].The variety of drugs that exist are for personalized medicine that depends on your cancer type, stage, chemical composition, or other. These can be used alone or in combination[20]. Drug development and the design of treatments will benefit from incorporating several hallmarks and multiple biochemical pathways[21]. Cancer cells have different hallmarks that make them unique. Effective anticancer drugs focus on targeting several hallmarks to induce cell death[21].

Significance

It is important to implement research in cancer since this past year, and the world experienced a pandemic due to COVD-19, which mainly attacks the immune system. A study from the United Kingdom explained that patients with blood cancer were at a greater risk of experiencing a severe case of COVID-19 compared to patients with other cancer types and had an even higher likelihood of dying from the virus if on chemotherapy[22].

Leukemia is the most common cancer in children and teens, accounting for almost one out of three incidences[23]. The survival rate of leukemia cancer is about 69 of 100 persons who typically live at least five years after diagnosis depending on the type [24]. The investigation of new treatments and drug discovery is necessary to increase survival rates and give patients other options in case of resistance. Cancer can persist and eventually re-grow after time, especially in late-stage tumors[25]. Dr. Mirna Rivera found a novel compound at the University of Puerto Rico at Humacao, which can target several cancers. The collaborator has not released many details of the compound, named LMDF5-7, since they are still working on published data, but anticancer properties have been observed. My research will contribute to this data as I evaluate the biochemical effects of this compound on cancer cells.

Specific Aims. My research focuses on testing the novel compound LMDF5-7 against cancer cells to investigate the cytotoxic effects. Dr. Aguilera's lab focuses on *in vitro* screening of several possible cancer drugs. This thesis aims to understand how the compound affects cancer and non-cancer cells while looking for a piece of better knowledge on the molecular and cellular level of cancer research. Cancer has several hallmarks, some therapies focus on a single target, but it may be more feasible to conduct multiple assays to check the efficiency of the compound.

The main goal is to identify the potential of this compound to be an anticancer drug and its molecular mechanism of action.

Specific Aim1. *Evaluation of the cytotoxic effects of LMDF5-7 in various cancer cell lines.* Investigate cytotoxic and selective effects on several cancer cells and non-cancer cells by the DNS assay.

Specific Aim 2. *Investigation of the potential of LMDF5-7 to induce apoptosis in a Leukemia cell line.* For this aim, we determine that the mode of action of LMDF5-7 is programmed cell death. Analyze the health of the mitochondrial membrane by the examination of depolarization of such. Additionally, we search for reactive oxygen species which can cause excess oxidative stress, cause damage to the mitochondria and induce cell death. Observe the activation of the caspase-3 enzyme due to its functional role in the apoptosis cascade. Finally, we examine how the compound can affect the progression of the cell cycle by a flow cytometer.

MATERIALS AND METHODS

Cell lines. All the cell clines are incubated at 37°C and humidified with a 5% CO_2 atmosphere. Lymphoma/leukemia (CEM, NALM-6, and RAMOS) cell lines were cultured in RPMI-1640 medium (Hyclone, Logan UT) supplemented with 100 U/mL of penicillin, 100 µg/ mL of streptomycin (Lonza, Walkersville, MD), and 10% fetal bovine serum (FBS; Hyclone). HL60 cells were grown in 20% of FBS and the same medium as the other leukemia cell lines mentioned before. The breast cancer (MDA-MB-231), pancreatic cancer (PANC-1), and fibroblast (HS-27) cell lines were grown in DMEM medium (Hyclone) supplemented with 10% FBS (Hyclone), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Lonza). Finally, MCF-10A cells were grown in DMEM F/12 media with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Furthermore, 20 ng/mL of epidermal growth factor (EGF), 0.5 µg/mL of hydrocortisone, and 10 µg/mL of insulin were added to the MCF-10A cell line.

Differential nuclear staining assay (DNS). The purpose of this assay is to examine the potential cytotoxicity of LMDF5-7. The DNS assay identifies live and dead cells using two different nucleic acid fluorescent dyes: Hoechst, a blue dye (Invitrogen, Eugene, OR, USA), and Propidium Iodide a red dye (PI; MP Biomedicals, Solon, OH, USA)[26]. Hoechst stains the nuclei of healthy and dead cells (total cells), and PI only stains the dead cells. Cells were seeded in 96-well microplates at a density of 10,000 cells per well in 100 µL of media and incubated overnight. Then, LMDF5-7 was added at different concentrations. The controls used for the experiment were the following: a vehicle (1% DMSO), a positive control (H202), and untreated cells. Two different incubation times, 48 and 72 h were used. Two hours before imaging, the mixture of fluorescent dyes (1 µg/mL final concentration) was added to each well and incubated before the analysis image. For the

captured images from the plate, the IN-Cell Analyzer 2000 system was used (GE Healthcare, Pittsburg PA, USA). Then the IN Cell Analyzer Workstation 3.2 software (GE Healthcare) was used to segment and analyze both living and dead cells. Before preparing the experimental plate, the cell viability (at least 95% viable) was determined using PI staining and the Gallios flow cytometer (Beckman Coulter, Miami, FL).

Cytotoxic concentration 50% (CC_{50}) and selective cytotoxicity index (SCI) values. The IN Cell Analyzer Workstation 3.2 software that was used for the DNS assay analysis gave the numbers for each cell subpopulation and from each individual well. Then, the CC_{50} and SCI could be calculated. The CC_{50} is the concentration needed to kill 50% of the cell population by the compound[27].The percentage of dead cells obtained from the vehicle control (DMSO) was subtracted from each individual experimental point to eliminate the background of dead cells. Next, a linear interpolation equation was used to determine the CC_{50} value. A selective cytotoxicity index (SCI) determines the selective toxicity of a drug in cancer cells compared to a non-cancerous cell line (Hs27 and MCF-10A). For the calculation, the CC_{50} for the non-cancerous control was divided by the CC_{50} of the cancer cells.

Cell cycle analysis by flow cytometer. Two 24-well plates with CEM cells at a concentration of 100,000 cells in 1 mL of culture media per well were used. Cells were treated with 0.06 μM (CC_{10}) ,0.13 μM (CC_{20}) and 0.20 μM (CC_{30}) of LMDF5-7 for 48 and 72 h. The controls included for this experiment were DMSO, untreated, and hydrogen peroxide as a positive control. Then, cells were collected in flow cytometry tubes and centrifuged at 262g for 5 min. Then, the supernatant was discarded and gently resuspended in 100 μL of culture media. Before using the

flow cytometer, we added 200 µL of nuclear isolation medium (NIM)-DAPI solution (NPE Systems, Inc. Pembroke Pines, FL, USA)[28]. Then we read the samples in the flow cytometer (10,000 events) and looked for the different stages of the cell cycle. The NIM-DAPI reagent can quantify DNA content at different stages using an FL-9 detector that detects the fluorescent signal and the 405-nm laser (Gallios).

Reactive oxygen species detection assay. We conducted via carboxy-H2 DCFDA (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate) indicator of oxidative using flow cytometry[29].CEM cells were seeded in 24-well plates at a concentration of 100,000 cells per well, 1 ml of media, and incubated overnight at F°C. Then, we added controls used before and LMDF5-7 compound and incubated it for 18h. Each well was collected in flow cytometer tubes, centrifuged for 5 min at 262g, and decanted the supernatant to keep the pellets. We resuspended in a mixture of pre-warmed PBS and the carboxy-H2 DCFDA with a final concentration of 10 µM (loading buffer). After that, the flow cytometer tubes with the cells were placed back into the incubator for 1 h at 37°C. When the incubation is over, remove the loading buffer, carefully spin down the tube, and add 500 µL of PBS. As a final step, we incubated for 30 min at 37°C. Immediately we read with the Gallios flow cytometer (Beckman Coulter), acquiring 10,000 cells per sample, and analyzed via Kaluza software (Beckman Coulter).

Analysis of mitochondrial membrane potential *via* JC-1 assay. The MitoProbe JC-1 assay kit (Molecular Probes, M34152) and flow cytometry were used to analyze the mitochondrial depolarization[28]. We plated CEM cells in a 24-well with 100,000 cells and 1 mL of cell culture

media per well. The CEM cells were treated with LMDF5-7 and the same controls mentioned before in triplicate for 5 h. Then, we collected cells in flow cytometer tubes and centrifuged them at 262g for 5 min. Next, we decanted the supernatant and resuspended with 500 µL media containing 5 µL (2 µM final concentration) of JC-1 reagent (5',6,6'-tetrachloro-1,1 ',3,3 '-tetraethylbenzimidazolylcar-bocyanine iodide) and incubated at 37 °C, 5% CO_2 for 45 min. Cells were washed once with pre-warmed PBS after incubation. Immediately analysis at the flow cytometry by the Kaluza software (Beckman Coulter).

Analysis of apoptosis/necrosis by Annexin V-FITC/PI assay. These experiments are used to explore whether LMDF5-7 induces apoptosis or necrosis as the mechanism of cell death. The Annexin V-FITC Kit was utilized (Beckman Coulter, Miami, FL, USA) with CEM cells seeded in 24-well plates at a concentration of 100,000 cells per well with 1 mL of culture media and incubated overnight at 37°C. Treatments were applied to cells in triplicate and incubated for an additional 24 h. After the incubation, cells were collected in flow cytometry tubes and centrifuged for 5 min at 262g. Each pellet was resuspended in 103.5 µL of the following mixture: 100 µL of the 1× binding buffer, 1 µL Annexin V-FITC, and 2.5 µL of propidium iodide[28].To facilitate the exposure of the fluorophores, we resuspended them again and kept them on ice for 20 min in the dark. Afterward, we added 300 µL of ice-cold binding buffer to each tube and analyzed *via* flow cytometry (Cytomics FC 500, Beckman Coulter) and the Kaluza software (Beckman Coulter).

Caspase-3 assay. For this assay, HL-60 cells were plated in 24-well plates (100,000 cells). The assay consists of a fluorogenic substrate which is cleaved by an active caspase-3 using NucView 488 caspase-3 substrate assay kit (Biotium, Hayward, CA), releasing a product that after binding

to the DNA emits a bright green fluorescence signal. The cells emitting a green signal are positive to caspase-3 active, meaning apoptotic cell; incubate at 6 h with controls and compound. After the incubation, cells were placed in flow cytometry tubes and centrifuged at 262g for 5 min. Cell pellets were gently disrupted by adding 200 μL of PBS containing 5 μL of NucView 488 Caspase-3 substrate (5 μM final concentration) and incubated 30 min at room temperature in the dark. Then we added 300 μL of PBS and analyzed it by flow cytometry. Data was of 10,000 events and analyzed by using Kaluza Flow Cytometry Software (Beckman Coulter).

Statistical analysis. Each experimental point signifies at least three independent measurements. All findings are shown as the multiple measurements with their corresponding standard deviations to denote our experimental variability. The statistical significance of the differences between two experimental samples was performed using a two-tailed Student's *t*-test, and a *P*-value equal to or less than 0.05 was considered significant.

RESULTS

Compound LMDF5-7 is cytotoxic and selective against cancer cells: The Differential Nuclear Staining assay can determine CC_{50}, which is the value concentration of the compound that causes 50% of cell death as compared to solvent-treated and how selective it is against cancer cells[30]. The Selective Cytotoxicity Index (SCI) determines how selective the toxicity of a compound is in cancer cells compared to non-cancerous cell lines. The equation for SCl= CC_{50} of the non-cancerous cell over CC_{50} of cancer cell and the values greater than 1 were deemed selective.

The compound was tested in 8 different cell lines, and the effects of each one are in **Table 1**. The best results obtained were in the CEM cell line with a CC_{50} of 0.33uM, and the second-best result was another leukemia cell line, HL-60, with a CC_{50} of 0.70 uM. The Selective Cytotoxic Index indicates the compound is highly selective against leukemia cell lines for both cell lines[28] . To calculate the Selective Cytotoxic Index (SCI), the CC_{50} of non-cancer cells is needed, and the two cell lines tested were Hs27, a fibroblast, and MDF-10A, a Fibrocystic Diseases. However, the only one used for the calculation was Hs27. We could not detect a CC_{50} lower than 100 uM with MCF-10A, and we could not go higher due to issues with dissolving the compound at a higher concentration.

Table 1: CC_{50} and SCI values of LMDF5-7-treated cancerous and non-cancerous cells.
Note: On the top of the third column (left to right) should read CC_{50} [µM]

Cell Line	Disease	Cc50[uM]	S.D.	SCI
CCRF-CEM	Acute Lymphocytic Leukemia	0.3341	0.0116	254.02
HL-60	Acute Promyelocytic Leukemia	.7045	0.0247	120.46
Hs27	None	84.87	1.820	-
MCF-10A	Fibrocystic Disease	>100	N/A	-
MDA-MB-231	Adenocarcinoma	8.146	1.2414	10.41
PANC-1	Epithelioid Carcinoma	1.335	1.0372	63.57
Ramos	Burkitt's Lymphoma	5.39	1.311	15.74
Nalm-6	Acute Lymphocytic Leukemia	5.178	.814	16.41

DNS is an assay applicable to high content primary screens for identifying cytotoxic activity, especially cancer drug screening to determine CC_{50} and SCI. After analyzing the results, we detected that the LMDF5-7 compound was more cytotoxic towards T-cell leukemia cell lines. CEM is the cell line selected to continue with the other assays because it is highly selective and has a lower CC_{50} than other cell lines.

Cell cycle in CEM cell line at 48 and 72 hours by flow cytometer. The quantitative fluorescent staining by NIM-DAPI can quantify DNA content at different stages. Flow cytometric (FCM) is a rapid analysis of cellular DNA and evaluates DNA contents for obtaining cell-cycle frequency distributions[31]. The assay was done at different time-lapses: 48 and 72 hours since the cell cycle needs more time to complete each stage. This assay aims to look for changes or alterations in the cell cycle and look for DNA fragmentation, one of the apoptosis hallmarks. For the cell cycle assay, the concentrations were CC_{10}, CC_{20}, and CC_{30} because, at lower concentrations, the cells are allowed to complete the cell cycle and not be as aggressive as CC_{50}.

Figure 1 shows the cell cycle at 48 hours, demonstrating that Sub-G0/G1(A) has DNA fragmentation compared to the H_2O_2 (our positive control). The percentages of the cell cycle phases look as DMSO or non-treated, meaning that the LMDF5-7 compound is not altering the cell cycle progression. However, the sub-G0/G1 subpopulation has significantly increased, suggesting that the LMDF5-7 compound can induce DNA fragmentation.

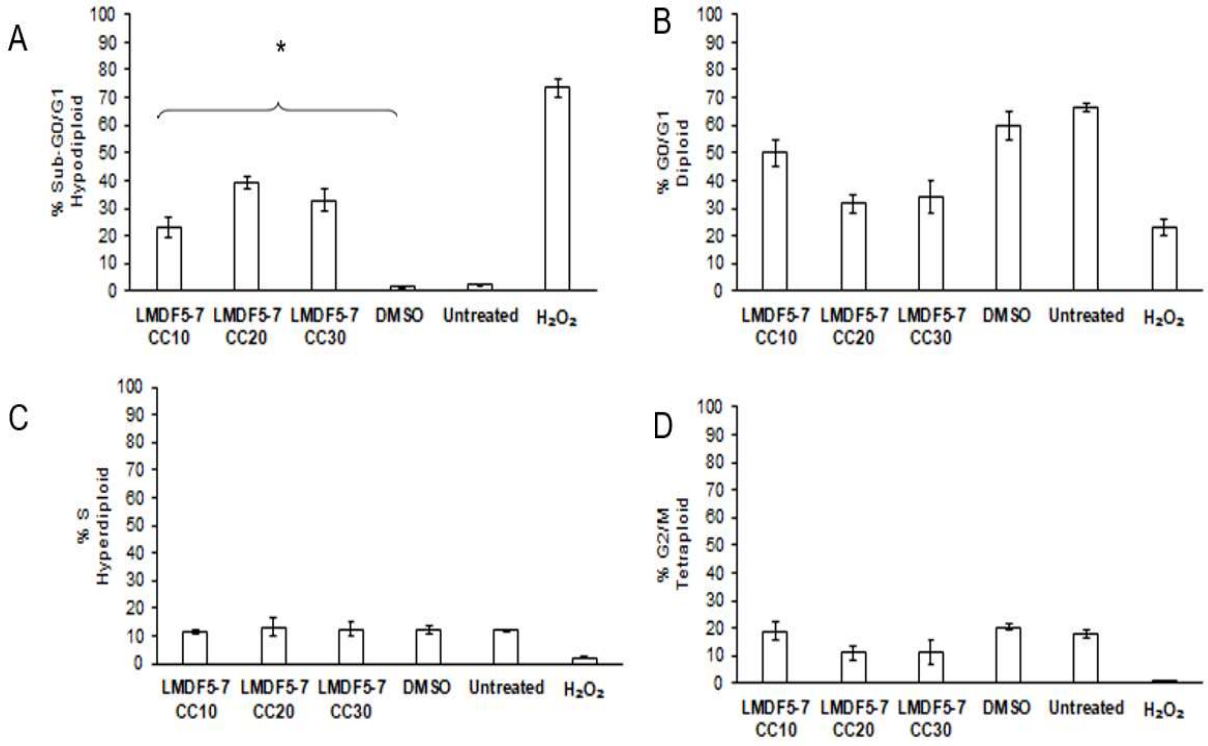

Figure 1. Analysis of CEM cell line in cell cycle profile by flow cytometry at 48 hours. Results show DNA fragmentation at Sub-G0/G1(A).
Note: It should read CC_{10}, CC_{20}, and CC_{30}. It is highly recommended to use Arial font in all the figures, numbers, and letters. Please, be consistent in all the Figures.

Results in **Figure 2** at 72 hours, the CEM cells have more time to reproduce and complete a cell cycle. The DNA fragmentation is higher in Sub-G0/G1(A), and similarly to the 48 h experiment, a non-discernible arrest was detected. After, the cell cycle looks disrupted, and the compound has a similar effect as our positive control H_2O_2.

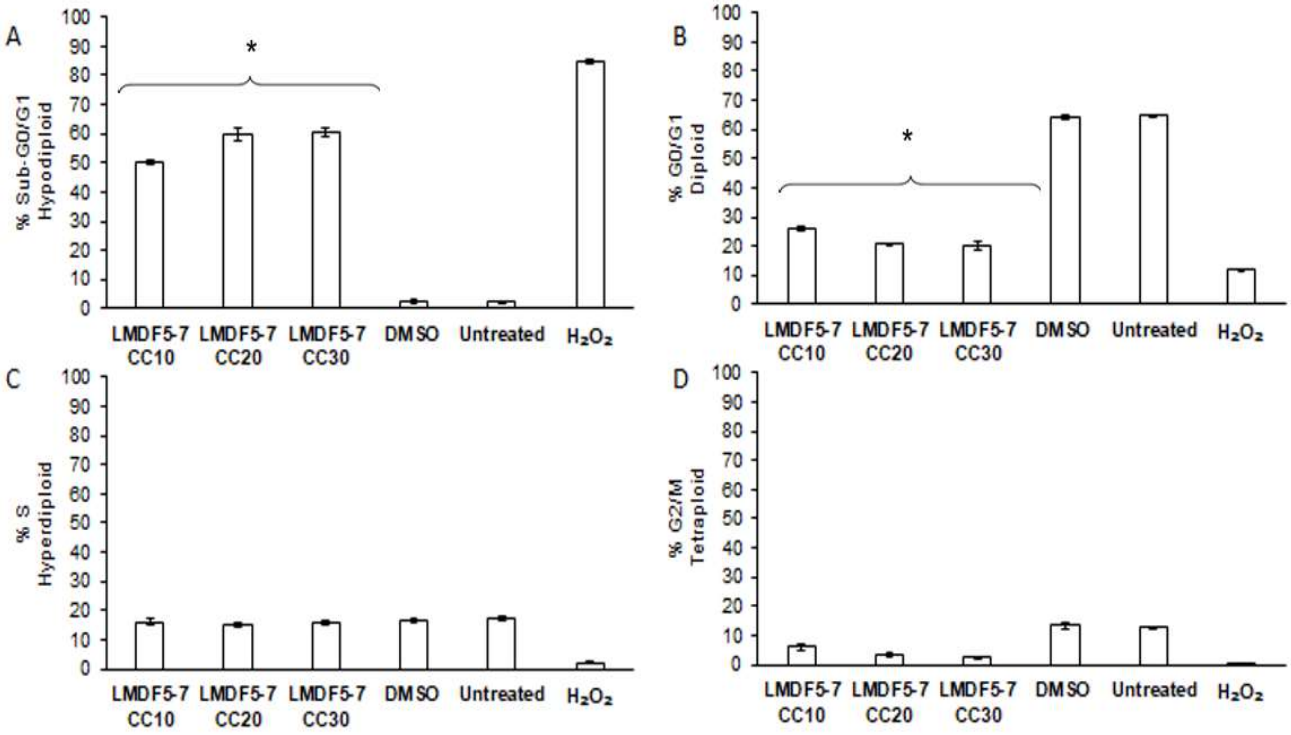

Figure 2. The cell cycle profile of CEM cell line after 72 hours of LMDF5-7 compound exposure. Demonstrate DNA fragmentation in Sub G0/G1(A).
Note: It should read CC_{10}, CC_{20}, and CC_{30}.

Apoptotic cells can be identified since the low molecular weight and content frequency histograms as cells with fractional sub-G0/G1[32]. Figure 1 shows a DNA fragmentation, but in comparison with Figure 2, the DNA fragmentation is more compatible with our positive control H_2O_2 and shows a change in the other phases. Both times demonstrate that LMDF5-7 causes

DNA fragmentation and distrust the continues of the cell cycle. The other phases, S and G2/M, in **Figure 1** and **Figure 2** show an unfavorable cell cycle progression.

Reactive Oxygen Stress (ROS) induced by LMDF5-7 in a Leukemia cell line. Reactive oxygen species have different signaling functions and are free/non-radicals, ions, or molecules with a single unpaired electron in their outermost shell of electrons[33]. Oxygen radicals are potent DNA-damaging agents that cause DNA strand breaks if an excess of ROS exists, induce cell death, and not let the proliferation continue[34].

For this assay, the probe that LMDF5-7 causes oxidative stress is a positive control. Hydrogen peroxide (H_2O_2) has been recognized as destructive molecules because they induce endogenous ROS[35]. Typically, if ROS is at low cellular concentrations, they allow proliferation and help have proper function of intracellular signaling and regulation[36]. **Figure 3** indicates accumulation and disturbs cellular homeostasis by ROS exposure. Excess of oxidative stress leading to cell death, known as apoptosis, tested in both compound's concentrations. LMDF5-7 demonstrates the hallmark oxidative stress and acting similarly to H_2O_2. DMSO and Untreated cells as negative control and p-value$\leq$0.05 (*) was deemed significant.

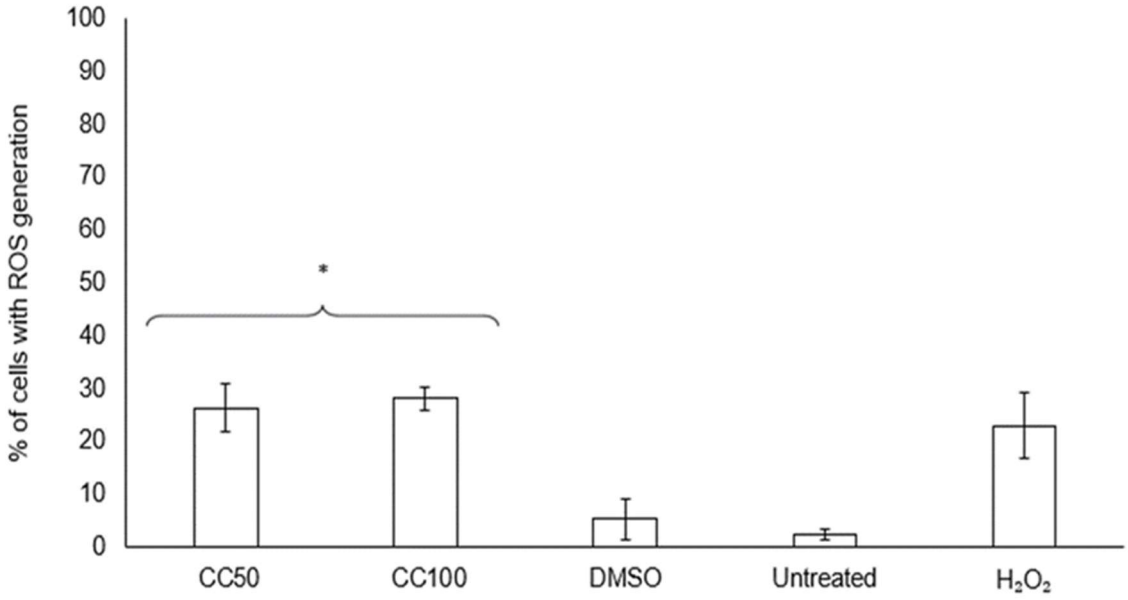

Figure 3. Accumulation to initiate cell death by Reactive Oxygen Stress (ROS) in CEM cell line for 18 hours with LMDF5-7 exposure at a concentration of 1 μM.
Note: It should read CC_{50}, and CC_{100}.

LMDF5-7 compound induces apoptosis. The Annexin V-FITC and propidium iodide (PI) (PI) kit is helpful to investigate whether the mechanism of cell death induced by LMDF5-7 compound is *via* apoptosis or necrosis. Cells experiencing apoptosis externalize the phosphatidylserine (PS) to the outer leaflet, which is typically confined to the plasma membrane's inner leaflet. Cells were monitored *via* flow cytometry after dual staining Annexin V-FITC and PI. The calcium-dependent binding of Annexin V can detect exposed PS, but in healthy cells, the PS is not detected since it is in the inner leaflet[37]. In necrotic cells, PI can penetrate the damaged plasma membrane and complex with nucleic acids. The fluorescence of Annexin V-FITC-PS complexes on only apoptotic cells was detected by flow cytometry after 24 hours of exposure.

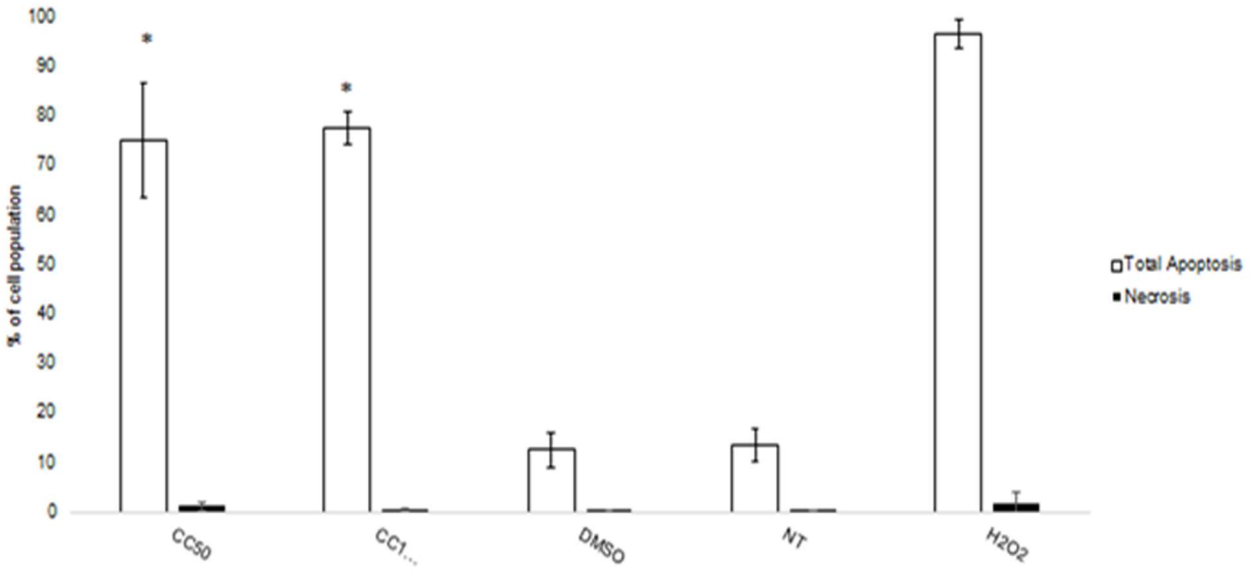

Figure 4. Phosphatidylserine externalization to identify apoptotic cells. Percentage of apoptotic cells (stained with Annexin V-FITC) and propidium iodide (PI) after 24-hour incubation with LMDF5-7 at a concentration of 1 µM
Note: The letters and numbers size font of the labels in the graph is tiny and fuzzy.

The results in **Figure 4** demonstrated programmed death was the mode of action for the compound LMDF5-7. The results do not detect necrosis. When comparing our compound with the other controls DMSO and untreated, a normal continuation of growth without cell death, we observed that LMDF5-7 caused apoptosis to the CEM cell line at the concentration CC_{50} and CC_{100} a P-value$\leq$0.0001 (*).

Analysis of mitochondrial membrane potential: The mitochondria use oxidizable substrates to produce an electrochemical proton gradient across the membrane used to produce energy (ATP)[38]. Also, it transports ions and proteins, which are necessary for healthy mitochondrial function. The mitochondrial membrane potential, known with the symbol $\Delta\Psi m$, maintains homeostasis by selective elimination of dysfunctional mitochondria[39]. If depolarization or

decreases of mitochondria membrane potential is present, meaning a dysfunctional or unhealthy mitochondrion, cell death can occur. Increased intrinsic ROS stress causes malfunction of the mitochondrial to let the depolarization happen and alters cellular function to induce apoptosis[40].

For the experiment know as JC-1, we look to see if the LMDF5-7 compound causes a depolarization as H_2O_2 to induce unhealthy mitochondria.

Figure 5 showed the compound increased dose-dependent mitochondrial depolarization that was statistically significant compared to the solvent ($P< 0.05$) and untreated controls. The CC_{100} results in a decrease of mitochondria membrane potential after 6 hours of incubation in the CEM cell line. As the ROS experiment demonstrated oxidative stress in the CEM cell line exposed to the LMDF5-7 compound, we believe there is a malfunction in the mitochondria. These indicate apoptosis was detected as the mode of action of LMDF5-7 and causes loss of membrane integrity.

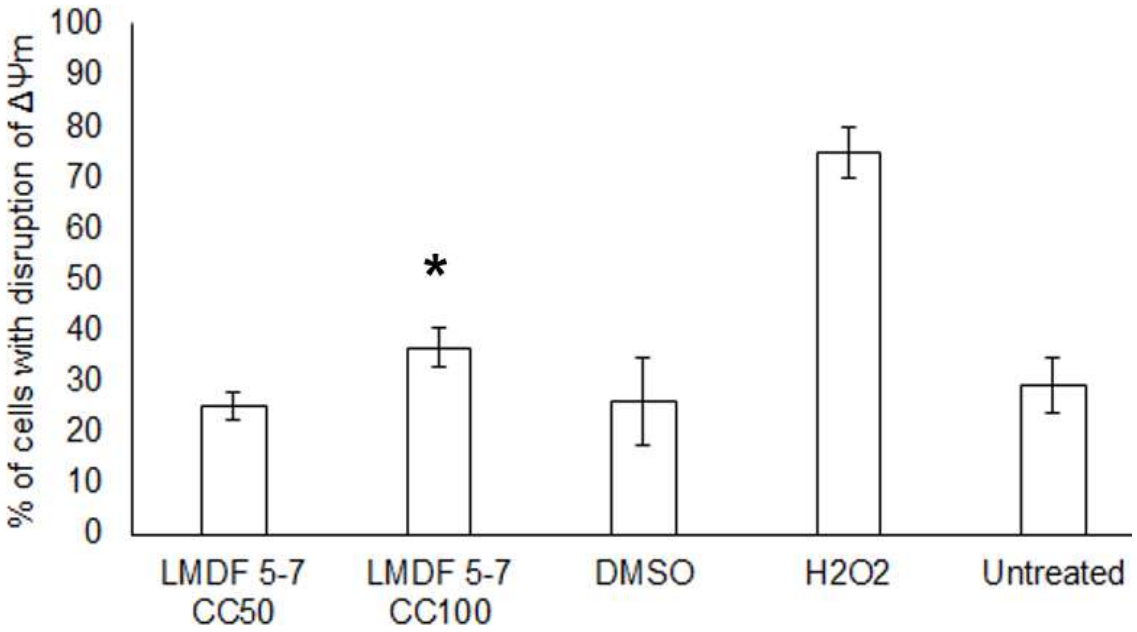

Figure 5. Analysis of cells with depolarized mitochondria after 6-hour treatment with 1 μM LMDF5-7 observed by JC-1 dye which emits a green fluorescence signal.
Note: It should read CC_{50}, CC_{100}, and H_2O_2.

LMDF5-7 Compound Caused Activation of Caspase-3 in Leukemia Cell line. Caspases family is known as mediators of programmed cell death (apoptosis), especially caspase-3, that activate death proteases and catalyze a specific cleavage of proteins[41]. Executioner caspase-3 is responsible for inhibiting electron transport through electrons and lowering the mitochondrial membrane potential ΔΨm to decrease ROS production due to apoptosis[42]. Therefore the detection of caspase-3 has a key role in detecting apoptosis as a mode of action created by LMDF5-7.

The experiment consists of a caspase-3 peptide cleaving the substrate DEVD, releasing the high-affinity DNA dye, which migrates to the cell nucleus and stains DNA with fluorescence. The caspase cleavage in the apoptotic cell and the dye released a fluorescent dye in the nucleus can be detected by flow cytometry[43].

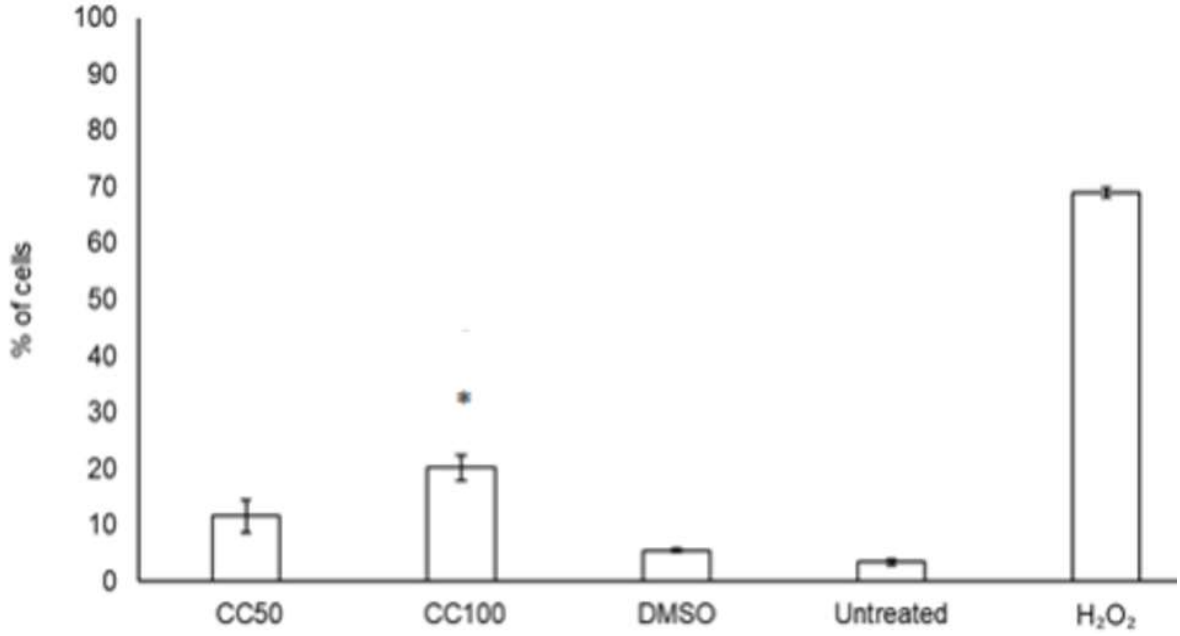

Figure 6. Activation caspase-3 was observed in HL 60 cells after treatment with 1 μM of the LMDF5-7 compound for 8 hours.
Note: It should read CC_{50}, and CC_{100}.

In **Figure 6**, we observed caspase-3 activation after 8 hours of incubation with the CC_{100} in HL60 cells. Demonstrating apoptosis as the mode of action and caspase-3 is essential to the compound's activity against the T-cell leukemia cell line.

DISCUSSION

Cancer has several hallmarks. Nowadays, effective anticancer compounds need to focus on several of these hallmarks to be more efficient. The LMDF5-7 compound has better results with T-cell leukemia and demonstrates anticancer properties. Our goal was to analyze cytotoxicity and if the compound induces the apoptosis pathway.

The first specific aim was to evaluate the cytotoxic effects of LMDF5-7 in various cancer cell lines. The experiment differential nuclear staining (DNS) was able to detect the concentration values of the compound required to reduce cell viability by 50% and detect the selectivity in cancer cells compared to non-cancerous cell lines. In the early discovery process, we must calculate the CC_{50} to evaluate the compound's suitability and performance[44]. We demonstrated leukemia, especially the CEM cell line, has better results after treatment with LMDF5-7. Then, to continue with the cytotoxic effects, we examined the cell cycle and its major phases: G0/G1, S, and G2/M, including Sub-G0/G1. Apoptotic cells often have fractional DNA content represented on the histograms by the sub-G0/G1[45]. Our **Figures 1 and 2** demonstrate DNA fragmentation in the Sub-G0/G1, meaning the compound is inducing cell death/damage. Also, disruption continues in the other stages and especially in **Figure 2,** the G0-G1 phase. The G1 phase plays an important role in cancer since it is in charge of signaling, cell growth, proliferation, stress management, and survival. It is also crucial in helping to define how cancer works and shows promising results in cancer therapies[46]. The last assay for the first specific aim to evaluate cytotoxic effects, was reactive oxygen species. The excess of ROS production is responsible for damages in DNA, proteins, and lipids and may have cell dysfunctions that result in apoptosis[29]. The overproduction of oxidative stress causes mitochondrial membrane depolarization, which initiates the activation of the intrinsic apoptotic pathway and impairing

protease activity[47]. The results show ROS overproduction in the CEM cell line. In summary, for our first specific aim, compound LMDF5-7 is cytotoxic to T-cell leukemia cell line and interferes with the proliferation, as demonstrated through the cell cycle and ROS.

The second specific aim was to investigate the potential of LMDF5-7 to induce apoptosis in a leukemia cell line. We used the Annexin/PI staining strategy to confirm whether the LMDF5-7 compound was using apoptosis or necrosis as its mechanism to inflict cell death. Our results demonstrated LMDF5-7 induces programmed death in the leukemia cell line. Next, was analyzed the mitochondrial membrane potential by using the JC-1 reagent. We revealed that LMDF5-7 is causing mitochondrial depolarization, indicating the activation of the intrinsic apoptotic pathway preceding membrane damage[48]. Mitochondrial membrane potential is the redox transformations, generators of ATP, metabolites for macromolecules, reactive oxygen species, and as an intermediate form of energy storage[49]. The results obtained reveal that the compound causes depolarization and decreases the mitochondrial membrane potential ($\Delta\Psi m$). For cancer research, an unhealthy mitochondrial is by depolarization that induces cell death[50]. It is concluded that apoptosis is cell-death programmed that is mediated by proteases called caspases and by proteins of the Bcl2 associate with the mitochondrial outer membrane[51]. The caspase-3 assay was done to conclude if the compound induces apoptosis. Caspase-3 can have multiple effects critical for cell death and implicating events such as cell shrinkage, blebbing, chromatin condensation, and fragmentation[52]. In cancer, higher levels of activated caspase 3 in tumor tissues lead to increased cell death[53] . Our compound was positive for caspase-3 activation in the leukemia cell line and indicated apoptosis. Moreover, during the cell cycle analysis, we have shown that LMDF5-7 induced DNA fragmentation, evidenced by an increment in the sub-G0/G1 subpopulation.

In summary, the novel compound LMDF5-7 induces cell death in cancer cells. The assays performed were phosphatidylserine externalization, mitochondrial depolarization, caspase-3 activation, DNA fragmentation, and disrupted cell cycle progression to demonstrate the effects of the LMDF5-7 compound.

CONCLUSION

Our data evidenced that LMDF5-7 is a novel compound that targets cancer cells, especially leukemia. It is cytotoxic and selective against cancer cells, and non-cancerous cells are more resistant to the LMDF5-7 compound. It interferes with the activity of the cell cycle and causes DNA fragmentation. In addition, LMDF5-7 causes accumulation of ROS, indicating the possibility of oxidative stress-induced cell death. Apoptosis was detected as the mode of action of LMDF5-7. It damages the mitochondrial membrane integrity by depolarization and activates Capsase-3 activation causing cell death/apoptosis.

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VITA

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