STUDY OF THE INTEGRATED STRESS RESPONSE PATHWAY INDUCED BY FLUORIZOLINE IN LEUKEMIA PRIMARY CELLS

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FINAL DEGREE PROJECT

BACHELOR'S DEGREE IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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This project is based on the results obtained in the external practicals carried out in Apoptosis and cancer research group at Department of Physiological Sciences from Faculty of Medicine and Health Science from University of Barcelona, authorized by Dr. Joan Gil Santano and Dr. Ana Mª Cosialls.

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To my friends, for a lifetime of friendship and for being my biggest support.

INDEX

ABBREVIATIONS

ABB: Annexin Binding Buffer

AML: Acute myeloid leukemia

APC: Allophycocyanin

ATF3: Activating transcription factor 3

ATF4: Activating transcription factor 4

BCL-2: B-cell lymphoma 2.

BID: BH3-interacting domain death agonist

BH: BCL-2 homology

BM: Bone marrow

CHOP: C/EBP homologous protein

CLL: Chronic lymphocytic leukemia

DISC: Death-inducing signaling complex

eIF2α: α subunit of the eukaryotic translation initiation factor 2

FADD: Fas-associated death domain

IMM: inner mitochondrial membrane

ISR: Integrated Stress Response

ISRIB: Integrated stress response inhibitor

MOM: mitochondrial outer membrane

MOMP: Mitochondrial outer membrane permeabilization

PHB: Prohibitin

PB: Peripheral blood

SEM: Standard error of the mean

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TRAIL: TNF-related apoptosis-inducing ligand.

ABSTRACT

Fluorizoline is a synthetic compound that leads to apoptosis by selectively binding to mitochondrial prohibitin (PHB) complex. The integrated stress response (ISR) is a conserved signalling pathway that responds to stress stimuli promoting cell survival or cell death, depending on the intensity and duration of the stress. Previous studies in cancer cells have shown that fluorizoline cause endoplasmic reticulum (ER) stress, leading to the activation of ISR. In this project, we have studied the ISR pathway induced by fluorizoline in chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) primary cells. Cellular viability and protein expression have been analysed and the obtained results demonstrated that fluorizoline treatment induced apoptosis and eIF2α phosphorylation, indicating that the ISR pathway could be activated by fluorizoline. The results of this preliminary study encourages us to perform more experimentation to better understand the fluorizoline-induced apoptosis mechanism as a treatment of cancer.

INTRODUCTION

According to the World Health Organization, cancer is the leading cause of death in the world, with almost 10 million deaths in 2020. Even though the progress in cancer research of the last decades, tumour therapies are not fully effective, in many cases just temporary, and curative only in a few (1).

Apoptosis and resistance to cell death have been described as one of the six hallmarks of cancer development and progression proposed by Hanahan and Weinberg in 2000 (2). Moreover, the generation of tumour resistance to treatment emerges, in most cases, from alterations in the apoptotic machinery. Therefore, apoptosis could be a promising target for anticancer therapy.

1. LEUKEMIA

Cancer is a generic term that englobes a variety of diseases that affect any part of the body. All cancer types have a common characteristic: the uncontrolled accumulation of abnormal cells (2).

Leukemia englobes a group of malignant disorders characterized by an increased number of abnormal leukocytes in the blood, the bone marrow and/or the lymphatic system (Figure 1). Leukemia represents 3% of all cancers, with 270.000 new cases diagnosed worldwide in 2020 (3).

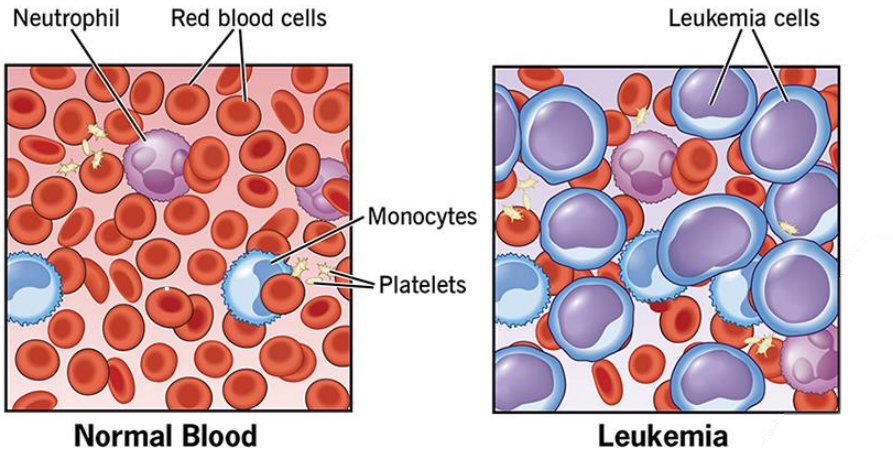

Figure 1. Comparison of the blood cell population in normal conditions and in leukemia disease. Adapted from Leukemia: Symptoms, Types, Causes & Treatments. Cleveland Clinic (2022). (4)

Leukemia can be classified according to the speed of disease development as acute (fast disease progress) or chronic (slower growing) and depending on the cell lineage, whether they start in myeloid cells or lymphoid cells (Figure 2) (5).

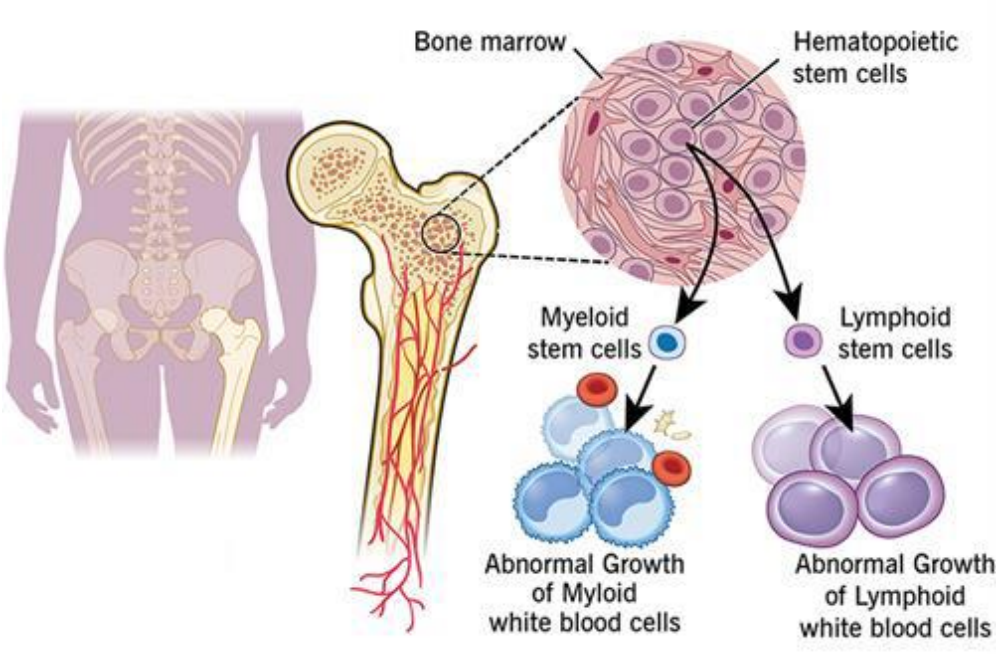

Figure 2. Types of leukemia depending on the cell type. Adapted from Leukemia: Symptoms, Types, Causes & Treatments. Cleveland Clinic (2022) (4).

1.1. Acute myeloid leukaemia (AML)

Acute myeloid leukaemia (AML) is a disorder of the hematopoietic stem cells caused by genetic alterations in myeloid precursors (myeloblasts). As a result, there is a clonal expansion of undifferentiated myeloblasts and an abnormal accumulation of these cells, most commonly in the bone marrow (BM) (6).

AML is the most common acute leukemia among the adult population, representing a 40% of all adult leukemia cases. The median age of diagnosis is 68 years, and the incidence increases with age (7,8). According to Carreras Foundation, about 15 new cases per million inhabitants are diagnosed every year in Spain (9).

AML is diagnosed by a morphological, immunophenotypic, cytogenetic and molecular analysis of peripheral blood (PB) and bone marrow (BM). The presence of at least 20% blasts in PB or BM (the blast count in normal bone marrow is 5% or less, while in blood is almost negligible) is diagnostic of AML. Specific diagnosis is confirmed by immunophenotyping surface type molecules like CD123, CD33, CD34, CD13 and CD117, among others (10). Moreover, AML is characterized by mutations in different genes involved in hematopoiesis as well as genomic aberrations. That allows the prognosis categorization into favourable risk, intermediate risk or adverse risk (8).

The standard treatment is based on polychemotherapy, which involves initial induction therapy and post-remission therapy. Hematopoietic stem cell transplantation is also a therapeutic strategy for young patients (11). Between 20% to 40% of patients fail to

achieve complete remission with induction chemotherapy, and between 50% and 70% of patients who achieve complete remission relapse within the first 3 years (12).

Despite all the advances in understanding the pathogenesis of AML and the new therapies in development, the treatment of this pathology has remained practically unchanged during the last 30 years. Therefore, it is necessary to identify new molecules that overcome the cellular resistance mechanisms to improve the overall survival of patients with AML.

1.2. Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder of monoclonal B cells ($CD5^+$, $CD19^+$, $CD20^+$ and $CD23^+$) that accumulate in the blood, bone marrow, lymph nodes, and other lymphoid tissues (13). As a result, CLL cells can represent up to 99% of circulating mononuclear cells in peripheral blood. CLL cells are morphologically mature but functionally immature, leading to a deficiency in the production of antibodies and favouring the appearance of infectious and autoimmune diseases.

CLL is the most common adult leukemia in west countries and represents 20-40% of all leukemia and 1.5% of all cancers. It is more common in men than in women, with a medium age of 70 years (14). According to the Carreras Foundation, about 30 new cases per million inhabitants are diagnosed every year in Spain (15).

CLL has been categorized into two prognostic groups based on the mutation of the *IGHV* gene. Moreover, the presence of some genomic aberrations such as deletions in 17p or 11q chromosomes are related to bad prognosis. Recent studies have focused on identifying mutated genes with important roles in the development of this leukemia, such as *SF3B1* (15%), *TP53* (13%), *ATM* (8%) and *MYD88* (8%) (16).

Treatment of CLL has experienced an important evolution during the last decade: new kinase inhibitors such as BTK inhibitor ibrutinib or PI3K inhibitor idelalisib, and BH3-mimetics such as venetoclax are now the standard front-line therapy for CLL (17). However, resistance to these drugs as well as relapse after treatment have appeared, suggesting that new drugs with novel mechanisms are needed.

2. APOPTOSIS

Apoptosis is a physiological highly conserved process of regulated cell death, required during embryogenesis, development of the immune system and tissue homeostasis, where defective, infected or unwanted cells are eliminated for the benefit of the whole tissue or organism (18).

The deregulation of apoptosis, either by excess or by defect, leads to severe clinical implications. On the one hand, the increased apoptosis may contribute to the development of immunodeficiency or neurodegenerative disorders, such Alzheimer's or Parkinson's diseases. On the other hand, uncontrolled cell proliferation would promote autoimmunity and tumorigenesis, as well as cell resistance to anti-cancer agents (Figure 3) (19).

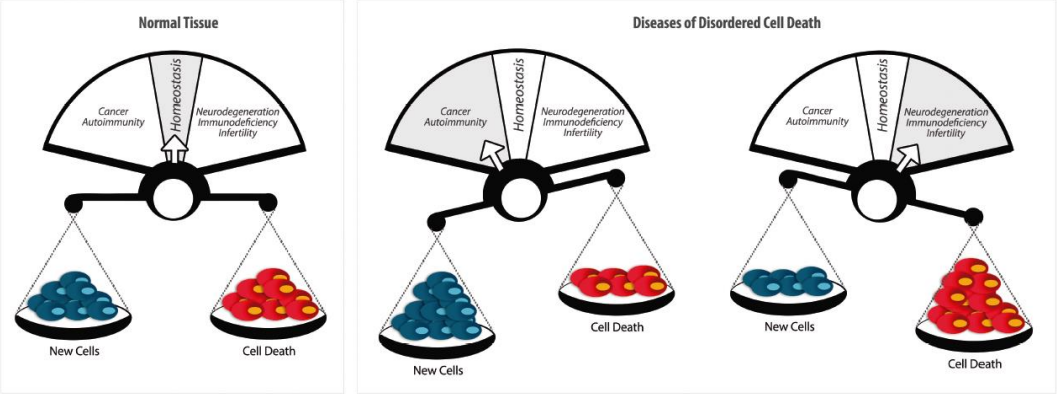

Figure 3. Apoptosis balance between proliferation and cell death to maintain tissue homeostasis. Adapted from Walensky, 2006 and modified in Pérez-Perarnau, 2013. (20)

Apoptosis is mediated by the activation of caspases, a class of cysteine proteins that cleave proteins responsible of cell death. The four initiator caspases-2, -8, -9 and -10 amplify the apoptotic signal by activating the effector or executioner caspases-3, -6 and -7 (18).

The activation of the effector caspases leads to the selective cleavage of cellular components such as cytoskeletal and nuclear proteins. As a result of the caspase enzymatic activity, cells undergo some morphological changes.

Apoptotic cells are characterized by cytoplasm shrinking, chromatin condensation, membrane exposition of phosphatidylserine (PS) on the extracellular side, and DNA fragmentation. In the latest stage of apoptosis, there is a modification of the cytoplasmic organelles and a loss of membrane integrity. The cell disassembles into apoptotic bodies, which are rapidly phagocytosed by the neighboring cells, where they will become degraded (Figure 4) (21,22).

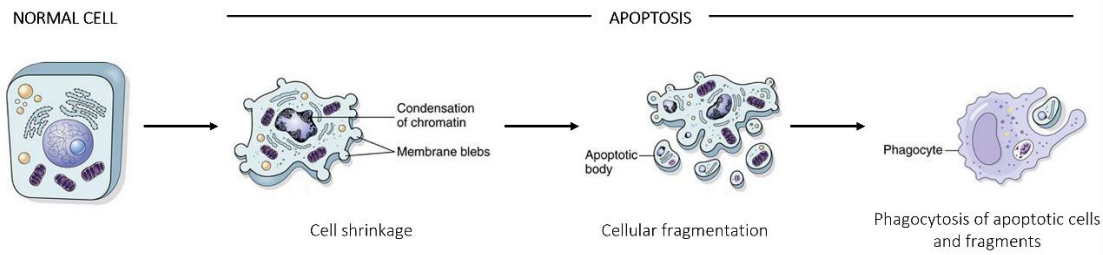

Figure 4. Morphological features of apoptosis. Apoptosis is characterized by the chromatin condensation, membrane blebbing and PS externalization, cell fragmentation and degradation. Adapted from Kumar V et al.: Robbins and Cotran pathologic basis of disease (2010).

2.1. Apoptotic pathways

Apoptosis is a regulated process that can take place through two different pathways, depending on the origin of the apoptotic signal.

2.1.1. Intrinsic pathway

The intrinsic pathway responds to intracellular signals and depends on the permeabilization of the outer mitochondrial membrane (OMM). In normal conditions, the OMM is permeable to molecules with a molecular weight of up to 5 kDa. In response to several pro-apoptotic signals, pores formed in the membrane allow the release of caspase activating factors from the mitochondria, initiating an escalating caspase cascade that commit the cells to die (23).

This pathway can be triggered by different stimuli, such as cells with damaged DNA or upregulated oncogenes, as well as growth factor deprivation, excessive Ca^{2+}, DNA-damaging molecules and oxidants (22).

The intrinsic pathway is regulated by B-cell lymphoma-2 (BCL-2) protein family. This family regulates both pro-apoptotic (BH3-only proteins) and anti-apoptotic (BCL-2 proteins) intrinsic pathways controlling the alteration in the permeabilization of the mitochondria. In response to pro-apoptotic stimuli, BH3-only proteins activate BAX and BAK, which oligomerize leading to mitochondrial outer membrane permeabilization (MOMP) (23). Apoptogenic factors, such as cytochrome *c*, disperse into the cytosol and activate mitochondria-dependent cell death. Cytochrome *c* binds to the apoptosis protease activating factor-1 (Apaf-1) and triggers the formation of the apoptosome complex. Apoptosome recruits the initiator pro-caspase-9, where it is activated and proteolyzed. Then, executor caspase-3, -6 and -7 are activated for cleavage of cellular substrates, ending to the apoptotic cell death (18).

2.1.2. Extrinsic pathway

The extrinsic pathway is activated by the binding of extracellular ligands to tumor necrosis factor (TNF) family death receptors integrated to the plasma membrane. Some death ligands include Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), tumor necrosis factor (TNF) (22). Death receptors recruit adaptor proteins, such as Fas-associated death domain (FADD), where initiator procaspases-8 and -10 bind, forming the death-inducing signaling complex (DISC) (18). This results in the activation of the initiator caspases-8 and -10, which activate the effector caspases-3, -6 and -7. The executioner caspases cleave proteins and cytoskeleton, leading to cell death.

Some cells do not die in response only to the extrinsic pathway and require an amplification step that converges into the intrinsic pathway. In this alternative situation, the caspase-8 targets BH3-interacting domain death agonist (BID),

generating the activated fragment tBID, which activates pro-apoptotic proteins responsible of MOMP. This point is where the intrinsic pathway continues (22).

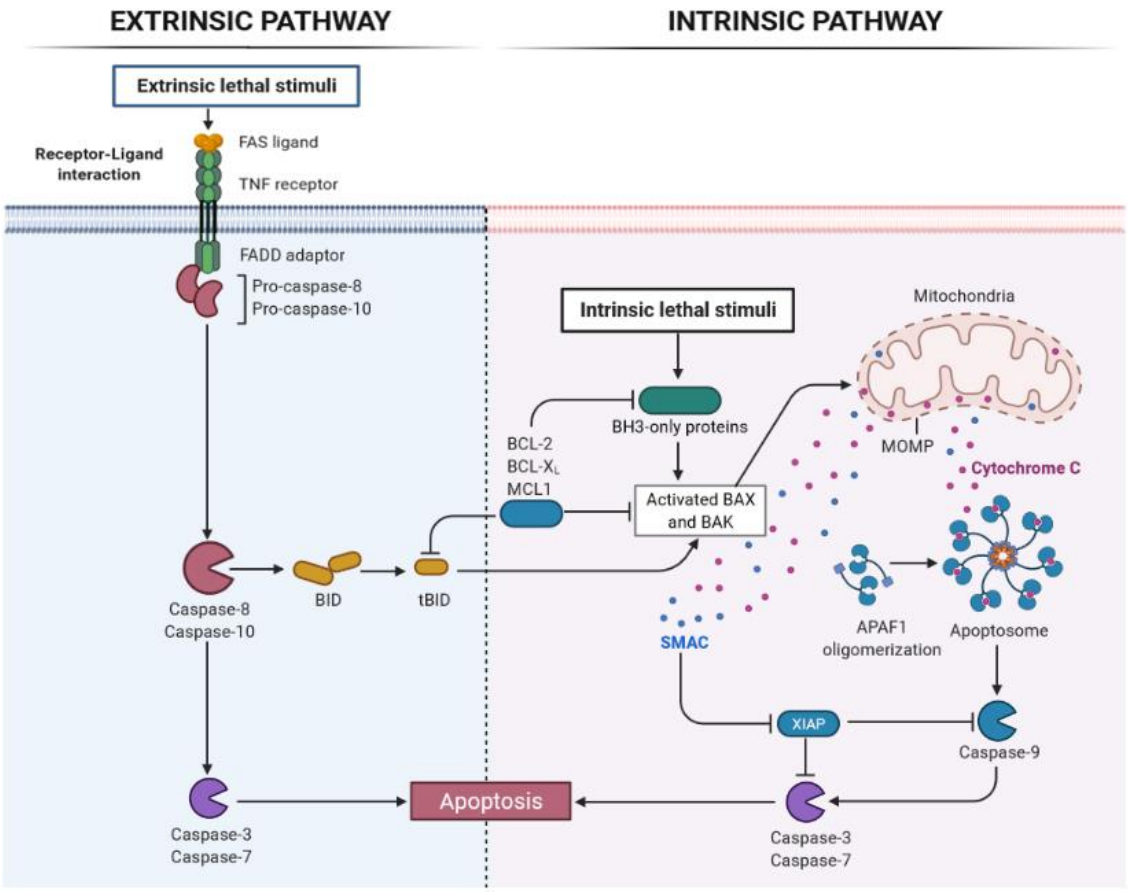

Figure 5. Extrinsic and intrinsic pathways of apoptosis. Apoptosis can be triggered by both pathways, depending on the stimuli received. They both converge in the activation of BH3-only proteins: BAX and BAK. Adapted from BioRender.

3. INTEGRATED STRESS RESPONSE (ISR)

The integrated stress response (ISR) is an evolutionary conserved signalling pathway that responds to different extracellular and intracellular stresses. The central event of this molecular pathway is the phosphorylation at Serine 51 of eukaryotic translation initiation factor (eIF) 2α, which leads to a reduction in protein translation (24).

The activation of eIF2α is mediated by four different kinases: PERK, PKR, HRI and GCN2. Each kinase is activated by different environmental and physiological stresses (ER stress, viral infection, heme deprivation and aminoacid depletion, respectively) (25). eIF2α phosphorylation causes a reduction of protein synthesis and activates the translation of specific mRNAs, including the activating transcription factor 4 (ATF4), to help the cell survival (Figure 6) (24).

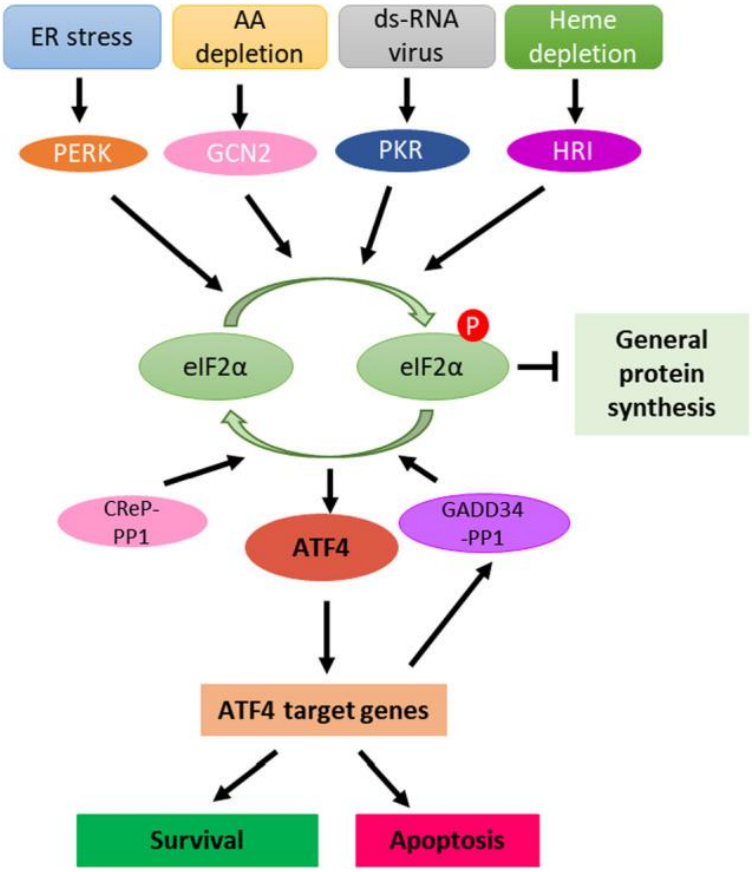

Figure 6. The Integrated Stress Response. The four kinases PERK, GCN2, PKR and HRI, mediate the ISR, which involves general protein synthesis inhibition and upregulation of ATF4 via phosphorylation of eIF2α. Adapted from Xiaobing Tian and Shengliang Zhang, 2021. (27)

ISR has a dual role depending on the intensity and the duration of the stress. In one hand, if the stress is moderate, the ISR acts as a pro-survival mechanism trying to alleviate and deal with the stress by blocking protein translation, which is beneficial in conditions of ER stress, viral infection or amino acid deprivation (24). On the other hand, if the stress is intense or prolongated and the cell is unable to restore its homeostasis despite the activation of stress response pathways, proapoptotic mechanisms can be activated. ISR is mainly regulated by the protein mediators ATF4, ATF3 and CHOP *(C/EBP-homologous protein)* (26).

ATF4 is a transcription factor that belongs to the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB family). ATF4 plays an important role in the communication of pro-survival and pro-apoptotic signals. It regulates transcriptional programs involved in cell survival, senescence and apoptosis, depending on the cell type, the kind of stress and its duration. The interactions of ATF4 with other transcription factors influence the outcome of ISR signalling: interactions with ATF3 enhance cellular efforts to re-establish homeostasis, while interactions with CHOP promote cell death upon ER stress (24,27).

ATF4 can also induce the transcriptional activation of the BH3-only gene *NOXA* (*Phorbol-12-myristate-13-acetate-induced protein 1*), either alone or by interaction with ATF3. CHOP induce the expression of apoptotic BH3-only proteins including PUMA (p53 upregulated modulator of apoptosis), NOXA and BIM (Bcl-2 interacting mediator of cell death), leading to cell death (Figure 7) (27).

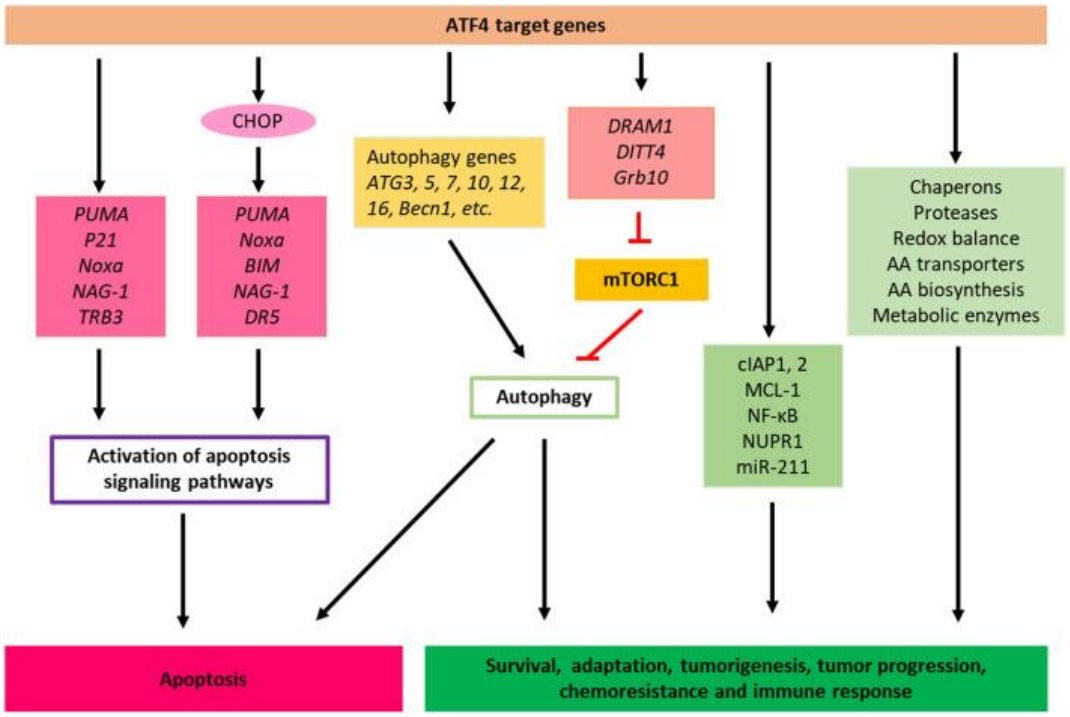

Figure 7. Final pathways of the ISR. ATF4 controls the activation of apoptosis, survival or tumour progression by regulating the transcription of a wide variety of genes. Adapted from Xiaobing Tian and Shengliang Zh, 2021. (27)

4. FLUORIZOLINE

Fluorizoline (Figure 8) is a novel pro-apoptotic compound that was first synthesized and described by the "*Apoptosi i càncer"* group at the IDIBELL-Universitat de Barcelona in collaboration with Dr. Fernando Albericio from Institut de Recerca Biomèdica de Barcelona and Dr. Rodolfo Lavilla from the Faculty of Pharmacy at the University of Barcelona.

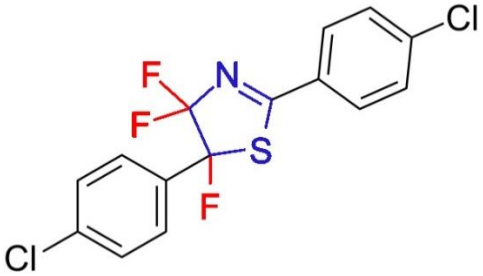

Figure 8. Fluorizoline chemical structure. Fluorizoline is composed by a trifluorated thiazoline. Adapted from Fluorizoline. Apoptosis Activator. MedChemExpress. (28)

Fluorizoline presents a fluorinated thiazoline scaffold and is able to induce apoptosis in a wide variety of cancer cell lines from different origins. The compound is quickly internalized and located in the mitochondria, where induces the intrinsic pathway of apoptosis (29).

Fluorizoline specifically binds to Prohibitin 1 (PHB1) and Prohibitin 2 (PHB2) (30,31), two conserved proteins located in the mitochondria, plasma membrane and nucleus that regulate a wide variety of cellular processes (Figure 9). In the mitochondria, PHB has been shown to be important in the maintenance of the cristae morphology. Importantly, PHB is essential for the apoptotic effect of fluorizoline (32,33).

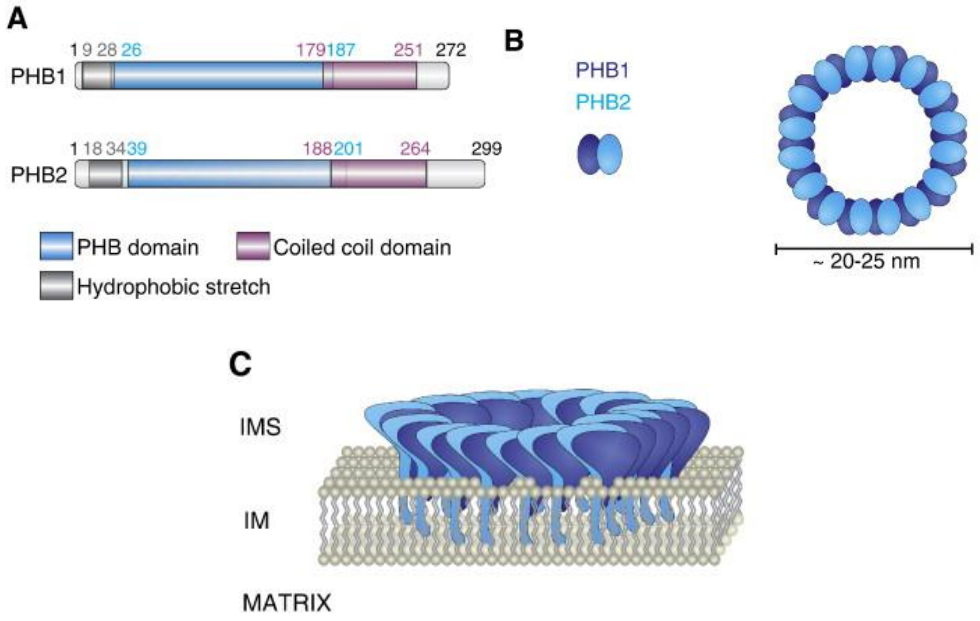

Figure 9. Schematic representation of PHB1 and PHB2 structure and prohibitin complex in mitochondria. (A) Domain structure of PHB. **(B)** PHB1 and PHB2 dimers forming the ring complex. **(C)** Suggested model of the PHB complex bound to the mitochondrial membrane. Adapted from Merkwirth and Langer, 2009. (34)

Recent studies with cell lines (HEK293T, U2OS and Jurkat cells) have shown that fluorizoline triggers ER stress, leading to activation of ISR and increases of ATF3, ATF4 and CHOP, involved in pro-survival response to this compound. In consequence, downregulation of ATF3 and ATF4 or inhibition of ISR results in increased resistance to fluorizoline-induced apoptosis (29).

However, studies with HeLa and HAP1 cells have shown that induction of ATF3 and ATF4 and activation of ISR due to fluorizoline treatment, resulted in apoptosis. ATF3 and ATF4 transcription factors bind to *NOXA* promoter, regulating its transcription and mediating apoptosis (30).

In primary cells of AML, CLL and multiple myeloma (MM) fluorizoline induces apoptosis and the BH3-only protein NOXA is involved in this process (35,36).

For all of these, it might be interesting to study whether fluorizoline treatment results in the activation of the ISR, and the involvement of this pathway in fluorizoline-induced apoptosis in primary cells. To this end, we propose to study the effect of fluorizoline on the cellular viability of primary cells obtained from CLL and AML patients. Besides, we will focus our analysis on the different proteins that integrate the ISR pathway, and how they are modulated after the drug treatment.

HYPOTESIS AND OBJECTIVES

The hypothesis of the project is that fluorizoline, a prohibitin-binding compound, activates the Integrated stress response (IRS) pathway, leading to apoptosis in primary cells.

The aim of this project is to investigate the mechanism of activation of the ISR caused by fluorizoline treatment in acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) primary cells. To this end, the main objective was divided into the following specific points:

1. Analyse the effect of fluorizoline treatment in cellular viability.
2. Study of the involvement of ATF3, ATF4, NOXA and eIF2α in fluorizoline-induced apoptosis in AML and CLL primary cells.
3. Analyse the effect of the inhibitor of the IRS pathway on the viability of cells and on the expression of the ISR proteins after treatment with fluorizoline

In order to accomplish the planned objectives, CLL and AML samples from patients will be processed, and the primary cells obtained will be cultivated and treated with fluorizoline. Then, the expression of the proteins of interest will be examined at different times. Finally, flux cytometry will be performed in order to obtain a quantification of the cellular viability.

MATERIALS AND METHODS

1. PRIMARY SAMPLES AND MONONUCLEAR CELLS ISOLATION

Peripheral blood (PB) samples and bone marrow (BM) aspirates from untreated patients were obtained after informed consent in accordance with protocols approved by the Human Research Ethics Committees of the Hospital ICO-Duran I Reynals, L'Hospitalet de Llobregat, Spain. Patients with chronic lymphocytic leukaemia and acute myeloid leukaemia were diagnosed according to standard clinical and laboratory criteria.

PB and BM mononuclear cells were isolated by centrifugation on a Biocoll isotonic separation solution (Biochrom AG, Berlin, Germany). PB and BM was diluted in phosphate buffered saline (PBS) to obtain a final volume of 8 mL. This dilution was slowly added on 4 mL of Biocoll solution, keeping the interphase. The preparation was centrifuged 20 minutes at 850 g. The deacceleration of the centrifuge was low, in order to not disturb the gradient.

In the resultant solution, polymorphonuclear cells and erythrocytes (high density) were located in the bottom of the tube. Above the Biocoll solution there were the mononuclear cells, forming a ring. Finally, the top of the solution was plasma and platelets diluted in PBS (Figure 10).

The ring of mononuclear cells was picked with a glass *pasteur* pipette and diluted in 30 mL of PBS. Cell count was performed using the Neubauer chamber. The solution was centrifuged 10 minutes at 480 g, and pellet was cryopreserved.

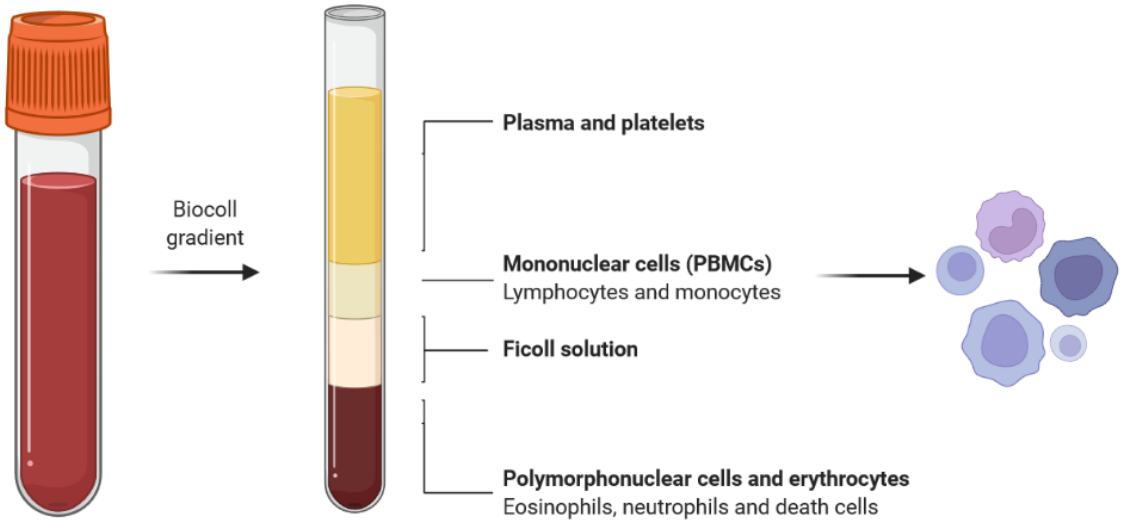

Figure 10. Diagram of the isolation of mononuclear cells. Mononuclear cells are located above the Biocoll solution, forming a ring. Adapted from BioRender.

2. FREEZING AND THAWING OF CELLS

Cryopreservation in liquid nitrogen (-196°C) allows long-term conservation of cells for later use as their characteristics and viability are maintained. The cryopreservation procedure was performed in cold DMSO as a membrane stabilizer.

2.1. Freezing

Cells were frozen in tubes at 20-30.0x10^6 cells/1.5 mL each tube. Cells were resuspended in 0.75 mL inactivated Fetal Bovine Serum (iFBS) (previously inactivated by heating at 60°C during 1 hour to avoid the activation of the complement proteins) and placed into ice. Next, 0.75 mL iFBS with 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich Inc, St Louis, MO, USA) were added dropwise into the resuspended cells, achieving a final concentration of 10% DMSO. Cryotubes were kept for 12-24 hours at -80 °C for a gradual decrease of temperature before stored into the liquid nitrogen tanks.

2.2. Thawing

Cryotubes were placed into a 37 °C bath, and before complete fusion of the medium, the tube content was poured on 30 mL of Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 20% iFBS to dilute DMSO concentration. Cells were centrifuged to 480 g for 5 minutes and the pellet of cells was resuspended in complete culture medium, and then cells were seeded into culture plates.

3. CELL CULTURE

Mononuclear cells were cultured immediately after thawing or isolation at a concentration of 1x10^6 cells/mL in RPMI 1640 culture medium supplemented with 10% iFBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 ng/mL streptomycin (all from Biological Industries, Kibbutz Beit HaEmek, Israel). All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

Manipulation of the cell lines was carried out in a Class II biological safety cabin.

4. PROTEIN EXTRACTION AND WESTERN BLOT

4.1. Total protein extraction

Cells were collected and centrifuged at 480 g for 5 minutes. Cellular pellets were washed with 1 mL of PBS and centrifuged at 480 g for 2 minutes.

Then, cells were lysed with reducing Laemmli Sample Buffer (LSB) at 95 °C. LSB contains 2% of sodium dodecyl sulphate (SDS), which ensures the complete lysis of the cell, including both plasma and nuclear membranes. Complete cell lysis was checked by centrifugation at top speed for 2 minutes. Protein lysates were frozen at -20 °C for later use.

4.2. Protein quantification

The total amount of protein in the samples was quantified with the Micro BCA Protein Assay Reagent kit (Thermo Scientific, Pierce, Rockford, IL, USA). in order to load equal amounts of protein in each condition.

Bovine serum albumin (BSA) was used as the protein standard and, after an incubation of 30 minutes at 60 °C, absorbance was measured in a plate reader at 550 nm.

4.3. Protein analysis by western blot

Western blot analysis allows us to measure protein modulations between samples and treatments mediating through the separation of proteins by molecular weight.

Protein samples (15-30 µg) were reduced by the addition of dithiothreitol (DTT) and then loaded into a polyacrylamide gel, and a 120-mV current was applied. The transfer was performed using Immobilion-P membranes (Milliopore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween® 20 and then incubated with the specific primary antibodies overnight at 4 °C. Then, primary antibodies were removed, and membranes were washed with TBS-Tween and then incubated with secondary antibodies conjugated with the horseradish peroxidase system (from GE Healthcare, Amersham, UK). Antibody binding was detected using enhanced chemiluminescence (ECL) detection system (GE Healthcare).

The primary antibodies used are listed in Table 1.

The chemiluminescence was detected in an Amersham Imager 680.

Table 1. Primary antibodies used in Western Blot and their characteristics.

PRIMARY ANTIBODY	CONCENTRATION	SOURCE	REFERENCE	COMPANY
p-eIF2α (Ser51)	1:1000	Rabbit	3398	Cell Signaling
eIF2α	1:3000	Rabbit	9722	Cell Signaling
ATF3	1:1000	Rabbit	sc-188	Santa Cruz
ATF4	1:1000	Rabbit	sc-200	Santa Cruz
CHOP	1:1000	Mouse	2895	Cell Signaling
NOXA	1:1000	Mouse	14766	Cell Signaling
BCL-2	1:1000	Mouse	M088729-2	Agilent Dako
β-actin	1:1000	Mouse	251 011	Sigma
α-tubulin	1:1000	Mouse	CP06	Calbiochem

5. FLOW CYTOMETRY

5.1. Cell viability analysis

Cell viability was measured by the exposure of phosphatidylserine and expressed as the percentage of annexin V negative cell population.

After incubation of $1.25x10^5$ cells with the indicated treatment and times, cells were washed in 1mL of annexin-binding buffer (ABB), and centrifuged 10 minutes at 480 g. The obtained pellet was resuspended in the corresponding stanning solutions.

AML samples were incubated in 50 µL ABB containing 0.5µL APC-Alexa Fluor 750 anti-human CD33 (Beckman Coulter, Marseille, France) or APC-Cy7 anti-human CD34 (Sony Biotechnology Inc., Champaing, IL, USA) for 10 minutes in the dark. Thereafter, samples were stained with 0.5 µL Annexin V-APC (eBiosciences, San Diego, CA, USA) incubated in 100 µL ABB, for 10 minutes in the dark (Figure 11).

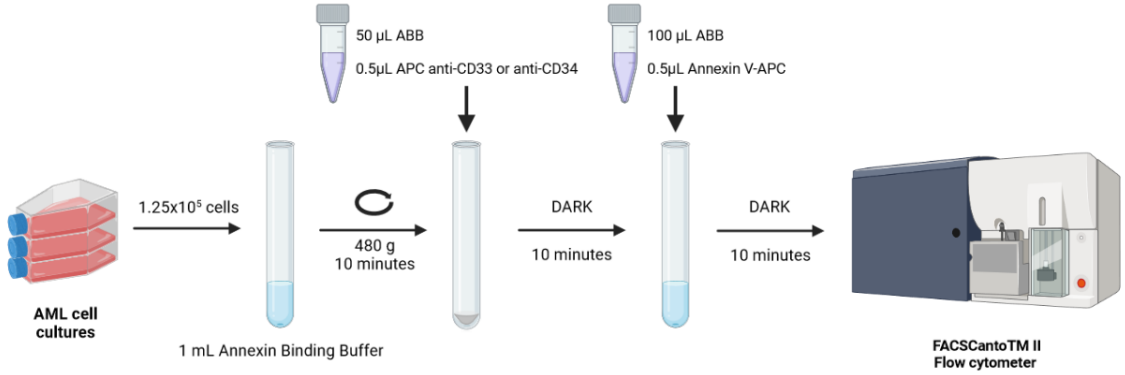

Figure 11. Diagram of AML samples preparation to cell viability analysis. Adapted from BioRender.

CLL samples were incubated in 50 µL ABB containing 0.5 µL Allophycocyanin (APC)-H7 against human CD19 (BD Biosciences, Franklin Lakes, NJ, USA) and 0.5 µL Peridinin Chlorophyll (PerCP)-labeled antibody against human CD3 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), for 10 minutes in the dark. Then samples were stained with 0.5 µL Annexin V-APC, also incubated in 100 µL ABB, for 10 minutes in the dark (Figure 12).

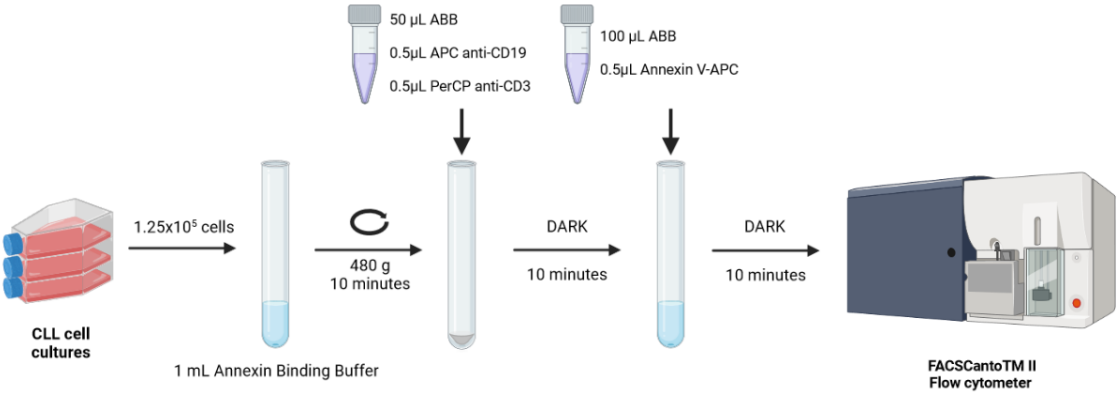

Figure 12. Diagram of CLL samples preparation to cell viability analysis. Adapted from BioRender.

Finally, cells were acquired using FACSCanto™ II flow cytometer and analyed using the FACSDiva™ software (Becton Dickinson, Franklin Lakes, NJ, USA).

RESULTS

1. PATIENT SAMPLES CHARACTERISTICS

This study was performed with bone marrow aspirates from AML patients and peripheral blood samples from CLL patients. The characteristics of the samples are summarized in Table 2 and Table 3.

Table 2. AML patients' characteristics.

Sample No	Blasts in BM (%)	CD33 expression (%)	CD34 expression (%)
1	60	95	0.2 (negative)
2	49	90	61 (positive)
3	86	98	3 (negative)

Table 3. CLL patients' characteristics.

Sample No	Leukocytes in PB (x10^6/mL)	Lymphocytes in PB (%)	$CD19^+$ (%)
1	46.04	92	85
2	245.18	93	91
3	26.35	76.8	80.3
4	197	45	77

2. STUDY OF THE INDUCTION OF THE ISR IN PRIMARY SAMPLES OF AML TREATED WITH FLUORIZOLINE

Previous results from the research group had shown that fluorizoline locates to the mitochondria, inducing mitochondrial fragmentation. Recent studies showed that mitochondrial stress induced by different stimuli triggered the ISR activation. This molecular pathway has as its core event the phosphorylation of eIF2α, which leads to a protein synthesis reduction.

It has been demonstrated that ISR participates in the early protective response against stress. However, if the stimuli are too strong or prolonged, it can lead to the induction of apoptosis. This pathway is characterized by an increase of NOXA expression through ATF3, ATF4, and CHOP expression, leading to the activation of apoptosis.

Considering the previous results in HeLa and HAP1 cells, as well as in HEK293T and U2OS cells, we analysed the status of the ISR after fluorizoline treatment in AML and CLL primary cells.

Isolated mononuclear cells (blasts cells) from 3 untreated AML patients were treated with 5 and 10 µM fluorizoline for different times: 1, 2, 4 and 24 hours. Then, protein expression was analysed by Western blot.

In order to demonstrate that the ISR pathway could be activated in AML cells, we used thapsigargin. Thapsigargin is a cell-permeable sesquiterpene lactone that induces the release of intracellular stored Ca^{2+} by inhibition of the endoplasmic reticulum Ca^{2+}-ATPase.

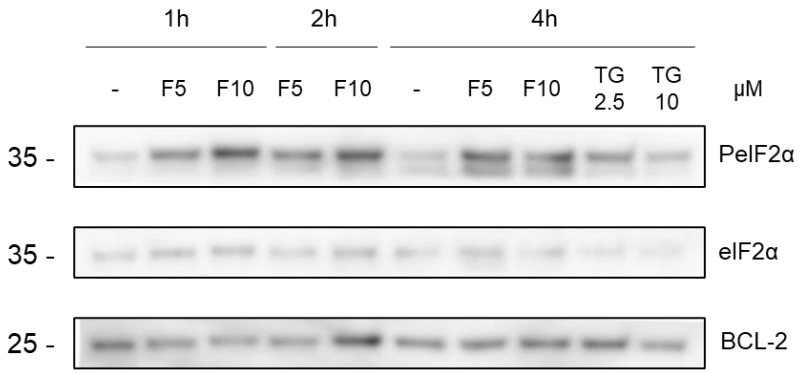

Figure 13. Effect of fluorizoline on eIF2α phosphorylation in a representative AML sample. AML cells (≥90% $CD33^+$) from patient 3 were treated for 1, 2 and 4 h without (-) or with 5 µM or 10 µM Fluorizoline (F) or 2.5 or 10 µM Thapsigargin (TG). Cells were lysed and analysed by Western blot. BCL-2 was used as loading control.The figure shows one representative patient out of 3 analysed.

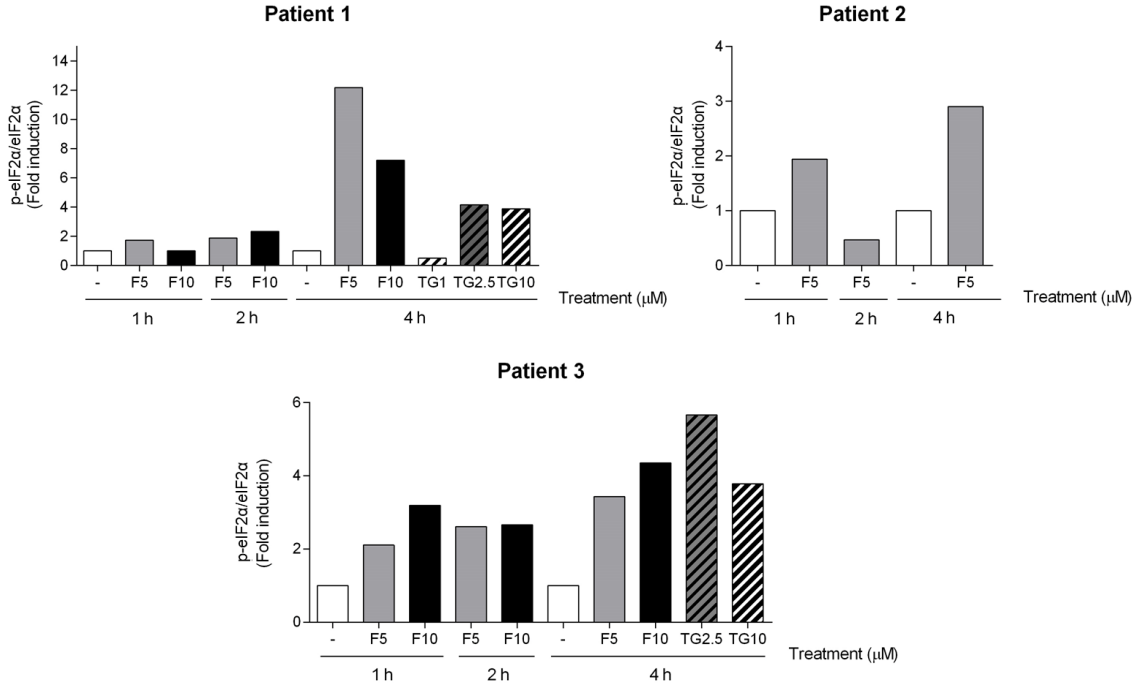

Figure 14. Quantification of relative phosphorylated (p-eIF2α) and total eIF2α band intensity in AML samples (≥90% $CD33^+$) from 3 patients. *AML samples were treated for 1, 2 and 4 hours with 5 µM or 10 µM fluorizoline (F) and 1 µM, 2.5 µM or 10 µM thapsigargin (TG). The results of protein expression are shown as the fold induction in fluorizoline-treated cells relative to untreated cells and referred to the protein expression levels of untreated cells.*

So as to verify the induction of ISR after fluorizoline treatment, the phosphorylation of eIF2α was evaluated by Western Blot. As shown in Figure 13, the phosphorylation of eIF2α showed an increment on its phosphorylation in a time-dependent manner.

The phosphorylation induction was detected from the early one hour of incubation with fluorizoline and was notably induced after four hours of incubation in all patients analysed (Figure 14). Important differences among patients in the degree of induction as well as in the time after treatment to achieve the highest phosphorylation status were found.

Next, we analysed whether eIF2α phosphorylation was accompanied by increases in other proteins related to the ISR. ATF4 is a key transcription factor in the stress response that activates the expression of other proteins such as transcription factor ATF3 and proteins related to apoptosis such as the BH3-only protein NOXA.

As shown in Figure 15, ATF4 and ATF3 do not show variations in the protein expression levels in any of the analysed samples. However, NOXA expression presents a slightly increase after 2 hours of incubation with fluorizoline in all the patients analysed.

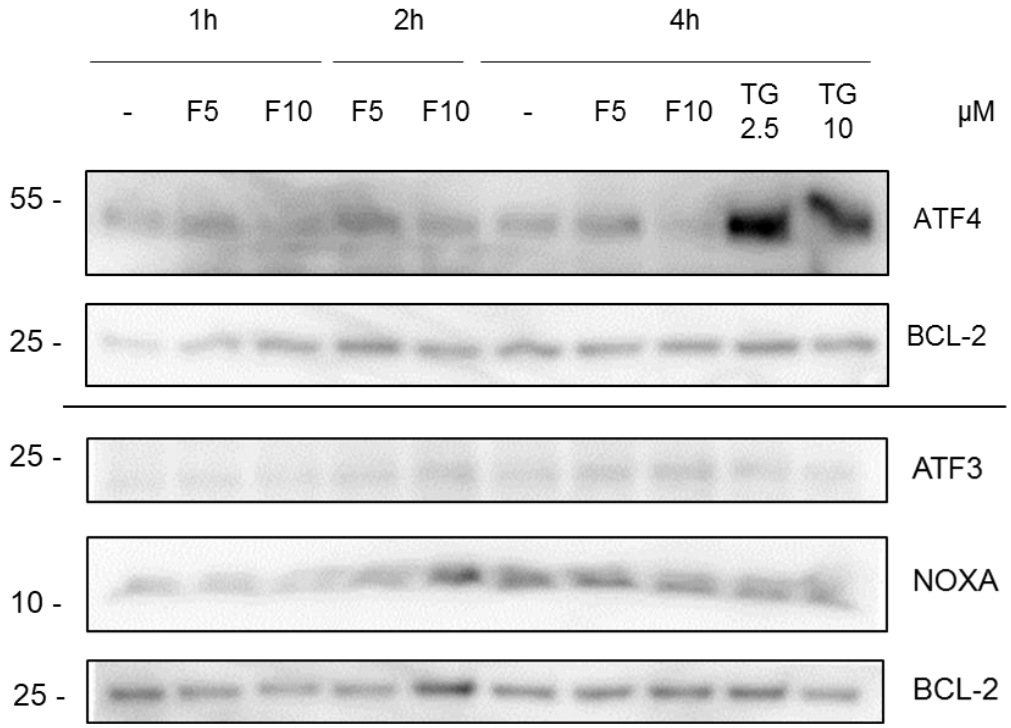

Figure 15. Effect of fluorizoline on ATF4, ATF3 and NOXA proteins in a representative AML sample. AML cells from patient 2 (≥90% $CD33^+$) were treated for 1, 2 and 4 h without (-) or with 5 µM or 10 µM Fluorizoline (F) or 2.5 or 10 µM Thapsigargin (TG). Cells were lysed and analysed by Western blot. BCL-2 was used as loading control. The figure shows one representative patient out of 3 analysed.

3. STUDY OF THE INDUCTION OF THE ISR IN PRIMARY SAMPLES OF CLL TREATED WITH FLUORIZOLINE

Isolated mononuclear cells from 4 untreated CLL patients were incubated with 5 µM and 10 µM fluorizoline for up to 24 hours and cellular viability was analysed by flow cytometry. Fluorizoline treatment induced the externalization of phosphatidylserine, a well-known marker of apoptosis (Figure 16A) and induced 11% and 28% of cell death at 5 and 10 µM, respectively (Figure 16B).

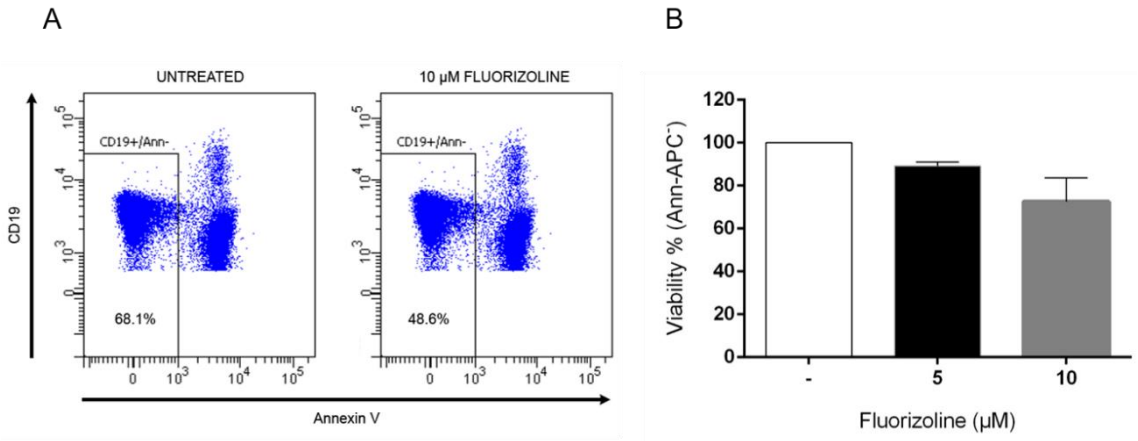

Figure 16. Viability of CLL samples treated with fluorizoline. (A) CLL sample 5 was untreated or treated with 10 µM Fluorizoline for 24 hours and then analysed by flow cytometry using anti-CD19 and Annexin V. Plots showing CD19 and Annexin V fluorescence channels are represented. Untreated cells were 68.1% of $CD19^+/Ann^-$. Cells treated with 10 µM fluorizoline were 48.6% of $CD19^+/Ann^-$. **(B)** The percentage of $CD19^+$ and non-apoptotic cells (annexin V-negative) was measured by flow cytometry and it is expressed as the mean ± SEM (n=4) of the percentage of the value of untreated (-) cells.

Moreover, cells from 3 untreated CLL patients were treated with 5 and 10 µM fluorizoline for different times: 1, 2, 4 and 24 hours. Then, protein expression was analysed by Western blot.

The phosphorylation of eIF2α was detected from the early one hour of fluorizoline incubation (Figure 17A) and was notably induced after four hours of incubation in all the analysed samples. In the case of sample 2, the highest level of induction was reached after 24 hours fluorizoline treatment (Figure 17B). All the results show important differences among patients in the degree of induction and in the time of treatment.

Protein expression of ATF4 and NOXA do not show any induction in response to fluorizoline treatment. ATF3 induction was only detected after two hours of fluorizoline induction (Figure 17A).

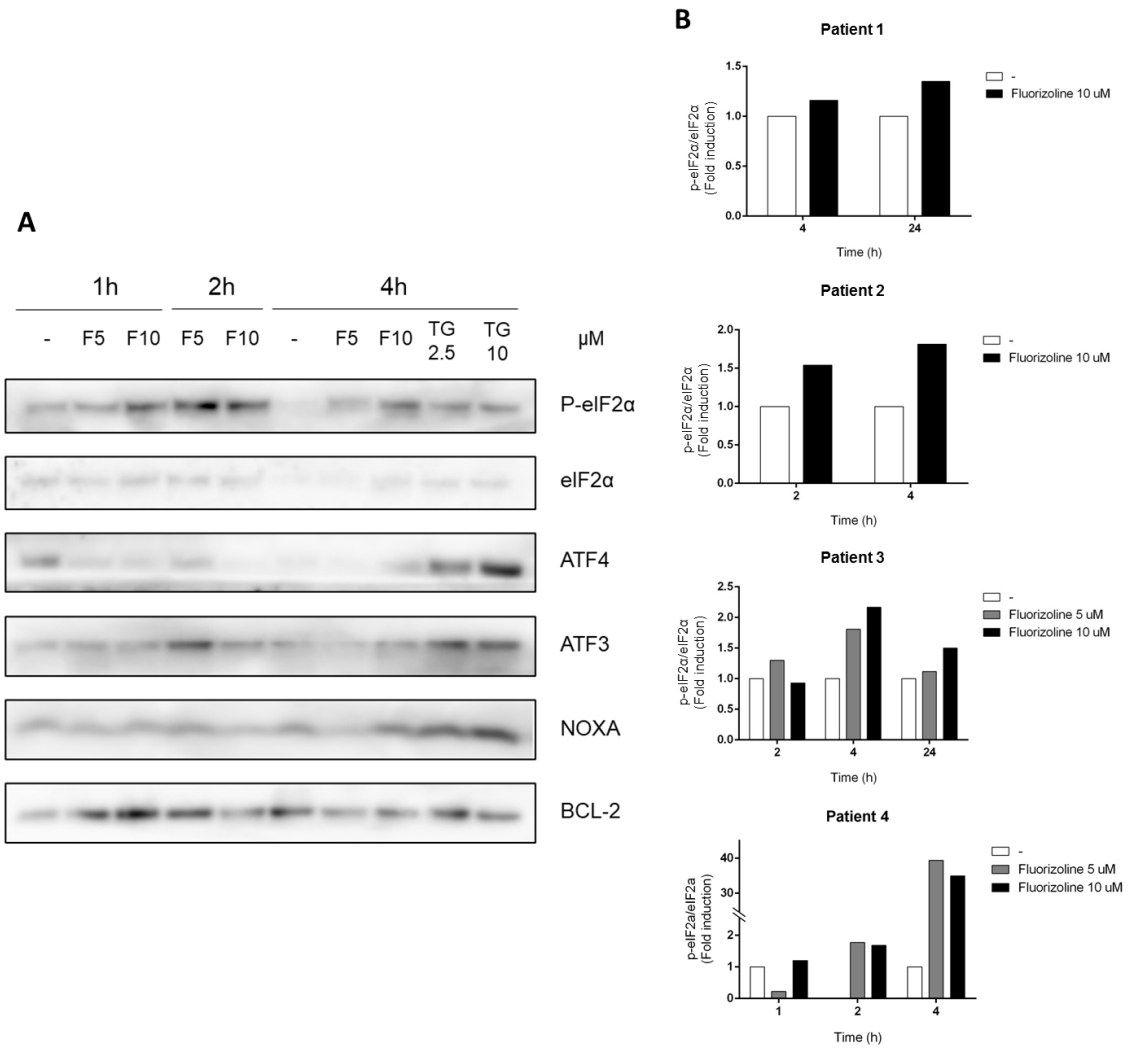

Figure 17. Induction of ISR pathway by fluorizoline in CLL cells. (A) Cells from CLL patient 4 were incubated for 1, 2 and 4 hours without (-) or with 5 µM and 10 µM fluorizoline (F), or 2.5 µM and 10 µM thapsigargin (TG). Cells were lysed and analysed by Western blot. BCL-2 was used for loading normalization. Panel A shows one representative patient out of 4 analysed. **(B)** CLL samples were treated for 1, 2, 4 and 24 hours without (-) or with 5 µM or 10 µM fluorizoline. The results of protein expression are shown as the fold induction in fluorizoline-treated cells relative to untreated cells.

4. STUDY OF THE EFFECT OF THE INHIBITION OF THE PHOSPHORYLATION OF eIF2α ON THE ISR PATHWAY AND ON FLUORIZOLINE-INDUCED APOPTOSIS IN CLL SAMPLES

In order to demonstrate that the protein increments were caused by the phosphorylation of eIF2α, we used the integrated stress response inhibitor (ISRIB). ISRIB is a drug-like molecule that reverses the effects of eIF2 phosphorylation by blocking the activity of eIF2B, a guanine nucleotide exchange factor for eIF2 (37).

We pre-treated cells with ISRIB for 1 hour, and then treated them with fluorizoline. We analysed how ISRIB affected protein modulations by Western blot and cell viability by flow cytometry. The effect of ISRIB on fluorizoline-induced apoptosis has been nearly insignificant. Although it seems to revert the fluorizoline-apoptotic effect in 10 µM fluorizoline treatment, the difference is almost negligible (Figure 18A). However, as expected, pretreatment with ISRIB reversed the phosphorylation of eIF2α (Figure 18B). Nevertheless, this inhibition did not affect the expression levels of the downstream transcription factor ATF3 in the CLL samples analysed (Figure 18C).

The reversion of the phosphorylation of eIF2α by ISRIB treatment was clearly achieved in three samples out of four analysed: in patient 1 reversion was detected both at 4 and 24 hours of incubation with fluorizoline, and in patient 2 and 4 at 24 hours. Unexpectedly, an increase of eIF2α phosphorylation was found in patient 3 after treatment with ISRIB (Figure 18D).

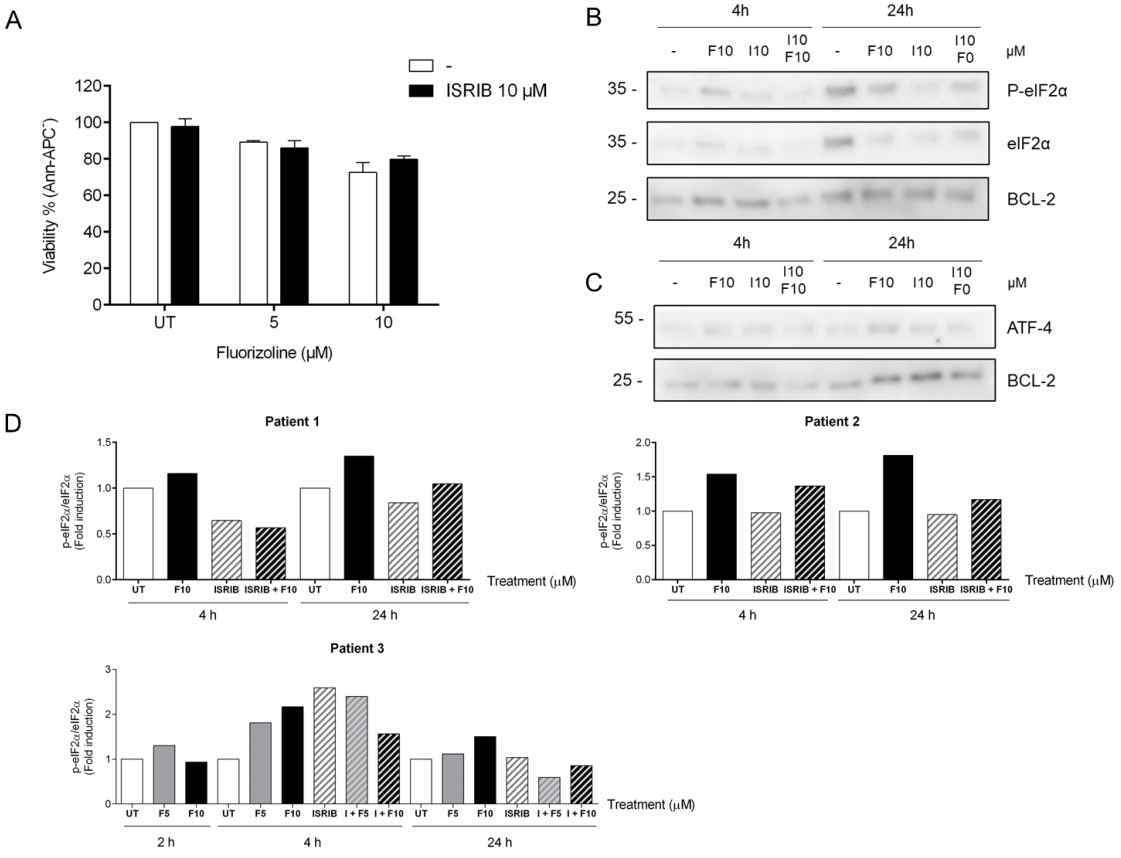

Figure 18. Effect of the inhibition of the ISR pathway on cell viability and protein expression in CLL cells treated with fluorizoline. Cells were preincubated during 1 hour with 10 µM ISRIB and then untreated (UT, -) or treated with 5 and 10 µM fluorizoline (F) at the times stated in the figure. **(A)** The percentage of non-apoptotic cells (annexin V-negative) was measured by flow cytometry, and it is expressed as the mean ± SEM (n=4) of the percentage of the value of untreated cells. **(B)** Cells from CLL patient 1 were lysed and analysed by Western blot. BCL-2 was used for loading normalization. Panel B shows one representative patient of 3 analysed. **(C)** Cells from CLL patient 2 were lysed and analysed by Western blot. BCL-2 was used for loading normalization. **(D)** The results of protein expression in 3 samples analysed are shown as the fold induction in fluorizoline-treated cells relative to untreated cells.

DISCUSSION

In this project it was performed a time-course assay to evaluate the induction of the ISR pathway in leukemia primary cells treated with fluorizoline.

In AML samples, eIF2α has showed an increment on its phosphorylation in a time-dependent manner. The levels of phosphorylated eIF2α increased in response to fluorizoline and thapsigargin treatment in all the treated samples. However, results show variability among patients in the induction level and in the time of treatment. Given that the phosphorylation of eIF2α is the core event of ISR, and after the obtained results, we could confirm that ISR pathway has been activated in AML cells treated with fluorizoline.

ATF4 protein has not shown an induction in the time-course proposed in AML primary cells. Previous results in cancer cell lines have shown an increase in ATF4 protein levels at 4 hours. However, the obtained results cannot be strictly extrapolated due to the fact that samples analysed in this project are primary cells (30,35). ATF3 had not shown an induction in any of the times analysed. Núñez-Vázquez et al. demonstrated that ATF3 and ATF4 act upstream and play a key role in NOXA upregulation (30). For this, it can be intuited that if ATF4 and ATF3 have not been induced after four hours of treatment in AML cells, NOXA would not be induced either.

In CLL samples, results of cell viability assay have shown a reduction in the percentage of non-apoptotic cells after 24 hours of fluorizoline treatment. However, levels of cell death are not comparable to previous results obtained in cell lines, were fluorizoline treatment reduced the cell viability from almost 100% to less than 20% (35). The obtained results suggest that the concentration of fluorizoline used in this study has been non-toxic for CLL cells.

Although quantification results in CLL samples present variability, they all show eIF2α phosphorylation induction after fluorizoline treatment demonstrating that fluorizoline induces the ISR pathway in CLL primary cells. Protein expression results reveal a slightly induction of ATF3 protein. However, protein levels of ATF4 and NOXA have not been modified. For all of these, we could not confirm that the time and/or the doses of the fluorizoline treatment proposed have a clear induction effect on ISR pathway in CLL cells. Further experiments are needed to adjust the doses and times of analysis.

The effect of ISRIB on fluorizoline-induced apoptosis in CLL cells has not been the expected. Previous results with cell lines revealed that pre-treatment with ISRIB slightly sensitized cells to fluorizoline-induced apoptosis, suggesting that this pathway plays a pro-survival role (26,29). Results in viability analysis shows that ISRIB treatment did not reversed cell death nor potentiates the effect of fluorizoline. However, as the apoptotic effect of fluorizoline was lower than the effects seen in precedent studies, it is difficult to see the death protective effect of ISRIB. Despite not having an effect on

the fluorizoline-induced apoptosis in CLL cells, ISRIB was able to reverse the phosphorylation of eIF2α.

In conclusion, all these results demonstrated that fluorizoline is able to induce the phosphorylation of eIF2α, a key event for the initiation of the ISR pathway, in AML and CLL primary cells. However, more experimentation is needed in order to find the specific treatment capable of inducing apoptosis.

All these results demonstrate the diversity of cancer disease, not only between different cancer types such as AML and CLL, but also among patients who suffer the same disease, highlighting the importance of personalized cancer therapies. For all of these, progress in cancer research would help to have a better understanding of the disease as a means to develop precise diagnostics and effective therapies.

CONCLUSIONS

After all these results have been discussed, we can extract the following conclusions:

1. Fluorizoline treatment induces eIF2α phosphorylation in both AML and CLL primary cells, suggesting that the integrated stress response (ISR) could be activated.
2. The proposed fluorizoline treatment has not been able to induce proteins involved in the ISR pathway: ATF4, ATF3 and NOXA, neither in AML nor in CLL primary cells.
3. Fluorizoline induces apoptosis in CLL primary cells although the time and doses of fluorizoline treatment had a low effect on cellular viability.
4. The ISR pathway inhibitor ISRIB did not protect CLL cells from fluorizoline apoptotic effect. However, ISRIB reverses the phosphorylation of eIF2α in most samples analysed.
5. The variability between primary samples complicates the interpretation of the results. This is a preliminary study, experimentation with a higher number of samples is necessary to obtain clear and full conclusions.

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