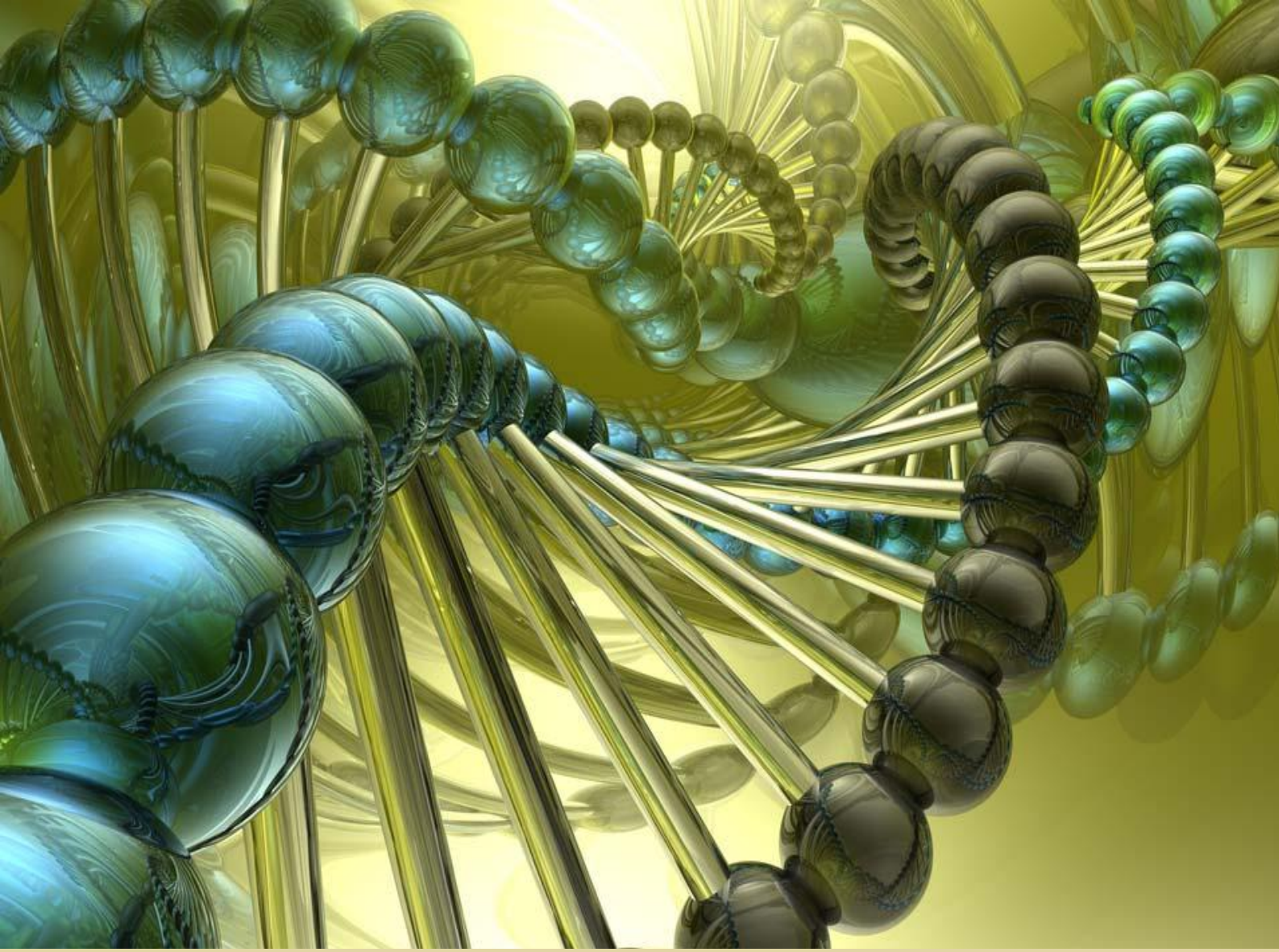

Sara Isabel Ferreira Veiga

Evaluation of the expression of microRNAs in the response to therapy in Chronic Myeloid Leukemia

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pela Professora Doutora Ana Bela Sarmento Ribeiro e pelo Professor Doutor João Nuno Moreira e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2014

· U C ·

Universidade de Coimbra

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Evaluation of the expression of microRNAs in the response to therapy in Chronic Myeloid Leukemia

Avaliação da expressão de microRNAs na resposta à terapêutica na Leucemia Mieloide Crónica

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Sara Isabel Ferreira Veiga, 2014

· U C ·

UNIVERSIDADE DE COIMBRA

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"Even the smallest person can
change the course of the future."

J.R.R. Tolkien

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Resumo

A Leucemia Mieloide Crónica (LMC) é uma doença clonal mieloproliferativa caracterizada pela existência da translocação t(9:22), da qual resulta o cromossoma Filadélfia. Esta translocação dá origem ao gene de fusão *BCR-ABL*, que codifica a oncoproteina BCR-ABL com atividade de tirosina cinase aumentada.

O Imatinib é um inibidor de tirosina cinase (TKI) que veio alterar a estratégia terapêutica habitualmente utilizada em doentes de LMC, o interferão alfa, e é atualmente o tratamento de primeira linha na doença. O Imatinib bloqueia especificamente a atividade da oncoproteina BCR-ABL e promove uma boa resposta por parte dos doentes.

No entanto são conhecidos casos de resistência, sendo a razão mais comum o aparecimento de mutações no domínio BCR-ABL que impedem que o Imatinib se ligue com sucesso e exerça a sua função. Para além disto, existem outros mecanismos moleculares envolvidos na resistência a este fármaco, como a alteração da expressão dos transportadores de influxo e efluxo e das enzimas envolvidas na metabolização do fármaco, entre outros.

Recentemente têm surgido várias terapias que atuam em vias de sinalização celular que se têm revelado cada vez com mais de interesse no tratamento da LMC. O Bortezomib, um inibidor do proteassoma, o Parthenolide, inibidor do NF-κB, e o Everolimos, inibidor do mTOR, são apenas alguns exemplos de novos fármacos que, devido ao seu mecanismo de ação podem revelar-se importantes no tratamento de doentes que apresentam resistência ao Imatinib.

Os microRNAs são pequenas moléculas de ARN não codificante, que regulam a expressão génica ao nível pós-transcripcional. Cada vez mais se tem verificado que a alteração da expressão dos microRNAs está envolvida em várias patologias, nomeadamente no cancro. Alguns microRNAs, como o miR-21, o miR-125b e o miR-155, têm sido relacionados com a aquisição de resistência a determinadas terapias em vários tipos de neoplasias.

Os objetivos deste trabalho foram avaliar a influência dos níveis de expressão dos microRNAs, miR-21, miR-125b e miR-155 na sensibilidade e resistência ao Imatinib em linhas celulares de LMC, bem como no potencial efeito terapêutico de novos fármacos

anticancerígenos dirigidos a alvos moleculares, em particular o Bortezomib, Parthenolide e Everolimus.

Para atingir estes objetivos utilizámos uma linha celular modelo de LMC sensível ao Imatinib, as células K562, e uma linha celular resistente a este TKI obtida a partir da linha celular anterior, as células K562 RC. Para avaliar o efeito dos diversos fármacos na viabilidade celular recorreu-se ao ensaio metabólico da rezasurina. A morte celular foi avaliada por citometria de fluxo com a dupla marcação anexina V e iodeto de protídeo, e por microscopia óptica através da coloração May-Grünwald-Giemsa. Alguns dos mecanismos envolvidos foram analisados através dos níveis de expressão de BAX e BCL-2, dos níveis intracelulares de NF-κB, de conjugados de ubiquitina e de p53 por citometria de fluxo recorrendo à marcação com anticorpos monoclonais. Avaliou-se também por *western blot* os níveis de expressão e ativação da proteína AKT, na ausência e na presença dos três compostos em estudo de forma a perceber a influência destes compostos nesta via de sinalização, devido ao facto de esta via estar relacionada com os mecanismos de ação dos três microRNAs em estudo. A avaliação da expressão dos miRNAs efetuou-se por PCR em tempo real com recurso a kits comerciais.

Os ensaios realizados com os fármacos em estudo revelaram que, o Bortezomib, o Parthenolide e o Everolimus, em monoterapia, induziram diminuição da viabilidade celular, de modo dependente da linha celular, da concentração, do tipo de fármaco e do tempo de incubação, induzindo morte celular preferencialmente por apoptose. Assim, nas células resistentes ao Imatinib (K562 RC) tratadas com Bortezomib e Parthenolide, observou-se um efeito citotóxico mais acentuado do que nas sensíveis, enquanto o Everolimus apresentou um efeito mais evidente nas células sensíveis K562 relativamente às células K562 RC.

Por outro lado, verificamos aumento da expressão dos miR-21 e miR-125b nas células K562 RC em relação as K562. Pelo contrário, os níveis de miR-155 são inferiores nas células K562 RC em relação às K562. Estas alterações podem significar que estes miRNAs desempenham algum papel na aquisição de resistência ao Imatinib, podendo eventualmente constituir novos biomarcadores preditivos de resposta e/ou de monitorização da terapêutica com este TKI.

Quando as células foram expostas a Bortezomib, Parthenolide e Everolimus, verificou-se alteração dos níveis de expressão dos microRNA nas células sensíveis e resistentes ao Imatinib. Nas células K562 verificou-se que o tratamento com os três compostos em estudo induziu um ligeiro aumento nos níveis de expressão dos miR-21 e

miR-155 em relação ao controlo, não se tendo observado nenhuma expressão do miR-125b. Por outro lado, nas células K562 RC, observou-se uma ligeira diminuição dos níveis de expressão do miR-21 e miR-125b, quando expostos aos três compostos em estudo. No entanto, os níveis de expressão do miR-155 apresentaram um ligeiro aumento com a exposição ao Bortezomib e ao Parthnolide e um decréscimo com a exposição ao Everolimus.

Assim, o Bortezomib, Parthenolide e Everolimus poderão constituir uma nova abordagem terapêutica em doentes de LMC resistentes à terapêutica com Imatinib, podendo os níveis de expressão do miR-21, miR-125b e miR-155 ser indicadores e/ou influenciar a resposta à terapêutica.

Palavras chave:

- miR-21
- miR-125b
- miR-155
- Leucemia Mieloide Crónica
- Resistência ao Imatinib

Abstract

Chronic Myeloid Leukemia (CML) is a clonal, myeloproliferative disorder characterized by the existence of the t(9:22) translocation which results in the Philadelphia Cromossome. This translocation results in the *BCR-ABL* fusion gene, which encodes the BCR-ABL oncoprotein with enhanced tyrosine-kinase activity.

Imatinib is a tyrosine kinase inhibitor (TKI) that modified the therapeutic strategy usually used in patients with CML, the interferon alfa, and nowadays is the first line treatment in the disease. Imatinib specifically blocks the activity of the BCR-ABL oncoprotein and promotes good response by patients.

However there are known cases of resistance, the most common reason being the onset of mutations in the *BCR-ABL* gene that prevent Imatinib to bind successfully and perform its function. Furthermore, there are other molecular mechanisms involved in resistance to this drug, as the altered expression of influx and efflux transporters and of the enzymes involved in the metabolism of the drug, among others.

Recently several therapies that act in pathways that have become increasingly interesting in CML therapy, have emerged. Bortezomib, a proteasome inhibitor, Parthenolide an NF-κB inhibitor and Everolimus, an mTOR inhibitor are just a few examples of novel therapies that, due to its mechanism of action may prove to be important in the treatment of patients who present resistance to Imatinib.

MicroRNAs are small noncoding RNA molecules that regulate gene expression at the post-trasncripcional level. Increasingly there has been observed that the altered expression of microRNAs is involved in several diseases, particularly in cancer. Some microRNAs, such as miR-21, miR-125b and miR-155, have been related to the acquisition of resistance to certain therapies in various types of malignancies.

The aims of this study were to evaluate the influence of the expression levels of miR-21, miR-125b and miR-155 in the response and resistance to Imatinib in CML cell lines, as well as the potencial therapeutic effect of new antineoplasic targeted drugs, particularly Bortezomib, Parthenolide and Everolimus.

For this, we used a CML cell line sensitive to Imatinib, K562 cells, and a cell line resistant to this TKI, obtained from the previous, K562 RC cells. To evaluate the effect of

various drugs on cell viability we used the resazurin metabolic assay. Cell death was assessed by flow cytometry, with annexin V and propidium iodide double staining and by optical microscopy through staining with May-Grünwald-Giemsa. Some of the mechanisms involved were analyzed by flow cytometry, trough the expression levels of BAX and BCL-2 and the intracellular levels of NF-kB, conjugated ubiquitin and p53, using specific antibodies. AKT expression and activation levels were assessed by western blot, in the absence and presence of the three compounds in study, in order to understand the influence of these compounds in this signaling pathway, due to the fact that this pathway is related with the mechanisms of action of the three miRNAs in study. Evaluation of the expression of miRNAs was performed by real-time PCR using the commercially available kits.

The assays performed with the drugs in study revealed that Bortezomib, Parthenolide and Everolimus, in monotherapy, induced decrease of cell viability, in time, dose, drug and cell line dependent manner, inducing death preferably by apoptosis. Therefore, cells resistant to Imatinib (K562 RC) treated with Bortezomib and Parthenolide presented a cytotoxic effect more pronounced than in sensitive cells, while Everolimus showed a less pronounced effect in K562 RC cells than in K562 cells.

Additionally, we observed increased expression of miR-21 and miR-125b in K562 RC cells compared to K562 cells. By contrast, the levels of miR-155 decreased in K562 RC relative to K562. These changes might mean that these miRNAs play some role in the acquisition of resistance to Imatinib, and may eventually constitute new biomarkers predictive of response and / or monitoring of therapy with this TKI. Upon exposure to Bortezomib, Parthenolide and Everolimus it was observed an alteration in the expression levels of the microRNAs in Imatinib-sensitive and resistant cells.

In K562 cells it was noted that treatment with the three compounds in study induced a slightly increase in the expression levels of miR-21 and miR-155, when compared to control, not having been noted any expression of miR-125b. Moreover, in K562 RC cells, it was observed a slightly decrease in the expression levels of miR-21 and miR-125b, when exposed to the three compounds in study. However, miR-155 expression levels presented a slightly increase upon exposure to Bortezomib and Parthenolide, and a decrease with Everolimus exposure.

Thus, Bortezomib, Parthenolide and Everolimus may constitute a novel therapeutic approach to CML patients resistant to treatment with Imatinib, and the expression levels of miR-21, miR-125b and miR-155 may be indicators and/or influence the therapeutic response.

Key words:

- miR-21
- miR-125b
- miR-155
- Chronic Myeloid Leukemia
- Imatinib resistance

Abbreviations

ABL – Abelson Murine Leukemia gene

Ago – Argonaute protein

AKT – Protein Kinase B

ALL – Acute Lymphoblastic Leukemia

AML – Acute Myeloid Leukemia

AP-1 – Activator Protein 1

ATCC – American Type Culture Collection

ATP - Adenosine Triphosphate

AV – Annexin V

BAK – BCL2 homologous Antagonist/Killer

B-CLL – B Cell Chronic Lymphoblastic Leukemia

BCR – Breakpoint Cluster Region gene

BIC – B cell Migration Cluster

BMDM – Bone Marrow-derived Macrophages

BMF – BCL2 Modifying Factor

BRCA1 – Breast Cancer 1

BTZ – Bortezomib

cDNA – Complementar DNA

CLL - Chronic Lymphoid Leulemia

CLP – Common Lymphoid Progenitor

CML – Chronic Myeloid Leukemia

CMP – Common Myeloid Progenitor

CpG – Cytosine-phosphate-Guanine Dinucleotide

CSC – Cancer Stem Cells

DL-BCL – Diffuse Large B Cell Lymphoma

DNA – Deoxyribonucleic Acid

DNMT – DNA Methyltransferases

DNR - Daunorubicin

DTT – Dithiothreitol

ECF – Enhanced Chemofluorescence Substrate

EPO - Erythropoietin

EVE – Everolimus

FADD – FAS Associated Death Domain

FBS – Fetal Bovine Serum

FITC - Fluorescein Isothiocyanate

G1 – *Gap 1*

G2 – *Gap 2*

GRK2 - G protein-coupled receptor kinase 2

HAT – Histone Acetyl Transferase

HDAC – Histone Deacetylase

HDM – Histone Demethylase

HDT – Histone Demethylase

HMT – Histone Methyl Transferase

HSC – Hematopoietic Stem Cells

HSPC – Hematopoietic Stem and Progenitor Cells

IC_{50} – Half Maximum Inhibitory Concentration

IFN- – Interferon

IGF-2 – Insuline-like Growth Factor 2

Ikk epsilon - I kappa B Kinase epsilon

IL-6 – Interleukin 6

IκK – Iκ B Kinase

K - Lysine

LPS - Lipopolysaccharides

M – *Mitosis*

MAGE – Melanoma Antigen Gene

MBD – Methyl-Binding Domain

MFI – Medium Fluorescence Intensity

MGMT – O^6-Methylguanine-DNA methyltransferase gene

miRNA or **miR** – micro Ribonucleic Acid

MLH1 – MutL Homolog 1

MPP – Multipotent Progenitor

mTOR – Mammalian target of Rapamycin

mTORC2 – mTOR Complex 2

ncRNA – non-coding RNA

NF-κB – Nuclear Factor κB

PACT – Protein ACTivator of the Interferon-induced protein kinase

PAX2 – Paired Box gene 2

PBS – Phosphate buffered saline

PDCD4 – Programmed Cell Death 4

PE - Phycoerythrin

PI – Propidium Iodide

PI3K – Phosphoinositide 3 Kinase

pre-miRNA – precursor microRNA

pri-miRNA – primary microRNA

PTEN – Phosphatase and Tensin Homolog

PTL – Parthenolide

PUMA - p53-upregulated modulator of apoptosis

PVDF – Polyvinylideno Fluoride

RAS – Rat Sarcoma

RB – Retinoblastoma gene

RISC – RNA induced silencing complex

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute

RT-PCR – Real-Time Polymerase Chain Reaction

S – *Synthesis*

SAM – S-Adenosil Methionine

SDS – Sodium Dodecyl Sulfate

SIRT1 – Sirtuin 1

Sp1 – Specificity Protein 1

STAT3 - Signal transducer and activator of transcription 3

TKI – Tirosine Kinase Inhibitors

TNF- – Tumor necrosis Factor

TP53BP1 – Tumor Suppressor P53 Binding Protein 1

TPO - Thrombopoietin

TRBP – HIV-1 TAR RNA binding protein

tsmiRNA – tumor supressor miRNA

TSS – Transcription Stat Sites

UTR – Untranslated Region

INTRODUCTION

1.1. Cancer

Human cells are regulated by complex mechanisms that allow cellular homeostasis. For most of the time, Deoxyribonucleic Acid (DNA) sequences are the most steady, unchangeable components of a cell, but nearly all of the other cellular apparatuses are in constant flux, being continuously created and recycled (Weinberg, 2007). The mechanisms of gene regulation determine which genes will be expressed and when, comprising the most robust defenses against the environment. If this complex regulatory circuit is disrupted it can lead to accumulation of genetic and epigenetic abnormalities, which result in transformation of normal cells into malignant derivatives (Pavet *et al.*, 2011).

Cancer can be described as a disease of abnormal gene function. Normal cells stop obeying the restraints imposed within differentiated cells and instead proliferate aberrantly and eventually turn malignant. Tumorigenesis is a complex, multistep process driven by a sequence of randomly occurring mutations and epigenetic alterations of DNA that affect the genes controlling cell proliferation, survival, differentiation and other traits associated with a malignant cell phenotype (Weinberg, 2007).

The danger posed by the disease comes from the combination of two properties: the ability of cells to proliferate in an uncontrolled way and their ability to spread through the body, invading areas reserved to other cells. Contrary to normal cells, that have a finite capacity to replicate, cancer cells can replicate indefinitely (Hayflick, 2003). It is suggested that at some point, during tumor progression, premalignant cell populations exhaust their amount of allowed doublings and can only continuing tumorigenesis by breaching the mortality barrier and acquire unlimited replicative potential (Hanahan and Weinberg, 2000).

Normal stem cells have the capacity to self renewal and differentiate into daughter cells that become tissue-specific and also have the potential to proliferate extensively. These three properties make stem cells unique (Jordan *et al.*, 2006, Lobo *et al.*, 2007). Recent studies enlighten the present of stem cells that have the exclusive ability to regenerate tumors. These cells are known as cancer stem cells (CSC) (Figure 1). Cancer stem cells are self-renewing cells within a tumor that have the exclusive ability to lead the growth and spread of an original tumor. Self-renewal enables these cells to produce daughter cancer cells that are more differentiated but also lack intrinsic regenerative potential, being destined to stop proliferating or die. Stem cells become more susceptible to initial oncogenic mutations because they are in constant division and are exposed to more genotoxic stress than their shorter-lived, differentiated progeny. (Lobo et al., 2007, O'Brien *et al.*, 2010).

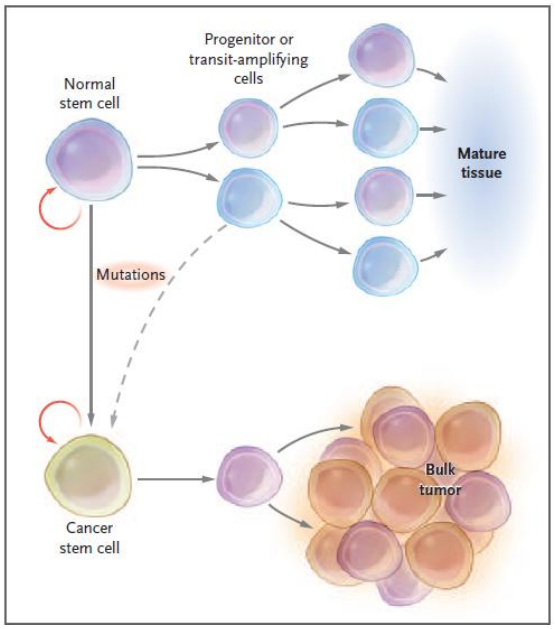

Figure 1. Normal and cancer stem cells. Normal stem cells have the ability to grow and differentiate into progenitor and mature cell populations, respectively. The figure show normal stem cells capacities: self-renewing (red curved arrow), differentiation into progenitors with multilineage potential (cells of different colors) having extensive proliferative capacity. Mutations can occur in normal stem cells and through this way cancer stem cells arise. Like normal stem cells, cancer stem cells can self-renew and have the ability to proliferate extensively forming new tumors (adapted from Jordan, 2006).

An important goal in cancer therapy is the development of treatments that target stem cells, once that, if these cells are not eradicated, the tumor may regrow and relapse (Figure 2).

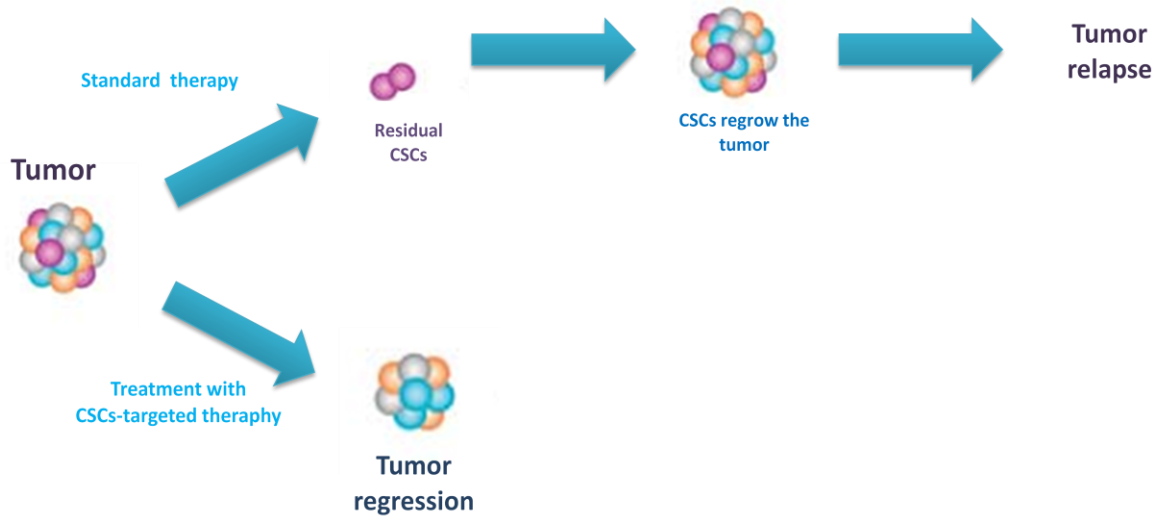

Figure 2. Development of effective anticancer therapies. Cancer stem cells (CSCs) are biologically distinct from bulk tumor cells. Current treatments for cancer can considerably diminish tumor burden, but have a decreased effect on CSCs, which are later capable of driving tumor recurrence and regrowth. To achieve cancer remission or cure, therefore, it will be necessary to develop novel therapies that target CSCs (adapted from Dick, 2008).

1.2. Hematopoietic System

Hematopoiesis in vertebrates is maintained during an organism's lifetime due to a strict regulation of the highly hierarchical hematopoietic system. Because mature blood cells have a very short life span, hematopoietic stem cells (HSCs) are required through life to maintain and continuously regenerate the entire blood supply, which is constantly being replaced. Hematopoietic stem cells, as all other stem cells, are capable of self-renewal – the production of additional HSCs – and differentiation, specifically into specialized blood cells that have important function in control homeostasis balance and response to microorganisms and inflammation (Hoffman and Calvi, 2014). HSCs reside within the bone marrow and depend on their microenvironment, the niche, for regulation of appropriate stem cell behavior. Within the microenvironment, HSC receive internal and external signals, which direct them to preserve balance between self-renewal and production of progeny to maintain hematopoiesis, while at the same time maintain a HSCs pool. Disruption of these signals can lead to stem cell depletion, altered hematopoiesis and malignancy (Hoffman and Calvi, 2014, Lo Celso and Scadden, 2011).

The process of formation of hematopoietic stem cells begins with the HSC that gives origin to another HSC by self-renewal and to multipotent progenitors (MPPs) which lose self-renewal potential but remain fully differentiates into all multilineages. The MPPs further give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). These progenitors differentiate into their restricted lineage commitment, according to the present stimulus: CMPs progress to erythrocytes, megakaryocytes (which give rise to platelets) and myeloblasts (which give rise to granulocytes and monocytes). CLPs advance to T Lymphocytes, B Lymphocytes and Natural Killer cells (Figure 3) (Chotinantakul and Leeanansaksiri, 2012).

Hematopoiesis is also supported by marrow space that contains also stromal cells which produce cytokines/growth factors, such as c-KIT ligand (among others), that stimulate stem cells and progenitors. Cytokines, including interleukins, thrombopoietin (Tpo), and erythropoietin (Epo), also influence progenitor function and survival (Orkin and Zon, 2008). All this growth factors activate several signaling pathways that stimulated hematopoietic cell proliferation, differentiation and death.

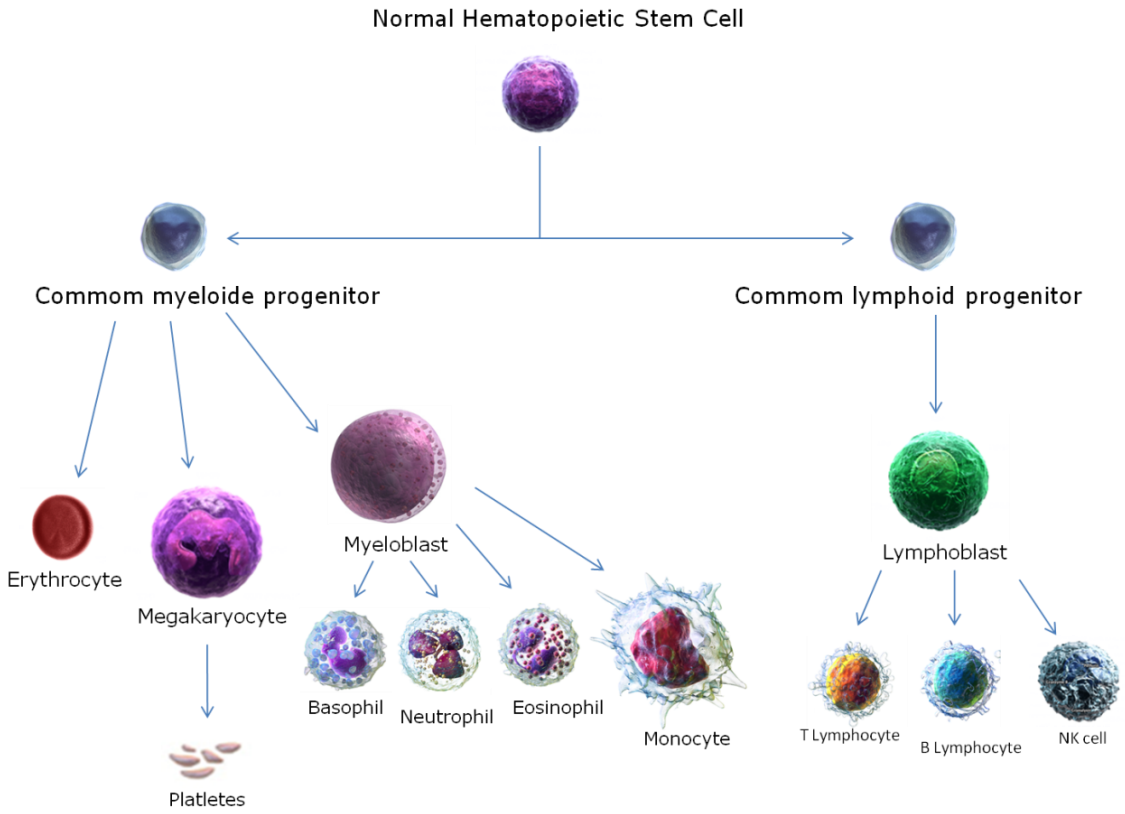

Figure 3. Normal Hematopoiesis. Hematopoietic Stem Cells self renewal and also give arise to commom progenitors of myeloid and lymphoid lineage. These cells will differentiate into the different blood elements (adapted fromhttp://www.biolegend.com/cell_markers).

1.3 Chronic Myeloid Leukemia

Chronic Myeloid Leukaemia (CML) is a clonal, myeloproliferative disorder characterized by the neoplasic transformation of hematopoietic stem cells. It was the first neoplastic disease associated with a chromosomal aberration, known as the Philadelphia chromosome (Figure 4). This chromosome is the result of a reciprocal translocation that lead to the juxtaposition of the breakpoint cluster region (BCR) gene on chromosome 22 and Abelson murine leukemia (ABL) gene on chromosome 9. This results in a fusion gene, the *BCR-ABL* gene, which codes BCR-ABL transcripts and fusion oncoproteins with unusual tyrosine kinase activity. The BCR-ABL transcript has constitutively tyrosine-kinase activity resulting in the activation of signaling pathways involving a cascade of proteins that control the cell cycle, confer a proliferative advantage, decreased adherence of the leukemic cells to the bone marrow stroma and also genomic instability, making the cell more susceptible to developing further genetic abnormalities (Hehlmann *et al.*, 2007).

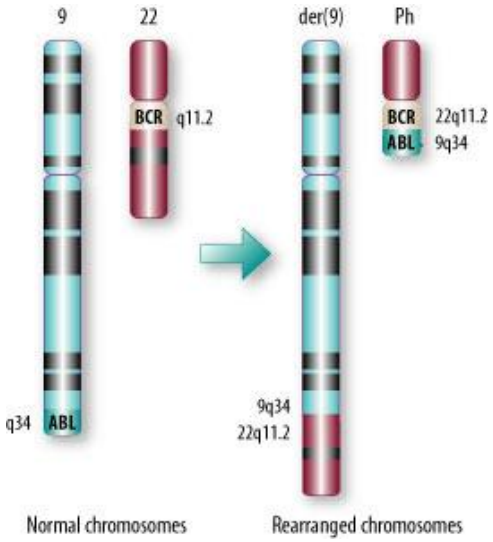

Figure 4. Philadelphia Chromosome. Translocation of chromosomes 9 and 22 at bands 9q34 and 22q11.2, respectively, results in the formation of Philadelphia Chromosome. The juxtaposition of the *ABL* oncogene from 9q34 and the *BCR* locus from 22q11.2 results in a chimeric gene on the derivative chromosome 22 (adapted from Armand, 2007).

1.3.1 Chronic Myeloid Leukemia Treatment

Chronic Myeloid Leukemia was the first to be treated with a biologic agent, the Interferon Alfa (IFN-α) (Alves, 2011). INF-α was the first effective therapy for CML. This agent remained the treatment of choice for CML patients, until a shift in therapeutic strategy after the arrival of the tyrosine kinase inhibitor, Imatinib (Frazer *et al.*, 2007).

CML was the first neoplasm for which knowledge of the genotype led to a rationally designed targeted therapy. Although, in recent years new tyrosine-kinase inhibitors (TKIs) such as Dasatinib or Nilotinib have emerged, allogenic stem cell transplantation is the only curative therapy for CML (Hehlmann et al., 2007).

1.3.1.1. Imatinib

Imatinib is a small-molecule drug that targets and inhibits the BCR-ABL tyrosine kinase by competitive binding at the ATP-binding site, making the target protein inactive. Imatinib produces a sustained cytogenetic response in the majority of patients and it is clinically well tolerated (Frazer et al., 2007). Despite its remarkable efficacy in treating CML, it does not have the capacity to eradicate residual disease related with the persistence of

stem cells, which are known to be resistant to Imatinb. Even in patients with undetectable BCR-ABL transcripts, relapses are seen after interruption of Imatinib, suggesting that the treatment has to be maintained indefinitely.

Resistance to Imatinib results most of the times of the development of mutations in the BCR-ABL kinase domain, thus preventing Imatinib binding successfully. Other mechanisms involve over expression of *BCR-ABL*; acquired clonal evolution, on account of addition of novel chromosomal aberrations, and mechanisms related with pharmacological mechanisms of drug transportation, resulting in the reduction in the quantity of available unbound Imatinib, culminating in suboptimal levels of Imatinib to achieve the desired effect (Frazer et al., 2007, Hehlmann et al.).

1.3.1.2. Novel therapies

To try to improve clinical response by patients and overcome resistance to Imatinib, novel therapies have emerged. Alternative therapeutic approaches may involve an attempt to obtain a synergistic effect through combination of BCR-ABL TKIs with inhibitors of non-BCR-ABL targets or targets found in chronically activated signaling pathways downstream of BCR-ABL (Melo and Chuah, 2008).

Proteasome inhibition has been an area of interest in CML therapy.
The ubiquitin-proteasome pathway is responsible for the degradation of several cellular proteins. Proteasomes have a dual role of "housekeeping" (disposing of damaged proteins) and regulation (degrading proteins involved in cell cycle regulation and tumor growth) within the cell. This latter property has driven the investigation of proteasome inhibitors as a new cancer therapy (Frazer *et al.*, 2007).

Inhibition of transcription activated by Nuclear Factor-κB (NF-κB) has been implicated as the mechanism responsible for the antitumor effect of proteasome inhibitors (Melo and Chuah, 2008). Bortezomib (PS341, Velcade) is a potent and selective proteasome inhibitor that has clinical activity in multiple myeloma and possibly lymphoproliferative disorders (Cortes *et al.*, 2004, Sarmento-Ribeiro *et al.*, 2005). It has shown to decrease proliferation, induce G2/M phase cell cycle arrest and promotes apoptosis in Imatinib sensitive and resistant CML cell lines. Despite simultaneous treatment of Imatinib sensitive CML cell lines with Bortezomib and Imatinib promoted and antagonistic interaction on growth inhibition, the sequential exposure to Bortezomib followed by Imatinib resulted in a

synergistic proapoptotic effect in Imatinib sensitive cells (Gatto *et al.*, 2003, Melo and Chuah, 2008).

In the same way that Bortezomib's NF-κB inhibition can be used to increase the response of cancer cells to chemotherapy, Parthenolide, a potent inhibitor of NF-κB, can also be used for the same effect. The mechanism of NF-κB down-regulation by Parthenolide appears to occur via inhibition of the Iκ B Kinase (IκK) complex. Parthenolide has shown to induce very effectively leukemic stem cells-specific cell death *in vitro* in acute myeloid leukemia (AML) (Guzman *et al.*, 2005).

BCR-ABL fusion protein also activates the PI3K/AKT/mTOR pathway (Alves, 2011). Mammalian target of Rapamycin (mTOR) is a serine-threonine kinase downstream of Phosphoinositide 3 kinase (PI3K) that is activated upon phosphorylation by Protein Kinase B (AKT). Everolimus, a derivate of rapamycin, is an inhibitor of mTOR (Melo and Chuah, 2008). It has been show that Everolimus revokes mTOR late re-activation in response to Imatinib (Mancini, M. *et al.*, 2010), an activation that is possibly responsible for the development of an Imatinib resistance phenotype (Melo and Chuah, 2008).

1.4. Epignetics

When Conrad Waddington first introduced the word "epigenetics" in the early 1940s, the term was used to explain "the casual interaction between genes and their products, which bring the phenotype into being" (Esteller, 2008). But, currently epigenetics refers specifically to the study of heritable changes in gene expression that do not involve any alteration in the DNA sequence. The disruption of such changes results in a wide variety of pathologies, including cancer (Rodriguez-Paredes and Esteller, 2011). It is known these non-genetic alternations are regulated by three major epigenetic modifications: DNA methylation, histone modifications (histone code) and miRNAs.

1.4.1. DNA methylation

In humans, DNA methylation takes place predominantly at the carbon-5 of a cytosine positioned within a 5'-CpG-3' dinucleotide resulting in methyl-CpG. Methylation is carried out by DNA methyltransferases (DNMTs) with S-adenosyl-methionine (SAM) as the methyl donor (Kristensen *et al.*, 2009) (Figure 5).

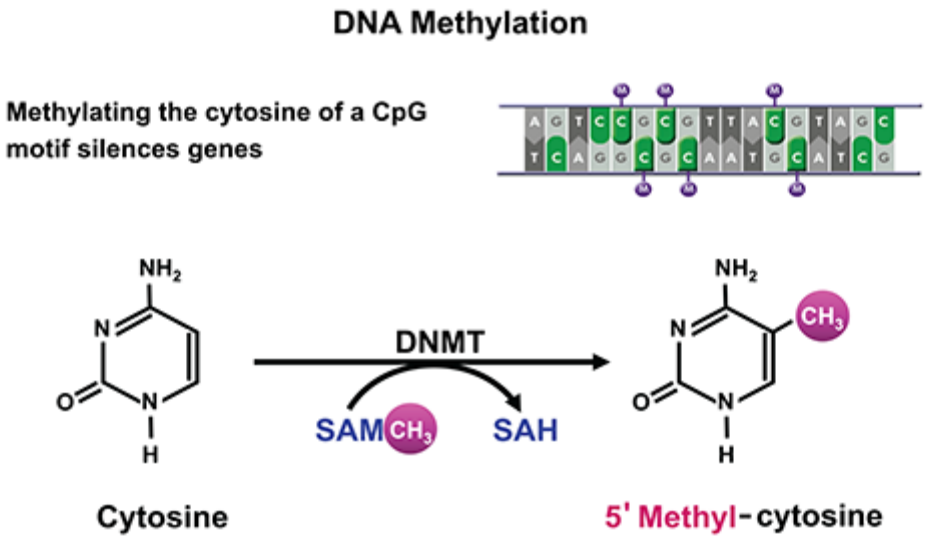

Figure 5. Schematic representation of DNA methylation. Cytosine is converted to 5'methyl-cytosine via the actions of DNA methyltransferase (DNMT). DNA methylation typically occurs at cytosines that are followed by a guanine (adapted from Zakhari, 2013).

There are three main DNA methyltransferases (DNMTs) that play major roles in establishing and maintaining DNA methylation patterns: DNMT1, which maintains the existing methylation patterns following DNA replication during S-phase, and DNMT3A and DNMT3B, enzymes responsible for *de novo* methylation that target previously unmethylated CpGs (Rodriguez-Paredes and Esteller, 2011). Transcription can be directly repressed by CpG methylation, preventing methylation-sensitive transcriptions factors from biding to their recognition sites. Methylated CpGs can also bind methyl-CpG-binding proteins through the methyl-biding domain (MBD), cutting off the binding sites of methylation-insensitive transcription factors and averting transcription, in addiction to recruiting repressive chromatin remodeling complexes (Rouhi *et al.*, 2008). CpG dinucleotides sites are not randomly distributed in the human genome; instead they are concentrated either in short CpG-rich DNA regions known as CpG islands, which are located in approximately 60% of human gene promoters, or in regions of large repetitive sequences such as centromeres and transposable elements (Rodriguez-Paredes and Esteller, 2011). Albeit in the latter case most of the CpGs are methylated to avoid chromosome instability and inappropriate transcription of repetitive elements leading to insertional mutagenesis, the majority of CpG islands are usually unmethylated in normal cells, and the associated genes are actively transcribed (Kristensen et al., 2009) (Figure 6).

It is thought that CpG islands escape methylation by binding of transcription factors and hence maintain their CpG density (Rouhi et al., 2008).

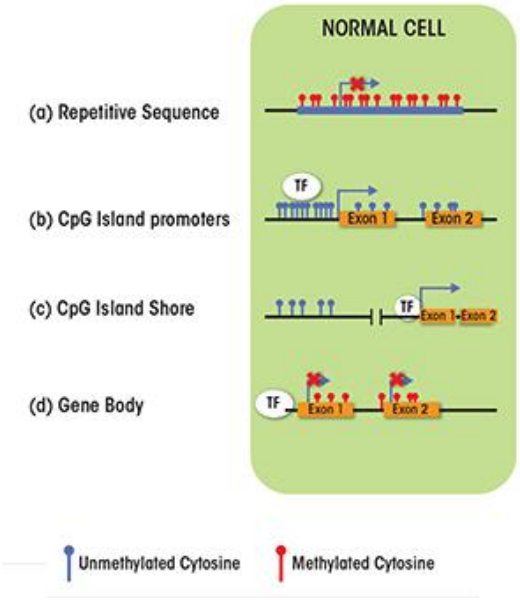

Figure 6. DNA methylation patterns in normal cells. DNA methylation can occur in different regions of the genome. (a) In normal cells, methylation of repetitive sequences prevents genomic instability and spurious transcription initiation, as aberrant transcription can occur from incorrect transcription start sites (TSSs). (b) and (c) CpG islands at promoter sites are usually unmethylated, allowing transcription. (d) CpGs located in gene bodies frequently are methylated in normal cells, preventing false transcriptions (adapted from Varela-Rey *et al.*, 2013).

However there are exceptions to this general trend as for instance in genomic imprinting where only genes from one of the two parental alleles are expressed. Hypermethylation of one allele results in its silencing and leads to monoallelic expression (Rodriguez-Paredes and Esteller, 2011). Genomic imprinting requires DNA hypermethylation at one of the two parental alleles of a gene to ensure monoallelic expression (Esteller, 2008). Other exceptions include hypermethylation of genes during X-chromosome inactivation in females and germ-line genes such as the MAGE (melanoma antigen gene) genes, which are hypermethylated and silence in almost all tissues except malignant tumors. (Kristensen *et al.*, 2009).

Besides DNA methylation, epigenetic activation or silencing is also accomplished through histone modifications (Rouhi *et al.*, 2008).

1.4.2. Histone Modifications

The principal structure of eukaryotic chromatin is the core nucleosome, consisting of an octomer of histones (two each of H2A, H2B, H3 and H4) around which the DNA winds (Kristensen et al., 2009). Chromatin not only serves as a way to condense DNA within the cellular nucleus, but also as a way to stabilize and control how the DNA is used.

In a simplified view chromatin can exist in two basic states: an open, transcriptionally competent euchromatin and a closed, more condensed and transcriptionally silent heterochromatin (Guil and Esteller, 2009).

Histones can undergo multiple post-transcriptional modifications such as acetylation, methylation, phosphorylation and ubiquitination that mainly occur at specific residues located predominantly but not exclusively in N-terminal tails. These modifications affect gene transcription and DNA repair. The most studied modifications are histone acetylation/deacetylation and methylation/demethylation (Figure 7).

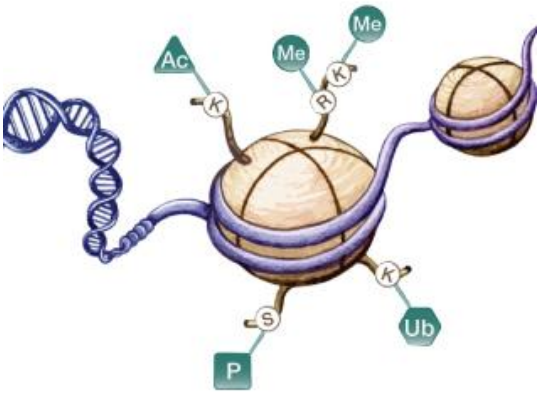

Figure 7. Histone modifications. Amino acids available for chemical modification in terminal tails of core histones are shown here. The modifications include acetylation of many different lysine residues, methylation of arginine and lysine residues, and phosphorylation. Modifications are made or removed by enzymes specialized for a particular modification (adapted from https://promega.files.wordpress.com/2014/01/histones_11340ta.jpg).

Histone acetylation of lysine (K) residues is associated with open chromatin and active transcription (Figure 8.A.), whereas the functional consequences of the methylation of histones on chromatin compaction and state of transcription depends on the type of residue – lysine or arginine – and the specific site that the methylation modifies (e.g. K4, K9 or K20) as well as the number of methyl groups covalently bound to the residue. Hence, methylation of H3 at K4 is related to transcriptional activation while methylation of H3 at K9 or K27 and of H4 at K20 is associated with transcriptional repression (Figure 8.B.) (Esteller, 2008, Rouhi et al., 2008).

A given locus may contain both transcription-promoting and repressive marks and the state of transcriptional activation is determinate by the balance of these marks. It has been proposed that distinct histone modifications form a "histone code" and that exert their effect on chromatin as a collective (Rouhi et al., 2008).

The histones-modifying enzymes most well characterized are: acetyl trasnferases (HATs), that add acetyl groups on lysine residues, and deacetylases (HDACs and sirtuins), that remove them, and also histone methyl transferases (HMTs) that add methyl groups on

lysine residues or on arginine residues and demethylases (HDTs) that reverse that process (Hatziapostolou and Iliopoulos, 2011).

Histone-modifying enzymes and methylated DNA interact. For instance, MBD proteins recruit to CpG-methylated loci nuclear corepressor complexes, such as SIN3 complex that contains histone deacetylases that remove acetyl groups from lysine residues present in the N-terminus of histones. This creates a more compact chromatin structure which is not accessible to the transcriptional machinery. In this way DNA methylation can directly and indirectly lead to the transcriptional repression of genes (Rouhi et al., 2008). On the other hand, the histones bound to unmethylated promoters are acetylated by HATs, which contribute to an open chromatin structure sheltering actively transcribed genes (Kristensen et al., 2009). Notably, all epigenetic processes work together to establish and maintain the global and local condensed or descondensed chromatin states that eventually determine gene expression (Rodriguez-Paredes and Esteller, 2011).

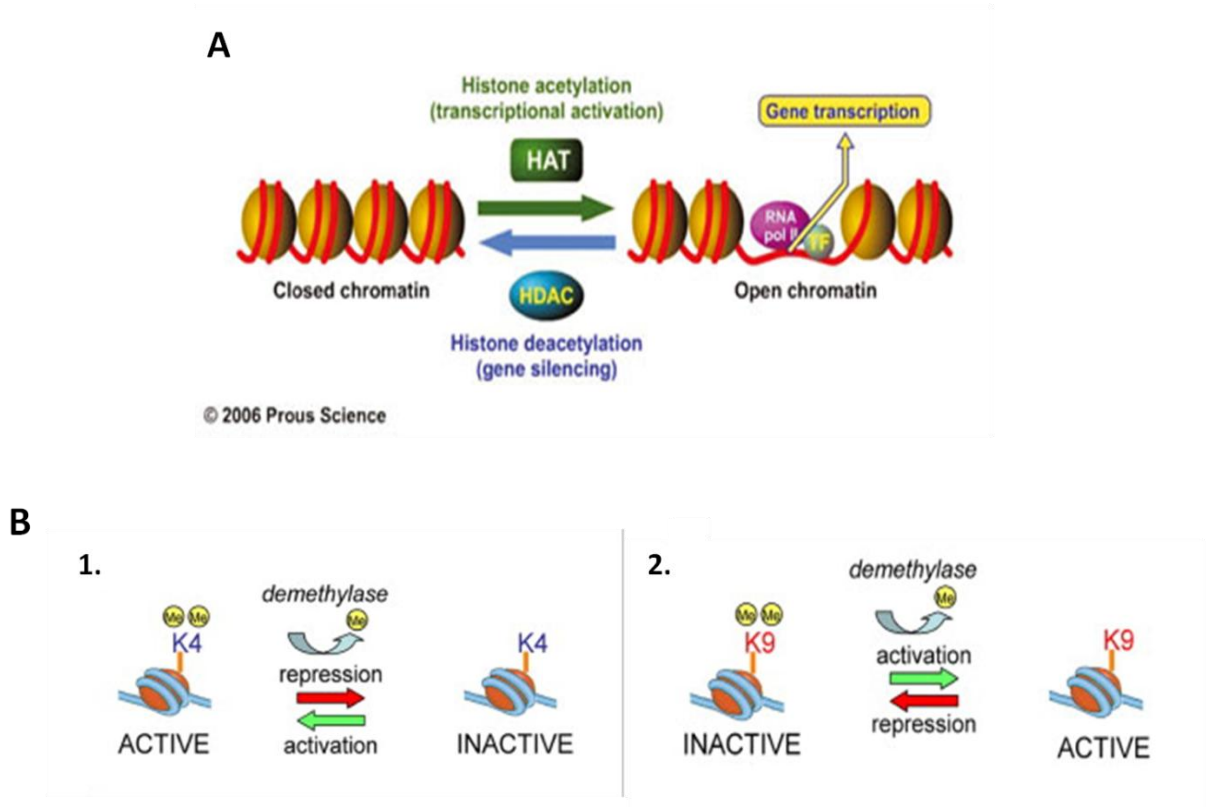

Figure 8. Histone acetylation (A) and Methylation (B). In **(A)** closed chromatin prevents transcription factors involved in gene expression to get to DNA, and gene expression does not occurs. Histone deacetylases (HDACs) are enzymes that remove the acetyl groups from histones, resulting in less accessible chromatin structure. There are also histone acetyltransferases (HATs) that add an acetyl group to histones, resulting in an open chromatin structure that allows gene transcription (adapted from Nicole A., 2013). In **(B) (1.)** methylation of H3 at K4 is related to transcriptional activation and conversely, its demethylation accompanies gene repression. In contrast in **(B) (2.),** methylation of H3 at K9 is associated with transcriptional repression; hence removal of H3K9 methyl marks coincides with gene activation (Wysocka *et al.*, 2005).

The aberrant functions of chromatin modifying enzymes, which can result in altered histone modification or DNA methylation patterns, are hallmarks of human diseases (Guil and Esteller, 2009).

1.5 Epigenetics and cancer

The occurrence of epigenetic alterations in cancer was discovered about 25 years ago with the demonstration of genome-wide DNA hypomethylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts (Rouhi et al., 2008).

The loss of methylation may contribute to tumorigenesis by inducing chromosomal instability, re-activation of transposable elements, loss of imprinting, as in the case of the *IGF2* gene (encoding insulin-like growth factor 2) in Wilms' tumor and colorectal cancer (Rodriguez-Paredes and Esteller, 2011, Wilson *et al.*, 2007). Besides that, can contribute to the activation of normally methylated oncogenes such as *PAX2* (a gene that encodes a transcription factor involved in cell proliferation and other important activities) (Esteller, 2008, Kristensen et al., 2009). The degree of hypomethylation increases through the evolution of a tumor, from a benign lesion to an invasive cancer. Nonetheless, it is not clear how important DNA hypomethylation is in tumorigenesis. Possibly it is only a real causative factor in some cancers and a modulator of cancer risk in others (Kristensen et al., 2009).

However, the most recognized epigenetic disruption in human tumors is the CpG island promoter hypermethylation associated with silencing of tumor suppressor genes (Figure 9) (Rodriguez-Paredes and Esteller, 2011).

The first tumor suppressor gene reported to undergo silencing as a result of promoter methylation was the Retinoblastoma gene (*RB)* gene in retinoblastoma tumors, followed by other important tumor suppressor genes, such as *MLH1* in colon cancer, *BRCA1* in breast cancer and *MGMT* in glioblastomas (Kristensen et al., 2009). The transcriptional inactivation caused by promoter hypermethylation affects genes involved in the main cellular pathways: DNA repair, Rat Sarcoma (RAS) signaling, cell cycle control, p53 network, cell-to-cell interaction and angiogenesis, among others (Portela and Esteller, 2010). Hypermethylation occurs at different stages in the development of cancer and in different cellular networks and the hypermethylation patterns in tumor suppressor genes are specific to a certain cancer type, as seen, for instance, by the exclusivity of *BRCA1* hypermethylation in breast and ovarian neoplasias (Ballestar and Esteller, 2005, Esteller, 2008).

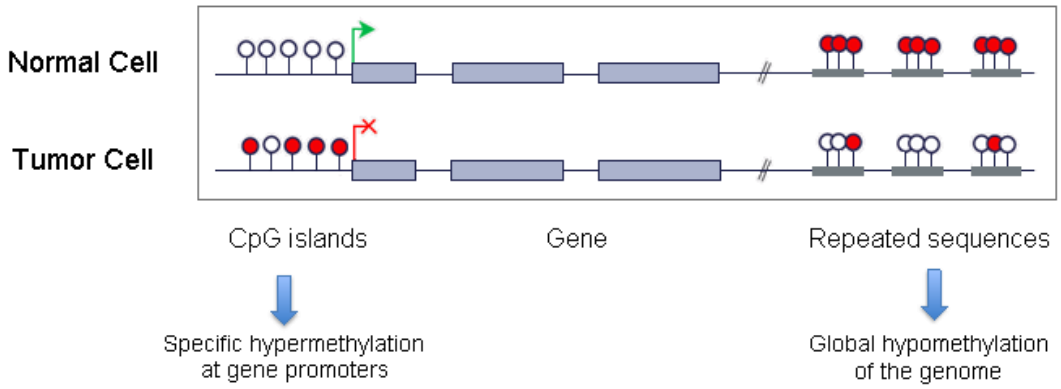

Figure 9. Aberrant DNA methylation profile in cancer cells. In normal cells, nearly all of the CpG dinucleotides are methylated, whereas CpG islands are unmethylated. In cancer cell, a global loss of DNA methylation at repeated sequences leading to global hypomethylation of the genome is observed along with DNA hypermethylation at specific sites, such as tumor suppressor genes promoters, inducing the silencing of their associated genes (adapted from Moison C, 2013).

Disruption of normal patterns of covalent histone modification is another hallmark of cancer. One of the most characteristic examples is the loss of acetylation at lysine 16 and tri-methylation at lysine 20 of histone H4, in association with hypomethylated repetitive DNA sequences present in many primary tumors (Figure 11). They have been found in breast and liver cancer (Hatziapostolou and Iliopoulos, 2011, Rodriguez-Paredes and Esteller, 2011). Silencing of tumor suppressor genes in cancer cells has been associated with a particular combination of histone marks: deacetylation of histones H3 e H4, loss of H3K4 trimethylation (active mark) and gain of H3K9 methylation and H327 trimethylation (repressive marks – silenced chromatin). The presence of hypo-acetylated and hypermethylated histones H3 and H4 silences certain genes with tumor suppressor like properties, despite the absence of hypermethylation of the CpG island (Figure 10) (Esteller, 2008).

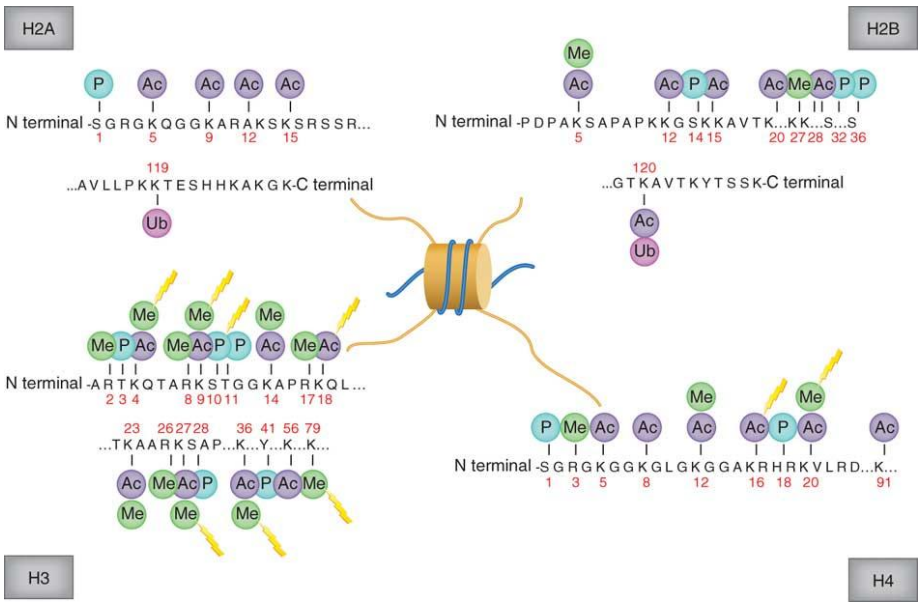

Figure 10. Histone modification paterns in normal and cancer cells. The Figure shows the main modifications of the four core histones in normal cells (type and position in the amino acid sequence). Additionally, and because disruption of their normal patterns is related to cancer, histone modifications typically associated with disease have been highlighted (adapted from Rodriguez-Paredes and Esteller, 2011).

The loss of acetylating is mediated by HDASs, which have been found overexpressed or mutated in different tumor types. Sirtuin family of proteins is the main class of HDACs implicated in this process. Sirtuin 1 (SIRT1) deacetylase activity and gene expression is upregulated in a wide variety of tumors and SIRT1 also interacts with DNMT1, affecting DNA methylation patterns as well. Several cancer types (e.g., colon, uterus, lung and leukemia) also bear translocations leading to the formation of aberrant fusion proteins, mutations or deletions in HATs and HAT-related genes, contributing this way to the global imbalance of histone acetylation (Portela and Esteller, 2010).

Cancer cells exhibit altered distribution of the histone methyl marks mainly due to the aberrant expression of both histone methyltransferases (HMT) and histone demethylases (HDMs). The histone methyltransferase EZH2 – which is the H3K27 specific HMT – enhances proliferation and neoplastic transformation and is overexpressed in several cancer types such as breast, colon and lung cancer. Recent discoveries also show inactivating mutations of EZH2 in follicular and diffuse large B cell lymphomas (Rodriguez-Paredes and Esteller, 2011). In addition to its histone methyltransferase activity, EZH2 interacts with DNMTs directly controlling DNA methylation (Portela and Esteller, 2010).

1.6 MicroRNAs

A vast diversity of RNAs expressed in the genome has diversified functions, other than being only the "messengers" from DNA to protein expression. These RNAs, named non-coding-RNAS (ncRNAs), were previously assumed to be "trash" but now are starting to be classified and carefully studied. Among these ncRNAs, microRNAs (miRNAs or miRs) have stood out (Teixeira, 2013).

The first miRNA, lin-4, was described in *Caenorhabditis elegans* in 1993 by Victor Ambros and his co-workers Rosalind Lee and Rhonda Feinbaum. Lin-4, a 22 nucleotide RNA product of the *LIN-4* gene, was shown to target lin-14 mRNA and negatively regulate the level of lin-14 protein (Lee *et al.*, 1993). This appeared to be unique until a second example of a similar small regulatory RNA in *C. elegans*, *let-7,* was discovered seven years later by Gary Ruvkun's lab (Piriyapongsa *et al.*, 2007, Reinhart *et al.*, 2000).

Since 2001, when the term miRNA was used for the first time, a great progress has been made in understanding the role of miRNAs and it has been demonstrated that miRNAs are involved in many biological processes including differentiation, proliferation and apoptosis (Vasilatou *et al.*, 2010).

1.6.1 MicroRNAs' biogenesis

MicroRNAs are small (19-24 nucleotides) single-stranded RNA molecules that negatively regulate gene expression at post-transcriptional level. The result is the degradation or inhibition of translation of their target mRNAs (Tuccoli *et al.*, 2006).

The miRNAs have a standard biogenesis that consists of three phases: transcription, maturation and assembly (Teixeira, 2013) (Figure 11). The biosynthesis of miRNAs starts in the nucleus with the transcription of long double stranded RNA molecules known as primary-microRNAs (pri-miRNA) by RNA polymerse II. These pri-miRNAs are characterized by a stem-loop structure and while still in the nucleus are cleaved into precursor RNA (pre-miRNA), a 70-100 nucleotide hairpin-shaped structure, by Drosha, an RNase III enzyme, and its interacting partner DGCR8. DGCR8 stabilizes Drosha and unlike the last can directly and stably interact with pri-miRNAs. Pre-miRNAs are exported to cytoplasm by exportin-5, and there occurs the next processing step. In the cytoplasm the loop of the pre-miRNAs is cleaved by Dicer (another RNse III enzyme) in association with

HIV-1 TAR RNA binding protein (TRBP), an essential RNA-binding protein and Protein ACTivator of the Interferon-induced protein kinase (PACT) (in humans) and pre-miRNAs are processed into a 19-24 nucleotide miRNA duplex (Vasilatou et al., 2010). After Dicer-mediated cleavage, Dicer and its interactors TRBP or PACT dissociate to from the miRNA duplex (Winter *et al.*, 2009). To form the active RNA induced silencing complex (RISC) that performs gene silencing, the double stranded miRNA is separated by a RNA helicase and the mature strand is loaded together with Argonaute (Ago) protein into the RISC, while the passenger strand is degraded.

The strand that remains stably bound to RISC represents the mature miRNA and is thought to be the one whose 5' end is thermodynamically more unstable, thus becoming more easily unwounded by the helicase (Teixeira, 2013). MiRNA loaded RISC recognizes 3'-Untranslated Region (UTR) regions of mRNAs and if there is full complementary between miRNA and mRNA degradation occurs, while partial complementarily causes translational repression. The miRNAs bind to the target transcript by their nucleotides 2-8, counted to the 5' end, which are called the "miRNA seed" (Figure 11) (Teixeira, 2013, Tuccoli *et al.*, 2006, Vasilatou *et al.*, 2010). This post-transcriptional inhibitory mechanism is of critical importance in fundamental cell processes, including development, proliferation, survival and death (Vasilatou *et al.*, 2010).

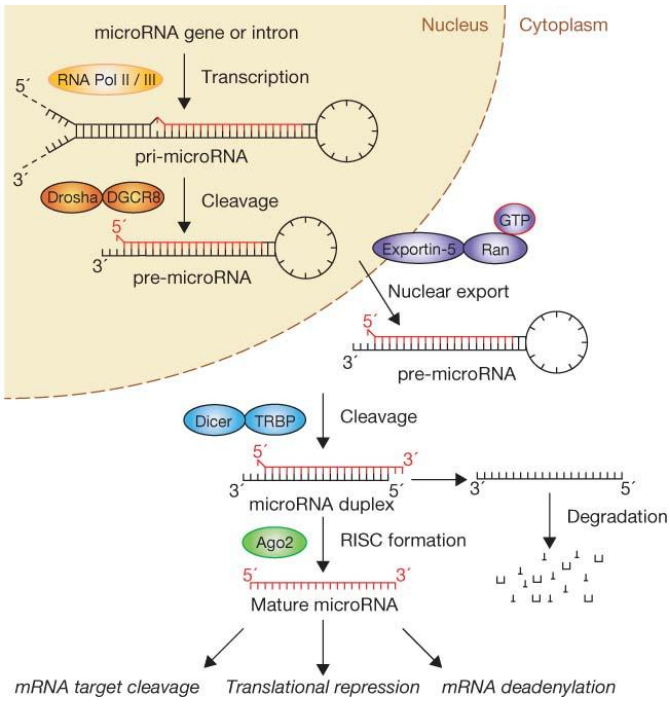

Figure 11. MicroRNA biogenesis. RNA polymerase II or III transcribes miRNA genes, generating long primary transcripts (pri-miRNAs) that are cleaved by the microprocessor complex Drosha-DGCR8 in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported by Exportin 5-Ran-GTP to the nucleus.

In the citoplasma, pre-miRNA is processed into mature miRNA by Dicer in a complex with double-stranded RNA-binding protein TRBP. The functional strand of mature miRNA is loaded together with Argonaute (Ago2) proteins into RISC (RNA-induced silencing complex). Once incorporated into RISC, the miRNA guides it to silence the target genes by degradation of the mRNA through direct cleavage, by inhibiting protein synthesis or by mRNA deadenylation (adapted from Winter *et al.*,2009).

1.6.2. Regulation of gene expression by miRNAs

The mechanism by which miRNAs regulate gene expression is still under investigation. Each miRNA can have one or more target mRNA while each mRNA may be regulated by one or more miRNAs (Vasilatou *et al.*, 2010). The great complexity of miRNA-mediated regulation relies on their ability to cooperate: various miRNAs can bind to the same mRNA and thereby regulate together the expression of a single mRNA, providing a stronger translational inhibition. Additionally, while some miRNA regulate the expression of various genes simultaneously others regulate specific individual targets. Many miRNAs are expressed in a tissue-specific or developmental-stage-specific manner. It comes out clear that miRNAs expression must be tightly regulated, because it has to be restricted to a particular development stage or cell type (Tuccoli *et al.*, 2006). Growing evidence suggests that miRNAs can be regulated at the levels of miRNA promoter transcription, methylation, miRNA processing, RNA editing and miRNA-target interactions (Breving and Esquela-Kerscher, 2010). Various studies have shown that miRNAs aberrant expression is associated with various pathological conditions including a wide variety of diseases broadening from diabetes, to cardiovascular or to various types of cancers (Teixeira, 2013).

1.6.3. Epigenetic control of miRNA expression

Compared to miRNA biogenesis that has been intensively studied and is well described, much less is known about the regulation of miRNAs expression. Genes encoding miRNAs are as tightly regulated as any other gene in the genome and in this way miRNAs expression is also submitted to similar epigenetic regulation. Epigenetic regulation of protein-coding genes can affect the expression of miRNAs as many of them are located in introns of protein coding genes and are regulated by the promoter of the host gene (Singh and Campbell, 2013). Approximately half of the human promoter regions contain CpG-rich regions, so it is not surprising that the expression of miRNAs can be affected by promoter hypermethylation or global hypomethylation (Figure 12) (Rouhi *et al.*, 2008).

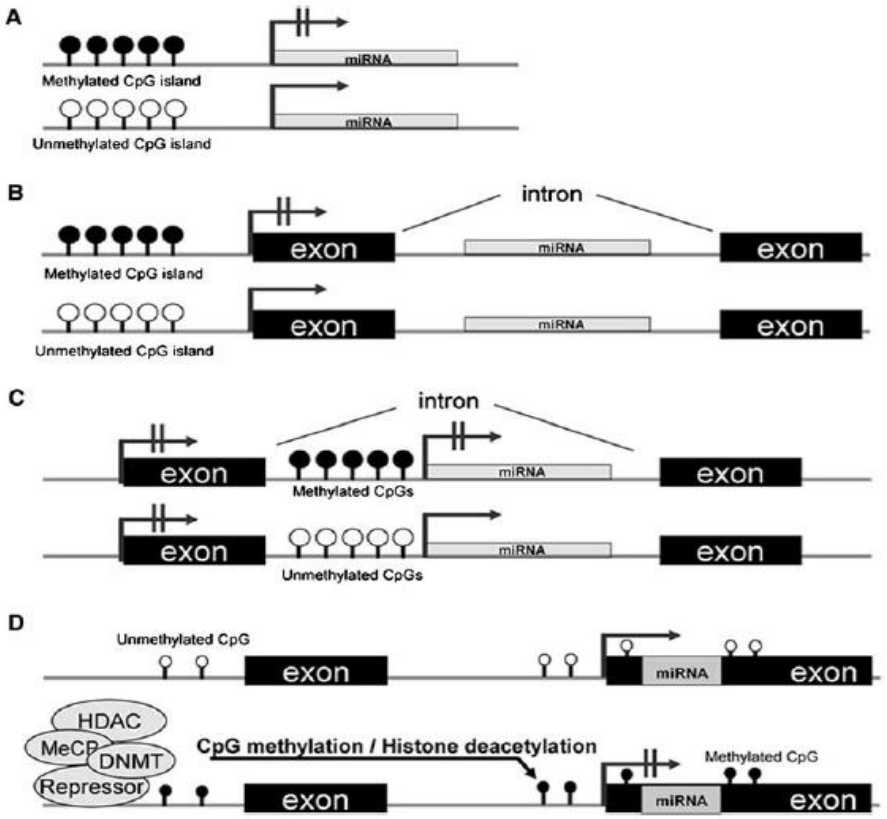

Figure 12. Epigenetic control mechanisms of miRNAs expression. (A) CpG island regulates transcription of an intergenic miRNA. **(B)** CpG island regulates transcription of a gene which hosts a miRNA. **(C)** CpGs regulate the transcriptional start site belonging to an intronic miRNA. **(D)** A transcription site recruits DNA and histone modifying enzymes to epigenetically regulate a miRNA within a gene which is surrounded by CpGs (adapted from Rouhi *et al.,*2008).

However methylation is not the only epigenetic mechanism that can affect miRNAs expression. Scott and collaborators (Scott *et al.*, 2006) showed that histone deacetylase inhibition is followed by extensive and rapid alteration of miRNAs levels (Iorio and Croce, 2012). In normal breast tissues, miR-155 was proposed to be epigenetically repressed by the wild-type *BRCA1*, which interacted with HDAC2 to deacetylate H2A and H3 on the miR-155 promoter. Nonetheless, the loss or mutation of *BRCA1*, resulted in miR-155 upregulation, as HDAC2 could not be recruited to the miR-155 promoter (Liu *et al.*, 2013).

1.6.4. MicroRNA control of epigenetic mechanisms

MicroRNAs can also control the epigenetic machinery including DNA methylation and histone modifications by regulating the expression of important epigenetic modifiers involved in the methylation or acetylation of DNA and histones.

These miRNAs are called "epi-miRNAs", and their aberrant expression is often related to development or progression of human cancer. First evidence of the existence of these epi-miRNAs was the discovery that miR-29 family is able to directly target the *de novo* DNA methyltransferases DNMT-3A and 3B in lung cancer. More recently, maintenance DNA methyltrasnfersase DNMT1 was also associated, although indirectly, through regulation of the transactivator Sp1. Introduction of miR-29s into lung cancers and AMLs resulted in reactivation of silenced tumor suppressor genes and inhibition of carcinogenesis (Iorio *et al.*, 2010). MiR-101 is another miRNA with tumor suppressive properties linked to epigenetic machinery, reported to be down-regulated in different tumor types. As recently shown (Friedman *et al.*, 2009), miR-101 is able to directly modulate EZH2 expression, which mediates epigenetic gene silencing by trimethyling histone H3 lysine 27 (H3K27me3) and is involved in silencing tumor suppressor genes in cancer. Transfection of miR-101 in bladder cancer cell lines and stable knockdown of EZH2 created a similar suppressive phenotype. The loss of miR-101 and consequent overexpression of EZH2 seems to alter the global chromatin structure in cancer. Enzymes regulating histone acetylation may also be directly regulated by epi-miRNAs, such as HDAC4, which is a direct target of miR-1 and miR-140 and HDAC1 regulation is mediated by miR-449a (Iorio *et al.*, 2010).

MicroRNAs can be considered part of a multilevel regulatory mechanism involved in modulation of gene expression. They are able to silence specific target molecules at post-transcriptional level, including members of the epigenetic machinery and are also strictly regulated by epigenetic events such as DNA methylation and histone modifications. An altered balance of the components of this network may lead to pathological conditions such as cancer (Iorio *et al.*, 2010).

1.7. Cancer and microRNAs

The first direct link between miRNAs and cancer was made by Carlo Croce and colleagues in 2002, describing that miR-15a and miR-16a, located on chromosome 13q14, are frequently deleted and/or down-regulated in patients with B cell chronic lymphocytic leukemia (B-CLL) (Calin *et al.*, 2002). This result provided the first evidence that miRNAs could be involved in the pathogenesis of human cancer as the deletion of chromosome 13q14 caused the loss of these two miRNAs (Calin et al, 2002). Indeed, study of a large

collection of chronic lymphocytic leukemias (CLLs) showed knock down or knock out of miR-15a and miR-16-1 in approximately 69% of CLL patients (Iorio and Croce, 2012).

This discovery triggered the investigation of the chromosomal location of miRNAs. Interestingly another study of Calin *et al.* (2004) showed that many human miRNAs genes are frequently located at fragile sites as well in genomic regions associated with cancer, suggesting that miRNAs are involved in the development of human cancer (Shenouda and Alahari, 2009).

There are many mechanisms and biological alterations that underlie the abnormal miRNA expression in cancer, including transcriptional deregulation, mutations, DNA copy number abnormalities and defects in the miRNA biogenesis machinery. Epigenetic changes like DNA methylation and histone modifications are also responsible for aberrant miRNA expression. Several miRNAs, such as miR-1, miR124a and Mir-127, are embedded in CpG island region and are epigenetically silenced by promoter hypermethylation and histone modifications, thus causing them to be under epigenetic control in human cancers (Hatziapostolou and Iliopoulos, 2011). In cancer, miRNAs function as regulatory molecules, acting as oncogenes (named oncomirs) or tumor suppressors (named tumor suppressors miRNAs). Therefore, miRNAs whose expression is increased in cancer and can down-regulate tumor suppressors or other genes involved in cell differentiation, conferring them an oncogenic role by simulating proliferation, angiogenesis and invasion, these miRNAs act as oncomirs. On the other hand, miRNAs whose expression is usually decreased in cancer and normally down-regulate different proteins with oncogenic activity act as tumor suppressors miRNAs (Figure 13) (Shenouda and Alahari, 2009). However this classification needs to be used with caution, as a miRNA can function as an oncomir or a tumor suppressor miRNA depending on the cellular context or tissue (Teixeira, 2013).

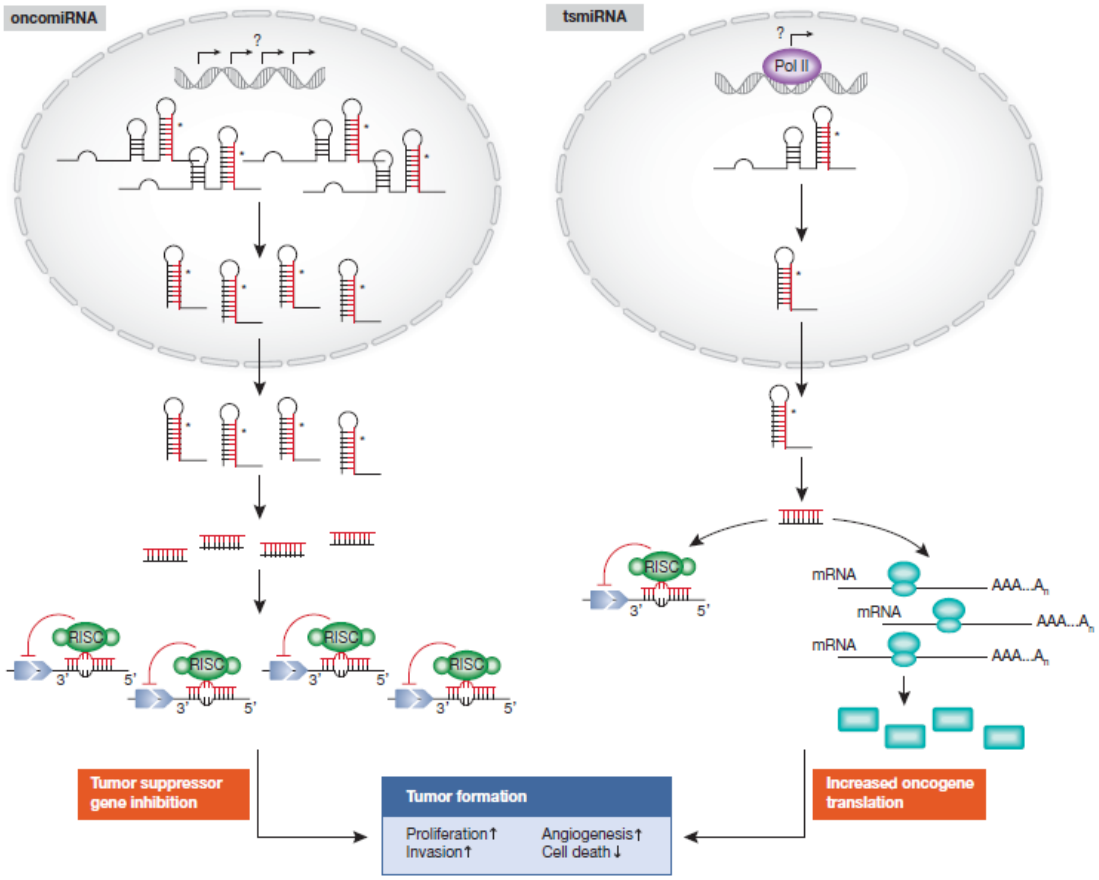

Figure 13. MicroRNAs as oncogenes and tumor suppressor genes. OncomiRNAs are actively transcribed in cancer and inhibit tumor suppressor genes, simulating proliferation and invasion. Tumor suppressor miRNAs (tsmiRNAs) expression is usually decreased in cancer and contributes to increase oncogene translation resulting in increase of angiogenesis and escape cell death. (adapted from Iorio and Croce, 2012).

In a study, Dong Wang and colleagues (Wang *et al.*, 2010) performed a comprehensive analysis of putative human miRNAs oncogenes and tumor suppressors. They found that oncogene and tumor suppressor miRNAs showed different patterns in function, expression, chromosome distribution, transcription factors and targets. Oncomirs were more frequently found in amplified regions in human cancers, whereas tumor suppressors miRNAs were located mainly in the deleted regions. This indicates that in human cancer oncogene miRNAs often gain function, while tumor suppressor miRNAs frequently lose function. Oncogene miRNAs tend to cleave target mRNAs more frequently than tumor suppressor miRNAs. These results indicate that these two types of cancer-associated miRNAs play different roles in cancer formation and development.

1.7.1 MiR-21

MiR-21 was one of the first miRNAs detected in the human genome and is frequently up-regulated in cancer having the majority of its reported targets as tumor suppressors. It has been involved in promoting tumor growth, cell proliferation, inflammation and angiogenesis. In particular, miR-21 is strongly involved in apoptosis regulation, being known to evade cell death (Feng *et al.*, 2010).

A study by Talotta and colleagues (Talotta *et al.*, 2009) showed that in response to RAS signaling, Activator Protein 1 (AP-1) induces miR-21 expression. MiR-21 induced by AP-1 triggers the downregulation of both Phosphatase and Tensin Homolog (PTEN) and Programmed Cell Death 4 (PDCD4), in response to the RAS oncoprotein. PDCD4, in turn, is necessary for the maximal stimulation of AP-1 activity in response to RAS signaling. Both, PTEN and PDCD4 dowregulation dependent of RAS is predominantly mediated by miR-21. The results point to miR-21 as a target and a regulator of AP-1, because it is at the same time controlled by AP-1 and necessary for the maximal induction of AP-1 activity in RAS-transformed cells (Figure 14).

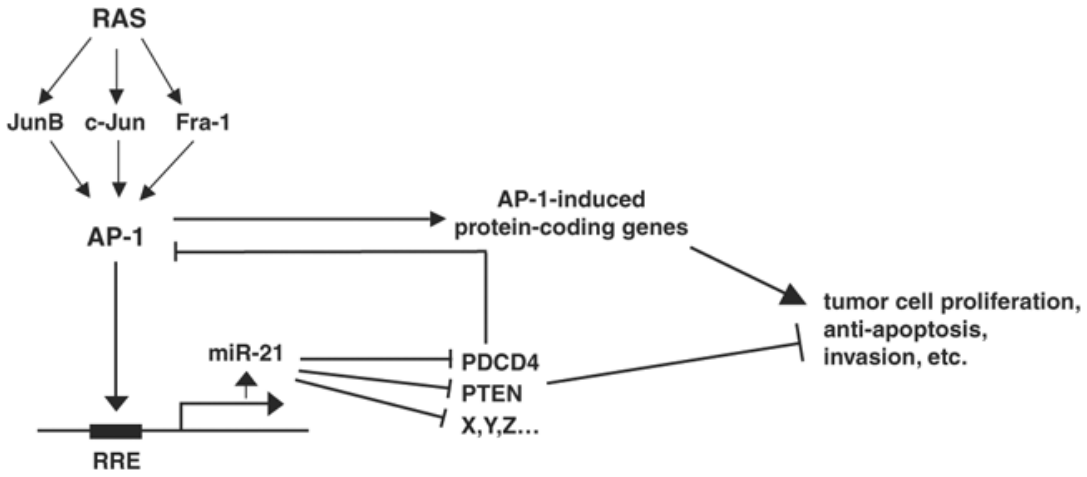

Figure 14. Scheme of the functional interactions between AP-1, miR-21 and PDCD4 in RAS dependent tumorigenesis. RRE indicates the AP-1-regulated RAS-responsive-enhancer of miR-21 promoter. X, Y, Z... indicates the many unidentified miR-21 target transcripts (adapted from Talotta *et al.*,2009).

PDCD4 has also been reported to be a mediator of Lipopolysaccharides (LPS)-induced apoptosis. The results presented by Sheedy *et al.* (2010), established that after LPS stimulation, PDCD4 induces apoptosis by inducing IL-6 and NF-κB. Besides inducing apoptosis NF-κB also induces miR-21 expression, by a process that requires MyD88 and NF-

κB subunit p65, since bone-marrow-derived macrophages (BMDMs) lacking MyD88 and mouse embryonic fibroblasts deficient in p65 failed to induce miR-21 in response to LPS. NF-κB by inducing miR-21 expression inhibits PDCD4 translation, what would lead to decrease of NF-κB activity, creating a negative feed-back loop. PDCD4 protein expression also decreases by a process that involves proteasome-dependent protein degradation, mediated by AKT/mTOR signaling (Young *et al.*, 2010). PDCD4 and miR-21 provide key links between inflammation and oncogenesis.

In the case of PTEN, miR-21 downregulation of this gene leads to increase activity of AKT and reduces apoptosis in cancer. PTEN inhibits AKT pathway by reversing the phosphorylation of PI3K. PTEN inhibits the activation of NF-κB. AKT is known to promote the NF-κB pathway, so miR-21 overexpression would result in increased NF-κB activation (Figure 15) (Choudhury and Li, 2012).

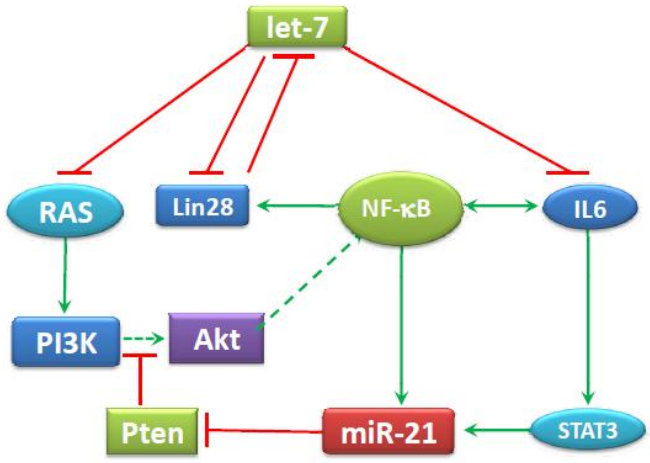

Figure 15. MiR-21 enhances the NF-κB signaling pathway. MiR-21 downregulates PTEN, a negative regulator of the PI3K/AKT pathway that elevates NF-κB activation, promoting in this way NF-κB activity (adapted from Choudhury and Li, 2012).

Recent studies have shown that miR-21 is overexpressed in various types of cancer and contributes to tumor resistance to chemotherapy. One of those studies by Haitao Bai and colleagues *(Bai et al., 2011)* demonstrates that resistance of the chemotherapeutic agent Daunorubicin (DNR) in K562 cell leukemic line is related to upregulation of miR-21 expression. MiR-21 may regulate survival of leukemia cell lines by downregulating PTEN expression and activating the PI3K/AKT pathway. The involvement of PDCD4 in DNR-induced resistance in K562 cells was also accessed. This group found that overexpression of miR-21 in K562 cells or knockdown of miR-21 in K562/DNR cells did not influence PDCD4

protein levels, suggesting that the later is not involved in drug resistance to DNR in this cell line.

1.7.2. MiR-125b

MiR-125b is a homologue of Caenorhabditis elegans miRNA lin-4, being one of the most conserved miRNAs. It can behave as either a tumor suppressor or an oncogenic miRNA, depending on the context *(*Tili *et al.*, 2012).

It has been reported that miR-125b expression decreases shortly after LPS stimulation of RAW264.7 macrophages, implying that it might have anti-inflammatory effects. The primary transcript that produces miR-125b is controlled in part by the AKT pathway *(*Androulidaki *et al.*, 2009, O'Connell *et al.*, 2011), which is activated by LPS (Figure 16). In contrast, miR-155 expression is increased by LPS and attending to the proinflammatory environment present in near 25% of tumors, the concurrent up-regulation of miR-155 and down-regulation of miR-125b can be involved in the onset of some neoplasias (Tili *et al.*, 2012).

Beyond these examples, there is evidence that miR-125b directly targets and down-regulates proapoptotic factors, such as BCL2 homologous Antagonist/Killer (BAK1), BCL2 Modifying Factor (BMF) and lin28 (Figure 16) (O'Connell *et al.*, 2011). Le *et al.* (2009) also demonstrated that miR-125b is a negative regulator of p53 in humans, suppressing apoptosis. Bousquet and colleagues (Bousquet *et al.*, 2010) showed that overexpression of miR-125b is able to accelerate the tumorigenicity of the BCR-ABL fusion protein and to confer a proliferative advantage to BCR-ABL leukemic cells. They also showed that miR-125b is able to induce primary lymphoid or myeloid leukemia in mice. Other studies also established a relation between miR-125b overexpression and fusion proteins such as the TEL-AML1 fusion protein in acute lymphocytic leukemia (ALL) patients and the translocation PML-RARA in pediatric AML (Bousquet *et al.*, 2010).

MiR-125b has been reported to contribute to tumor resistance to chemotherapy. Overexpression of miR-125b was showed to induce resistance in K562, THP-1 and Jurkat cells to the drug DNR by reducing apoptosis, while the suppression of miR-125b was found to enhance DNR cytotoxicity in REH cells. Furthermore, it was observed that miR-125b mediated DNR resistance in leukemia cell lines through decreasing expression of G protein-coupled receptor kinase 2 (GRK2) and p53-upregulated modulator of apoptosis (PUMA), which were shown to be direct targets of miR-125b (Zhou *et al.*, 2014).

Interestingly, miR-125b was found to confer resistance to Paclitaxel in breast cancer cells and to Cislatin in ovarian cancer cells, through the same mechanism: by targeting pro-apoptotic BCL-2 antagonist killer 1, and suppressing its expression (Kong *et al.*, 2011, Zhou *et al.*, 2010).

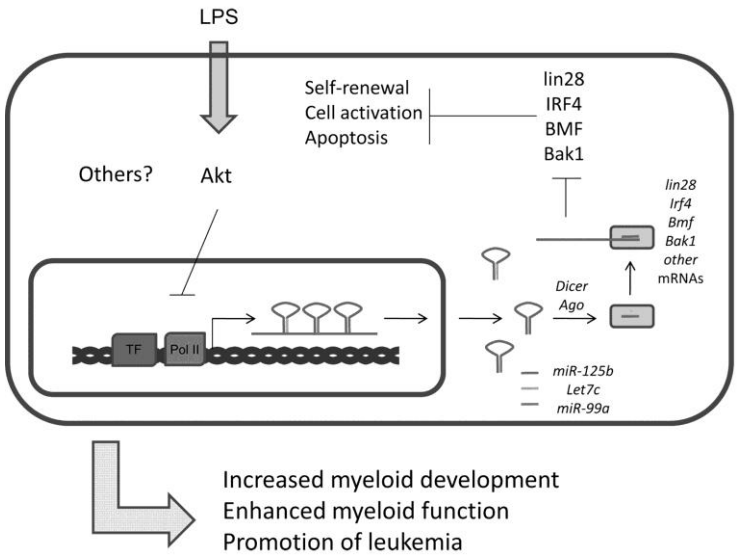

Figure 16. MiR-125b functions. MiR-125b transcription is controlled by the AKT pathway. Important targets of mir-125b include lin28, BAK1 and BMF, which are important in cell survival and apoptosis (adapted from O'Connel *et al.*, 2011).

1.7.3. MiR-155

MiR-155 was one of the first miRNAs identified in the hematopoietic system due to its high expression levels in certain types of lymphomas (O'Connell *et al.*, 2011). MiR-155 is a product of B-cell integration cluster (*BIC*) transcript. The presence of the double-stranded fold-back motif on *BIC* RNA would later be recognized as the precursor hairpin encoding miR-155, showing that this miRNA was produced from *BIC* transcripts. In matter of fact, miR-155 is only found in cells expressing *BIC* transcripts. The only evolutionarily conserved sequence of the *BIC* gene is the hairpin from which miR-155 is processed, suggesting that *BIC* oncogenic functions are through miR-155. In humans, both miR-155 and *BIC* transcript levels are elevated in different malignancies such as diffuse large B cell lymphoma (DL-BCL), Hodgkin lymphoma, B-CLL and also acute myelomonocytic leukemia. Besides hematological malignancies, miR-155 was described to be up-regulated in solid tumors such as breast, colon and lung cancers (Teng and Papavasiliou, 2009, Tili *et al.*, 2009). MiR-155 is expressed at low levels in most hematopoietic cells, under steady-state conditions, with basal expression higher in hematopoietic stem and progenitor cells (HSPCs) when compared with other more mature bone marrow lineages (O'Connell *et al.,* 2011).

The oncogenic properties of miR-155 are associated to its capacity to enhance cellular proliferation and its inhibition of apoptosis through down-repression of caspase-3 activity and also the targeting of FADD (FAS associated death domain), TP53BP1 (Tumor suppressor p53-binding protein 1) and Ikappa B kinase epsilon (IKKepsilon) (Tili *et al.*, 2009).

Recent studies (Lee *et al.*, 2012) show that miR-155 is associated with the loss of SHIP-1, an enzyme involved in the PI3K/AKT pathway. The *BIC* transcript regulation is under the transcriptional control of NF-κB and AP-1 when myeloid cells are exposed to inflammatory stimuli. While these factors increase *BIC* transcription, *BIC* transcription levels appear to be down-regulated by Signal transducer and activator of transcription 3 (STAT3) and AKT (Figure 17) (O'Connell *et al.*, 2011, Tili *et al.*, 2009). Using proteasome inhibitors of NF-κB activity, Tili and colleagues (Tili *et al.*, 2007) observed that miR-155 might be at least transiently under the direct control of NF-κB transcriptional activity. Overall, NF-κB, under particular circumstances, might control the expression of miR-155.

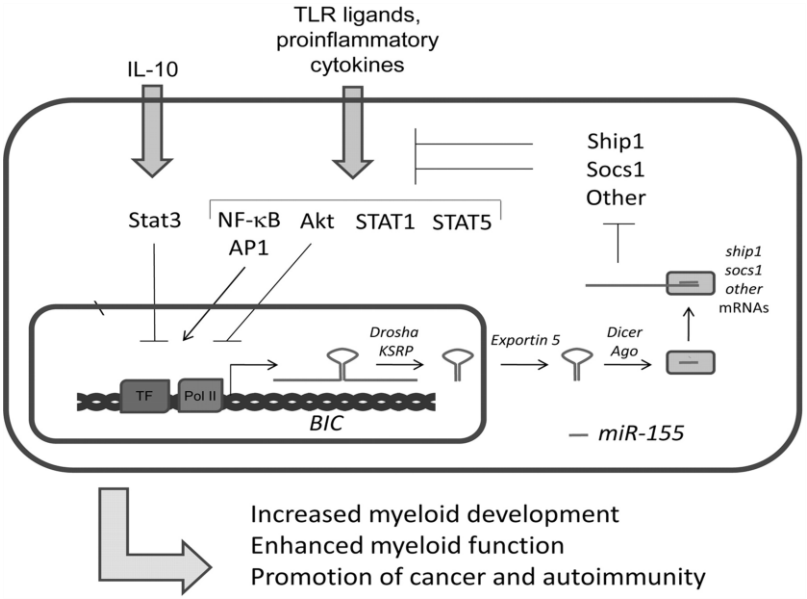

Figure 17. MiR-155 regulation and functions. MiR-155 targets mRNAs encoding SHIP1, SOCS1 and other proteins. In turn, SHIP and SOCS1 normally inhibit inflammatory responses that lead to the activation of AKT dependent pathways (that inhibit miR-155 transcription) and NF-κB (that up-regulates miR-155 transcription) (adapted from O'Connell *et al.,* 2011).

MiR-155 has been associated to drug-resistance in osteossarcoma (Chen *et al.*, 2014) and to inhibition of the sensitivity of lung cancer cells to the therapeutic agent Cisplatin (Zang *et al.*, 2012).

In conclusion, in this continuously changing field, there have been numerous discoveries that have allowed uncovering the miRNAs functions in the most diverse areas, including myeloid biology. Because they act on the pre-existing mRNA, miRNAs functions are highly dependent in a lot of factors, such as the cell type in which are induced (O'Connell et al., 2011). MicroRNAs represent a new layer of genetic regulation in the central dogma of molecular biology (DNA-mRNA-protein) (Rouhi *et al.*, 2008). Understanding the regulatory mechanisms of the miRNA pathway will allow the development of new biomarkers and therapeutic strategies based on the target genes of the miRNA that will enable treating diseases such as cancer through the regulation of oncogenic or tumor suppressor pathways in human patients (Breving and Esquela-Kerscher, 2010) and also help to prevent resistance to chemotherapeutic drugs by identifying the patients more suitable for a certain therapy.

However, the role of miRNAs in CML, in particular in monitoring therapy response, in resistance to TKI and how to circumvent it is not totally clarified.

Aims:

What we pretend with this work is:

- To evaluate the expression levels of miRNAs (miR-21, miR-125b and miR-155) in K562 cells (a model of CML *in vitro*) sensitive and resistant to Imatinib.
- To evaluate the therapeutic potential of Bortezomib, Parthenolide and Everolimus in K562 cells sensitive and resistant to Imatinib.
- See how Bortezomib, Parthenolide and Everolimus modulate miRNAs expression levels and correlate them with the response to therapy.

Expected results:

We hope to contribute to clarify the role of these miRNAs in the response to Imatinib, namely in Imatinib resistance, and to new targeted drugs, that translated to clinical practice could be used as new prognostic biomarkers to predict and monitoring drug response in CML patients.

SCIENTIFIC PAPER

Evaluation of the expression of microRNAs in the response to therapy in Chronic Myeloid Leukemia

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Abstract

Imatinib (IMA), the first specific inhibitor of the tyrosine kinase BCR-ABL1, is at present, the first-choice treatment for patients Chronic Myeloid Leukemia (CML). Despite the good results obtained, some patients show primary resistance or relapse after an initial response (secondary or acquired resistance). In an attempt to respond to IMA resistance, novel therapies have emerged aiming to improve patients clinical response and overcome resistance to IMA. Recent studies have shown that microRNAs (miRNAs) altered expression may be involved in several types of cancer and may also contribute to tumor resistance to chemotherapy. However, their role in IMA resistance and in the efficacy of new drugs in circumventing this resistance is not well known.

In this study, we investigated the basal expression levels of miR-21, miR-125b and miR-155 in an IMA sensitive CML cell line, the K562 cells, and in a derivative cell line resistant to IMA therapy, the K562 RC cells. We also evaluated whether Bortezomib, Parthenolide and Everolimus mediated a cytotoxic effect in these cell lines and how these drugs modulated the miRNAs expression levels.

Our results show d that miR-21 and miR-125b were upregulated in the IMA resistant cell line K562 RC compared to its parental line K562. Upon exposure to the compounds we verified that expression levels were altered from K562 cells to K562 RC cells, with the decrease in the expression of miR-21 and 125b upon exposure to all compounds. Everolimus reduced expression levels in miR-155 contrary to the other compounds. We can hypothesize that the compounds in study present themselves as alternatives in the therapeutic approach in patients with CML resistant to IMA therapy. Additionally, miRNAs expression levels in CML patients could be used has biomarkers to predict an evaluated the response to IMA therapy or to select the better drug to the right patient.

1.Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder characterized by a chromosomal translocation, known as the Philadelphia chromosome. The result of this translocation is a fusion gene, the *BCR-ABL*, which encodes a fusion oncoprotein with unusual tyrosine kinase activity (Hehlmann *et al.*, 2007). This oncoprotein was the starting point for the development of Imatinb, the first line therapy of CML, a specific tyrosine kynase inhibitor that targets and inhibits the BCR-ABL tyrosine kinase activity (Frazer *et al.,* 2007). However, drug resistance is considered to be a multifactorial phenomenon that involves several major mechanisms. Resistance to Imatinib has been associated with mutations in the BCR-ABL kinase domain, reduced apoptosis, changes in drug metabolism, and in membrane drug transporters (increase in efflux or decrease in influx) (Alves, 2011).

MicroRNAs (miRNAs) are small, non-coding RNAs that act post-transcriptionally and negatively regulate the expression of target genes by binding to the 3′UTR of mRNA. If complementary between miRNA and mRNA is complete, degradation occurs, while partial complementary only causes translational repression, decreasing the proteins levels they encode (Tuccoli *et al.*, 2006). This post-transcriptional inhibitory mechanism is of critical importance in fundamental cell processes, including development, proliferation, survival and death (Vasilatou *et al.,* 2010).

In particular, miR-21, miR-125b and miR-155 have shown to have influence in oncogenesis and drug resistance (Bai *et al.*, 2011, Chen *et al.*, 2014, Zhou *et al.*, 2010). MiR-21 and miR-155 are frequently upregulated in cancer (Buscaglia and Li, 2011, Tili *et al.*, 2009) while miR-125b can behave either as a tumor suppressor or as an oncogenic miRNA, depending on the context (Tili *et al.*, 2012). All these three miRNAs are in some way involved with PI3K/AKT pathway, because they regulate some of its components, and/or are regulated by them (Choudhury and Li, 2012, O'Connell *et al.,* 2011). Recently, miRNAs have been related to resistance to Imatinib (Firatligil *et al.*, 2013, Li *et al.*, 2013).

Additionally, to try to improve patient's clinical response and overcome Imatinib resistance, novel therapies have emerged. Bortezomib, Parthelonide and Everolimus are therapeutic agents that act at different levels in ubiquitin-proteasome and PI3K/AKT pathways. Bortezomib is proteasome inhibitor (Cortes *et al.*, 2004), Parthenolide is a NF-κB inhibitor that acts through inhibition of IκK complex (Guzman *et al.*, 2005) and Everolimus is an inhibitor of mTOR (Melo and Chuah, 2008).

In this work, we evaluated the expression of miR-21, miR-125b and miR-155 in cell lines sensitive and resistant to Imatinib. In the same cell lines we also examined the therapeutic potential of novel therapies as Bortezomib, Parthenolide and Everolimus. Moreover, we correlate the levels of miRNAs with the response to these novel therapeutics.

2. Materials and Methods

2.1. Cell culture

The K562 cells, a Chronic Myeloid Leukemia cell line, established from the bone marrow of a 53 years old female patient with Chronic Myeloid Leukemia in blast crises, was purchased at American Type Culture Collection (ATCC). The cell line was routinely grown in *Roswell Park Memorial Institute* 1640 (RPMI1640) medium (L-glutamine 2 mM, HEPES-Na 25 mM, NaHCO 1.5 g/L, penicillin 100 U/mL and streptomicyn 100 µg/mL), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen), at 37°C and with 5% CO2 in an humidified incubator. The K562 cells were always seeded at an initial density of 0.5 million cells/mL (10^6 cel/mL).

The Imatinib resistant cell line, named K562 RC, was established in our lab after gradual and continued exposition to Imatinib,and maintained in culture under the same conditions of K562 cell line, but supplemented with 250 nM of Imatinib. The IC_{50} of Imatinib in this resistant cell line is 8 times higher than in K562 cells.

2.2. Incubation of the CML cell lines with antineoplasic drugs

The K562 and K562 RC cells were maintained in culture as described in the 1.1 section in the absence and presence of antineoplasic drugs: proteasome inhibitor – Bortezomib (BTZ) (5 nM to 100 nM); IκK inhibitor – Parthenolide (PTL) (1 µM to 20 µM) and mTOR inhibitor – Everolimus (EVE) (5 µM to 50 µM). All the studies were performed with an initial density of $0.5x10^6$ cel/mL, during a period of 72 hours.

2.3. Evaluation of cellular viability with resazurin metabolic assay

Cell viability was assessed by the resazurin assay. Briefly, rezasurin is a non-fluorescence blue compound (oxidated form) that penetrates the cells and acts as an electron acceptor. The oxidation-reduction reaction originates a pink fluorescent compound, resofurin. This color alteration is the result of the cellular metabolism and represents an indicator of cell viability and proliferation (Zhang *et al.*, 2004).

At each 24h, resazurin was added at final concentration of 10 µg/mL. The absorbance was analyzed at 570 nm (correspondent to the oxidated form) and at 600 nm

(correspondent to reduced form), being the difference between them a measurement of cell viability. The results represent the mean ± standard deviation (SD) of 3 to 6 independent experiences.

2.4 MicroRNAs quantification by q-RT-PCR

To evaluate microRNAs expression we used TaqMan MicroRNA Assays from Applied Biossystems. It is constituted by two steps: first we synthesize a cDNA chain from a specific primer of the microRNA with help of a reverse transcriptase (RT), and then occur an amplification of the sequence correspondent to a specific microRNA for every RT-PCR reaction. In the cDNA synthesis, we used a specific primer of RT-PCR, miRNA from *TaqMan MicroRNA* assays (Applied Biossystems) and reagents from *TaqMan MicroRNA Reverse Transcription Kit*. After the cDNA synthesis, we proceeded to the amplification of miR-21, miR-125b, miR-155 and RNU6B (endogenous control) by q-RT-PCR. For each miRNA, we used one *TaqMan MicroRNA Assay* with specific probes and primers. The miRNA quantification was determined in untreated cells and in cells exposed to 2.5 nM of BTZ, 5 μM of PTL and 5 μM of EVE for 48h.

2.5. Cell death evaluation

For all conditions tested, the cell death analysis was performed by optical microscopy, through morphological assessment of May-Grünwald-Giemsa stained slides, and by flow cytometry, using the Annexin V (AV) and Propidium Iodide (PI) double staining.

For the morphological evaluation, the cells were transferred to slides and fixed with FBS, stained and evaluated under light microscopy, using a Zeiss Axioskop2 equipped with Zeiss Axiocam ICc3. The images acquired were processed by Zeiss program.

The Annexin V and PI double staining allow the discrimination between live cells (AV-/PI-), early apoptotic cells (AV+/PI-), necrotic cells (AV-/PI+) and late apoptotic or necrotic cells (AV+/PI+). In resume, in the beginning of apoptosis phosphatidylserine, a phospholipid with negative charge, suffers a translocation from the inner side of the membrane to the outer side. AV is a molecule able to bind to phospholipids with negative charge in presence of calcium. As AV is bind to a fluorochrome, it enables to determine the location of phosphatidylserine in the membrane. This way, we can identify cells in early apoptosis. IP is a

DNA intercalator molecule that, within DNA chain, emits fluorescence. In early apoptosis cell membrane is still intact and IP is not capable to enter the cell and bind to DNA chain. In later stages of apoptosis and in necrosis, the nuclear envelope becomes discontinuous allowing IP to intercalate with DNA (Goncalves *et al.*, 2013). To perform this flow cytometry assay, cells were collected after 48h of treatment, washed with PBS (centrifuged at 500 xg for 5 min), resuspended in 100 µl of binding buffer and incubated with 1 µg of AV (BD Pharmingen) and 1 µg of IP (BioLegends) for 15 min in the dark. After incubation time, cells were diluted in 300 µl of binding buffer and analyzed using a FACScalibur flow cytometer. The experiments were performed in triplicated and the result analysis was performed using the Paint-a-Gate^{TM} program.

2.6. Assessment of BAX, BCL-2, p53, NF-κB and ubiquitin expression levels

The modulation of BAX, BCL-2, p53, NF-κB and ubiquitin conjugated proteins expression levels were analyzed in cells cultured in the absence and presence of Bortezomib, Parthenolide and Everolimus. Since they are intracellular molecules, it was necessary to fix and permeabilize the cellular membrane. Briefly, the cells were fixed using the kit IntraCell (ImunnoStep) by incubation with 100 µL of solution A for 15 min. After centrifugation, the cells were permeabilized with 100 µL of solution B (kit IntraCell) and incubated with 1 µg of anti-BAX (PE), 1 µg of anti-BCL-2 (FITC), 1 µg of anti-p53 (FITC), 1 µg of anti-phospho NF-κB (FITC) or anti-ubiquitin conjugated (PE) (Santa Cruz Biotechnology), for 15 min in the dark at room temperature. All the experiments were performed in triplicate and analyzed using a FACScalibur flow cytometer. The results were express as Mean Fluorescence Intensity (MFI) arbitrary units. This value represents the medium fluorescence intensity detected in the cells, which is proportional to the number of molecules labeled by the antibody.

2.7. Evaluation of Cell Cycle by flow cytometry

The cell cycle progression analysis was performed by flow cytometry, using detection kit PI/RNase (ImmunoStep). For that, cells were collected and washed with PBS for 5 min at 300 xg. The pellet was resuspended in 200 µL of cold 70% ethanol, during vortex agitation, being incubated during 30 min on cold. After incubation time, cells were washed again and resuspended in 300 µL of PI/RNase solution.

Cells were evaluated through CellQuest™ and data analyzed by Modfit™. Results are expressed by the percentage of cells in each phase of cell cycle with a mean ± SD of at least three independent experiments.

2.8. Study of AKT expression levels by western blot

We evaluated the effect of Bortezomib, Parthenolide and Everolimus on intracellular signaling through the expression of AKT, in total and in the phosphorylated form by western blot. First, cells were incubated with 2.5 nM of BTZ, 5 μM of PTL and 5 μM of EVE for 48h. After this period of time, we performed cell lysates with RIPA buffer (50 mM Tris HCl at pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 2 mM EDTA) supplemented with protease and phosphatase inhibitors [Complete Mini (Roche) and PhosphoSTOP (Roche)] and DTT. For that, cells were centrifuged at 12 000 xg for 10 min at 4°C to remove the nucleus and cellular debris. The protein content of each sample was assessed and then the proteins were denatured. For this purpose, the protein extracts were boiled at 95°C for 5 min in a denaturation buffer (Tris 0.5 mM, pH 6.8; 50% glycerol, 10% SDS, 10% 2β-mercaptoethanol and blue bromophenol). For the western blotting assay, protein was separated by adding 30 μg of total protein to each well of a 12% SDS-PAGE gel, and electrophoresis was performed during 60 min at 130 V. Posteriorly proteins were transferred to a PVDF membrane that was incubated overnight with the primary antibody against p-AKT, total AKT and β-actin. This enabled the detection of the immunocomplexes that were later quantified using enhanced chemifluorescence detection (ECF) reagent. Total AKT was used as a loading control to p-AKT and β-actin was used loading control to total AKT.

3. Results

3.1 Bortezomib, Parthenolide and Everolimus decrease cell viability of K562 cell lines sensitive and resistant to Imatinib inducing apoptosis.

Initially we investigated if BTZ (5 nM to 100 nM), PTL (1 μM to 20 μM) and EVE (5 μM to 50 μM), influenced the proliferation and survival of K562 and K562 RC cells. The exposure of K562 and K562 RC cells to BTZ, PTL and EVE induced a decrease on cell viability in a dose and time dependent manner, as shown in Figure 1. After 48h of exposure, the effect of BTZ and PTL was slightly more pronounced in K562 resistant cell line than in their sensitive counterpart. On the other hand, the effect of EVE was more pronounced in the sensitive cells than in the resistant ones. At this time, reduction of cell viability to values close to 50% was achieved between 25 nM and 100nM of BTZ, between 10 μM and 15 μM of PTL and between 15 μM and 25 μM of EVE, in K562 sensitive cell line. In K562 RC cells to achieve the same effect lower doses were necessary for BTZ and PTL (25-50 nM and 5-10 μM, respectively) and in the case of EVE the doses were equal or slightly higher (15-25 μM). In both sensitive and resistant cells after 48h the effect is less dose-dependent and there is a reverse of the effect, more pronounced in the resistant cell line.

A non-linear regression analysis was performed to more accurately determinate IC_{50} after 48h (Table 1), which confirm that K562 RC cells are more sensitive to BTZ and PTL and slightly less sensitive to EVE than K562 cells.

Table1. Analysis of IC_{50} of Bortezomib, Parthenolide and Everolimus in Chronic Myeloid Leukemia cell lines sensitive (K562) and resistant (K562 RC) to Imatinib.

Cell line	IC_{50} BTZ	IC_{50} PTL	IC_{50} EVE
K562	28.10 nM	12.20 μM	20.9 μM
K562 RC	23.41 nM	5.7 μM	25.9 μM

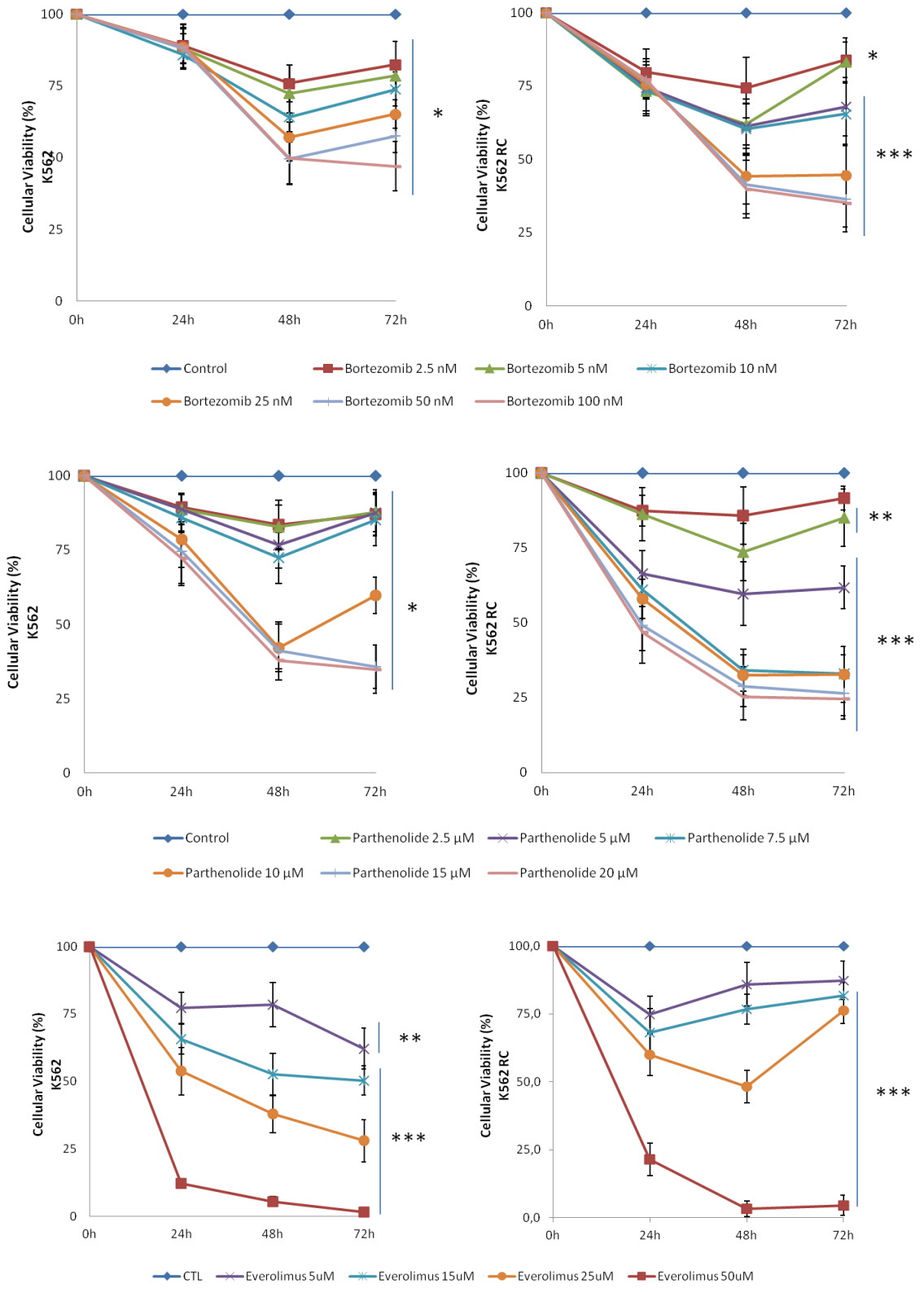

Figure 1. Viabilitty dose response curves in K562 and K562 RC cell lines. Cells were incubated at a density of 0.5 x 10^6 cells/ml, during 72h, in the absence or presence of increasing concentrations of Bortezomib (BTZ), Parthenolide (PTL) and Everolimus (EVE), as indicated in figure. Cell viability was established by resazurin method, as described in Materials and Methods. Cell viability is expressed in percentage (%), Data are expressed as mean±SD obtained from 3 to 8 independent experiments. Statistic analysis was performed by comparison with control by Tukey test: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

The type of cell dead induced by the compounds was analyzed by flow cytometry using annexin V/propidium iodide incorporation, as shown in Figure 2, and by optical morphology (Figure 3).

We can observe that BTZ, PTL and EVE induce a decrease in the percentage of live cells in both K562 cell lines, and leads preferentially to an increase in the apoptotic cells that is especially patent in the higher doses of the compounds BTZ 10 nM, PTL 10 µM and EVE 25 µM. In resistant cells, higher doses of PTL induce also high rates of necrosis, indicating that in this case dead is mediated by apoptosis and necrosis and there is activation of both cell death processes. Moreover, these results indicate that cell death is dose and cell type dependent.

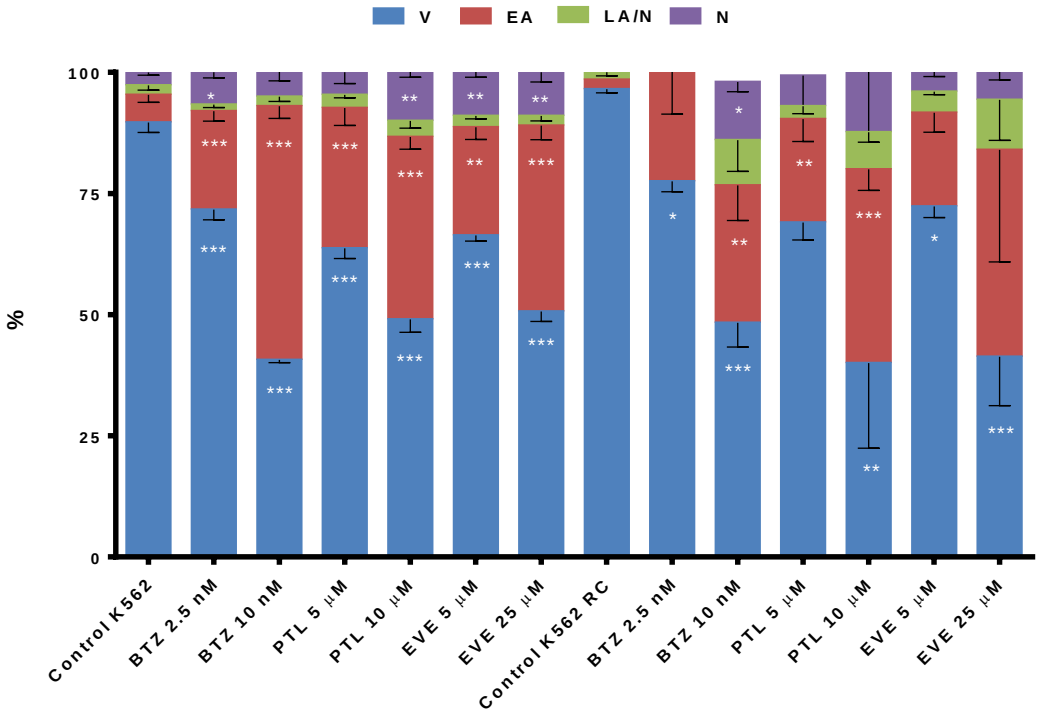

Figure 2. Analisys of cell death induced by Bortezomib, Parthenolide and Everolimus in K562 and K562 RC cells by flow cytometry. K562 and K562 RC cells were incubated in a density of 0.5 x 10^6 cells/ml, during 48h, in the absence and in the presence of BTZ, PTL and EVE in the concentrations indicated in figure. Cell death was detected by annexin v and propidium iodide staining and analyzed by flow cytometry. The results presented are expressed as percentage (%) of viable cells (V), early apoptotic cells (EA) late apoptotic/necrotic cells (LA/N) and necrosis (N), and represent the mean±SD obtained from three independent experiments. Statistic analysis was performed by comparison with control by Tukey test: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Morphologic features of K562 sensitive and resistant cell lines treated with BTZ, PTL and EVE confirm the results obtain by flow cytometry (Figure 3). In both cell lines, were

observed morphologic characteristics of apoptotic cell death, such has "blebbing", nuclear fragmentation and abnormal mitosis. Characteristics of necrosis, such as cell membrane ruptured were also observed, especially in the resistant cells treated with PTL. The great majority of cells depicted also shown vacuolization that can be the result of the toxicity induced by the drugs or the beginning of the autophagic process (Figure 3).

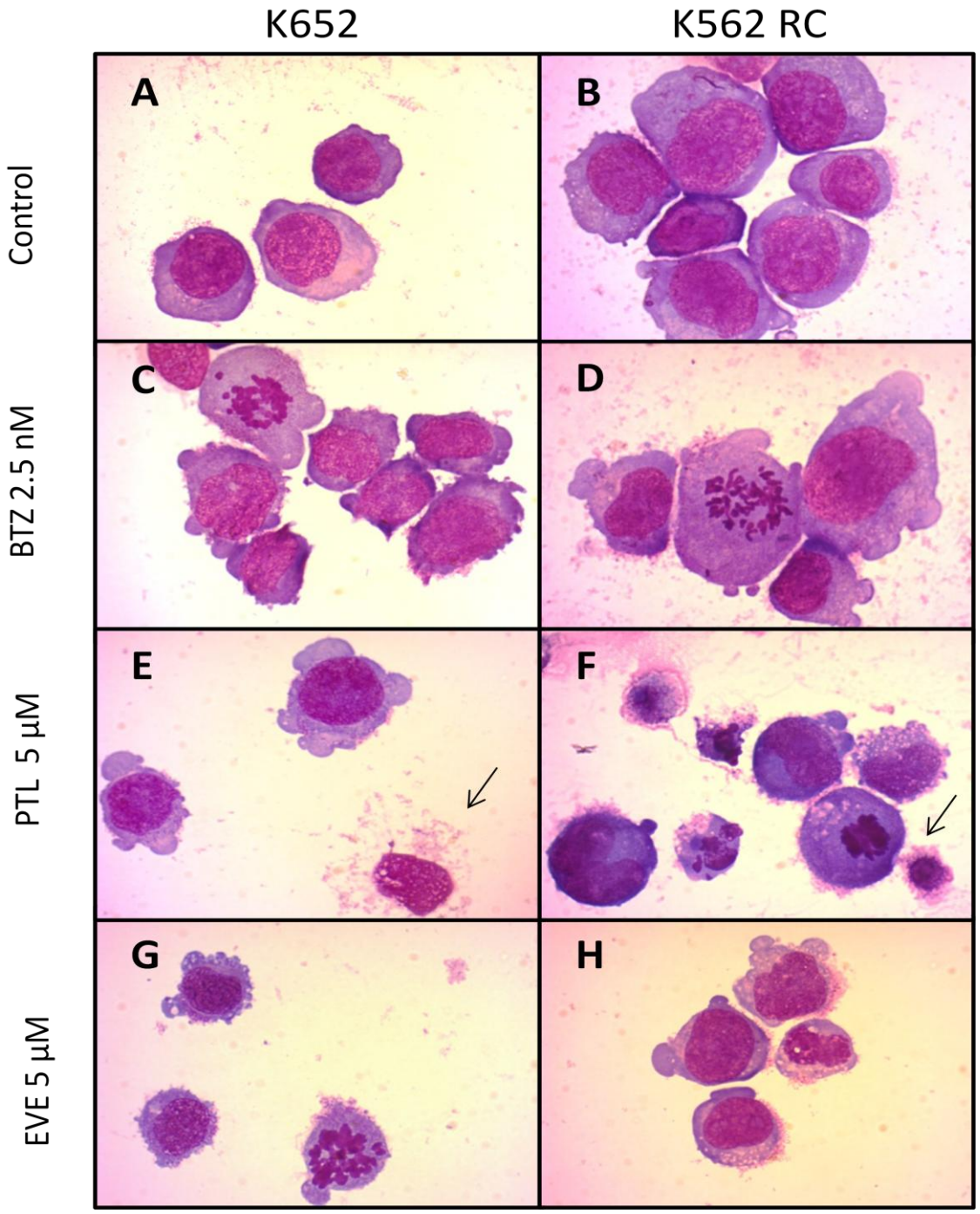

Figure 3. Morphological aspects of K562 and K562 RC cells treated with Bortezomib, Parthenolide and Everolimus. Control cells (A, B) and cells treated with BTZ (C, D), PTL (E, F) and EVE (G, H) were stained with Grünwald-Giemsa after 48h of incubation. Cells smears show morphologic features of apoptosis, such as blebbing, nuclear fragmentation, catastrophic mitosis and apoptotic bodies. Necrosis is also shown (black arrows). Amplification 500x.

In order to know how BTZ, PTL and EVE modulate apoptotic proteins, we analyzed the expression levels of p53, BCL-2 and BAX.

As shown in Figure 4, the p53 intracellular levels decrease in K562 RC cell line treated with PTL and EVE, and that decrease was more accentuated at lower doses of both compounds (5 μM). The exposure of the sensitive cell line to PTL resulted in a decrease of p53 expression levels. However, these sensitive cell line show an increase of the expression of p53 in the presence of both concentrations of EVE and with the lower concentration of BTZ, but a decrease of the levels with the higher concentration of this compound was also observed. However, the observed differences were not significant.

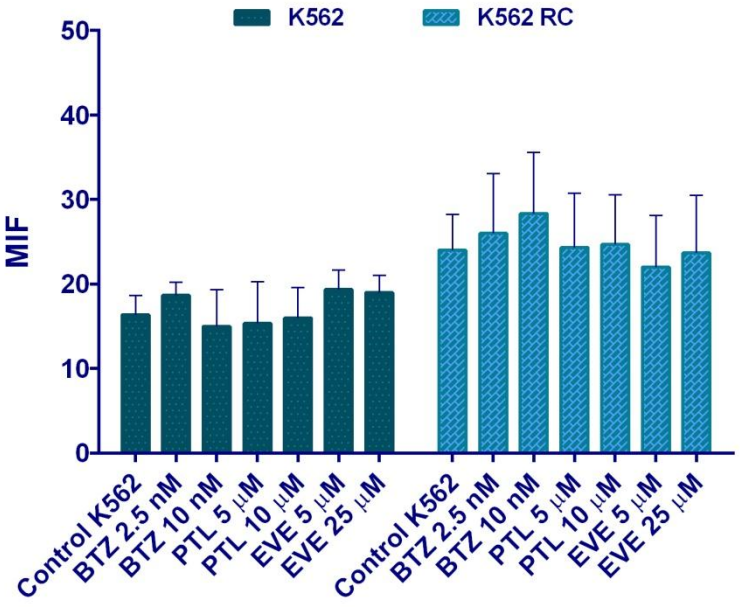

Figure 4. ntracellular expression levels of p53 in K562 and K562 RC cells expose to Bortezomib, Parthenolide and Everolimus by flow cytometry. p53 levels were analyzed by flow cytometry using anti-p53 (FITC). K562 cells and K562 RC cells were incubated during 48h with BTZ, PTL and EVE. Results are expressed as medium fluorescence intensity (MFI) and represent mean±SD of fluorescence intensity detected in three independent experiments. Statistic analysis was performed by comparison with control by Tukey test.

As represented in Figure 5A, a significant increase in intracellular BAX is observed in both cell lines when treated with the three compounds, separately. The increase is very marked in the resistant cell line when treated with BTZ or EVE. Moreover, we also observed a significant decrease in BCL-2 (Figure 5B) expression levels in both cell lines treated with BTZ, PTL and EVE, with an exception for the resistant cell line treated with BTZ, in which BCL-2 levels increase as well as BAX levels, however with a significant decrease in BCL-2/BAX ratio (Figure 5C).

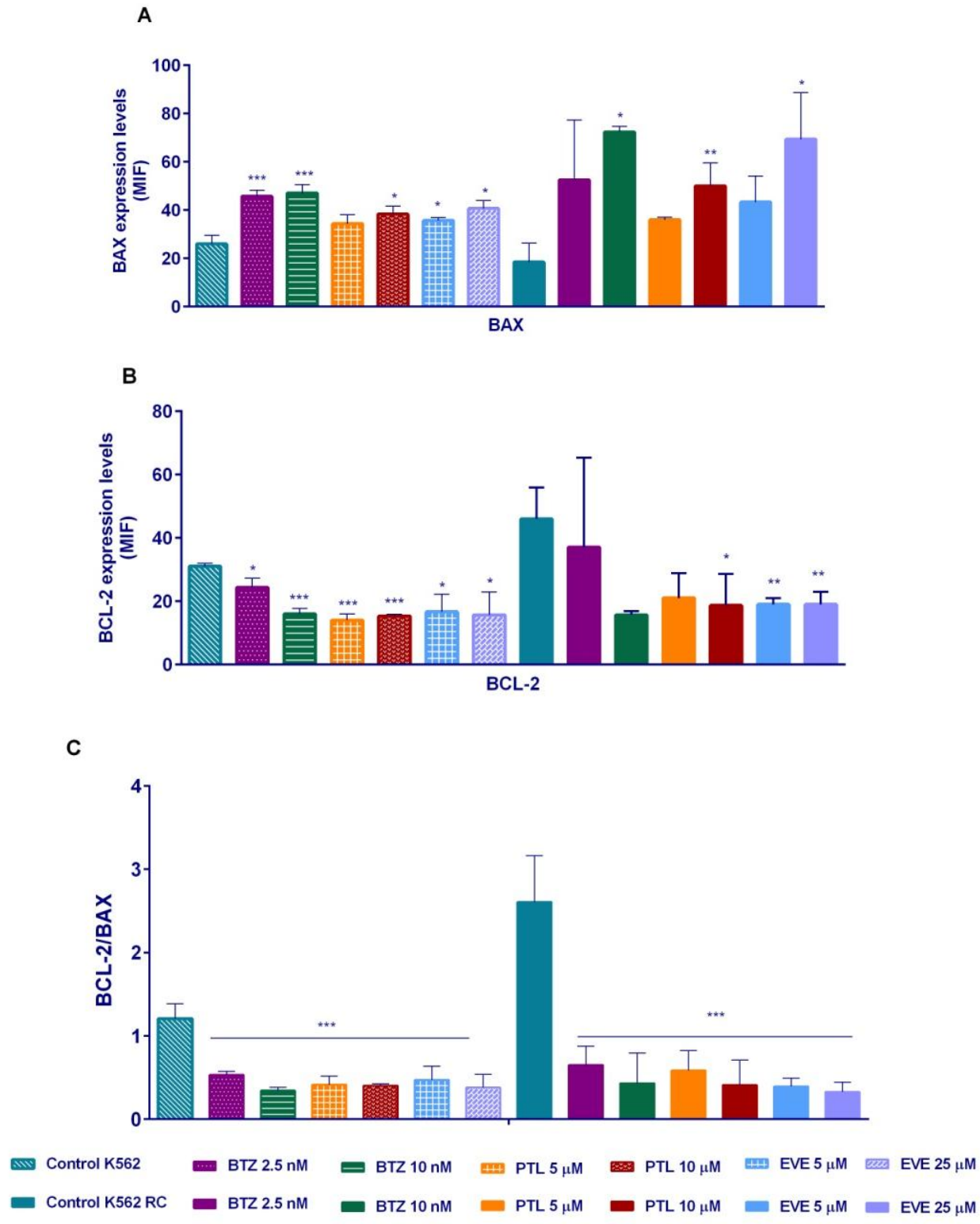

Figure 5. Intracellular expression of BAX (A) and BCL-2 (B) and BAX/BCL-2 ratio (C) in K562 and K562 RC cells expose to Bortezomib, Parthenolide and Everolimus by flow cytometry. BCL-2 and BAX levels were analyzed by flow cytometry using monoclonal antibodies. K562 cells (pattern) and K562 RC cells (fill) were incubated during 48h with BTZ, PTL and EVE in the concentrations indicated in figure. Results are expressed as medium intensity fluorescence (MIF) and represent mean±SD of fluorescence intensity detected in three independent experiments. BAX/BCL-2 ratio was estimated as the ratio between the MIF of each molecule, in the different study conditions. Statistic analysis was performed by comparison with control by Tukey test: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.2. Cell cycle analysis

With the intent to understand if the effects of the compounds studied are due to the activation of cellular check points, thereby inducing cell cycle arrest (and confirming the antiproliferative effect), we evaluated the distribution of cells trough the different cell phases.

As we can see in Table 2, in K562 sensitive cells treated with the higher dose of BTZ there is an increase in the percentage of cells that are arrested at G0/G1 phase, however the cells treated with the lower dose have no significant differences compared with control. In cells treated with the lower dose of PTL, cell arrest occurs at S phase. But, for the ones treated with the higher dose, we observe an increase in percentage of cells in G0/G1 phase. In the case of K562 cells sensitive to Imatinib treated with EVE, we can also observe a significant increase in the percentage of cells arrested at G0/G1. In K562 RC cells, exposure to BTZ provoked cell arrest at G2/M. On the other hand, PTL induced cell arrest at S phase and EVE at G0/G1 (Table 2).

Furthermore, in Table 2 we also can observe an increase in percentage of cells in sub G0/G1 peak in both cell lines treated with all drugs, confirming apoptosis previously described by other techniques.

Table 2. Evaluation of the effect of BTZ, PTL and EVE in cell cycle in K562 and K562 RC cells.

		Sub G0/G1	G0/G1	S	G2/M
K562	Control	0,0 ± 0,0	36,7 ± 0,6	54,7 ± 2,9	8,7 ± 3,2
	BTZ 2.5 nM	1,0 ± 1,0	35,7 ± 2,1	56,3 ± 1,5	8,0 ± 3,6
	BTZ 10 nM	13,0 ± 3,6 ****	42,3 ± 2,9	46,0 ± 7,2	11,7 ± 4,5
	PRT 5 µM	8,7 ± 1,2	34,0 ± 3,5	60,0 ± 7,8	6,0 ± 4,4
	PRT 10 µM	22,0 ± 2,6 ***	44,3 ± 1,2 *	43,4 ± 1,5	12,3 ± 0,6
	EVE 5 µM	3,3 ± 2,3 **	44,7 ± 1,5 **	50,0 ± 3,0	5,0 ± 4,4
	EVE 25 µM	10,7 ± 0,6 ****	66,3 ± 2,5 ***	27,7 ± 3,2 ****	5,3 ± 0,6
K562 RC	Control	0,5 ± 0,7	44,5 ± 3,5	41,0 ± 7,1	14,5 ± 3,5
	BTZ 2.5 nM	5 ± 0,0	34,5 ± 14,8	41,5 ± 2,1	15,0 ± 0,0
	BTZ 10 nM	21,7 ± 7,6	24,0 ± 8,7	38,3 ± 3,5	37,7 ± 11,8
	PRT 5 µM	2,7 ± 0,6	37,3 ± 2,5	51,0 ± 7,8	11,7 ± 5,5
	PRT 10 µM	20,3 ± 7,6	28,5 ± 2,1	54,0 ± 0,0	17,5 ± 2,1
	EVE 5 µM	5,3 ± 1,5	56,7 ± 0,6	33,7 ± 1,2	9,7 ± 0,6
	EVE 25 µM	38,0 ± 1,4 *	65,3 ± 0,6	29,3 ± 8,1	5,3 ± 8,4

The results presented are expressed as percentage (%) of cells present in each cycle phase, and represent the mean±SD obtained from three independent experiments. Statistic analysis was performed by comparison with control by Tukey test: * $p<0.05$, ** $p<0.01$,*** $p<0.001$ and **** $p<0,0001$.

3.3. Evaluation of NF-κB, ubiquitin conjugates and p-AKT levels

The NF-κB is an important molecule in the mechanism of action of Parthenolide, which is a NF-κB inhibitor through IKK inhibition. NF-κB is also related with the three miRNAs we were studying so we also analyzed the expression levels of phosphorylated NF-κB (NF-kB-p) to see how BTZ, PTL and EVE affected its intracellular levels.

In Figure 6A, we noted that in the sensitive cell line, the higher dose of PTL and EVE results in an increase of NF-κB levels, opposed to a decrease observed with the both doses of BTZ in the same cell line. This result with PTL higher dose is a very interesting result considering that PTL main gold is to prevent NF-κB activation. However, in K562 RC cells phosphorylated NF-κB levels decrease to values around 16 MIF, when compared to control (22 MIF) with the administration of both doses of BTZ and PTL. With EVE the values are 21.7 MIF to 5 μM and 19MIF to 25 μM, so it cannot be considered a significant decrease.

We also assed ubiquitin conjugates levels (Figure 6B), owing to the fact that one of the drugs under study, Bortezomib, is a proteasome inhibitor, and ubiquitin intracellular levels can shed light on proteasome activity. We also could obtain some results about how these drugs affect the ubiquitin proteasome pathway.

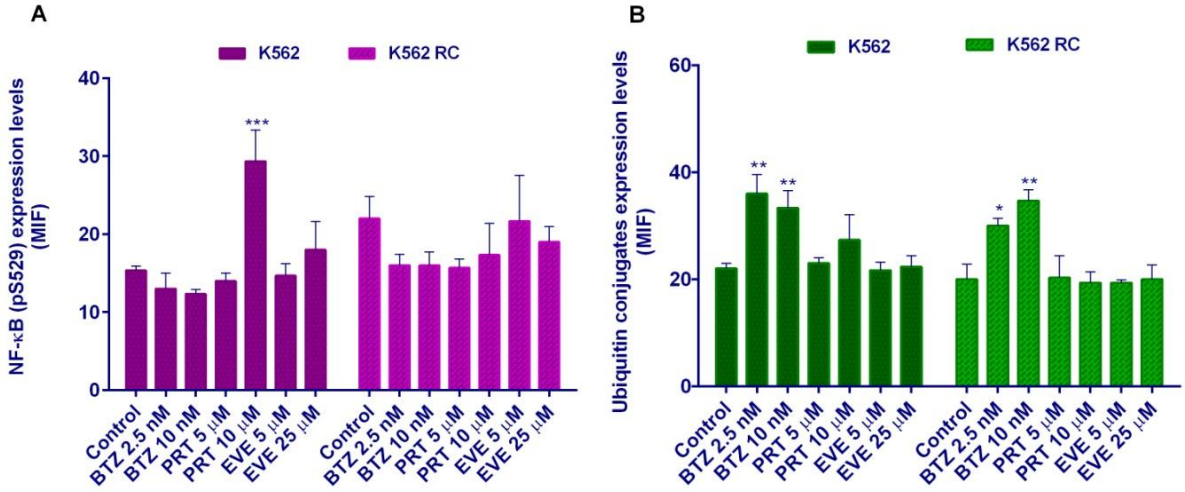

Figure 6. Analysis of intracellular expression levels of NF-κB (A) and Ubiquitin (B) in K562 and K562 RC cells upon exposure to Bortezomib, Parthenolide and Everolimus by flow cytometry. NF-κB and ubiquitin levels were analyzed by flow cytometry using monoclonal antibodies. K562 cells and K562 RC cells were incubated during 48h with BTZ, PTL and EVE. Results are expressed as medium intensity fluorescence (MIF) and represent mean±SD of fluorescence intensity detected in three independent experiments. Statistic analysis was performed by comparison with control by Tukey test: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

The major difference in ubiquitin intracellular levels, as expected, was noted with the exposure to BTZ in both cell lines K562 and K562 RC cells. This drug increases significantly the level of intracellular ubiquitin conjugates, however in a dose and cell type dependent manner. The ubiquitin levels are higher in K562 cells treated with the lower concentration of BTZ and andthe inverse happens in K562 RC cells, in which the higher levels of ubiquitin are observed upon exposure to the highest concentration of BTZ.

To access the effect of BTZ, PTL and EVE in AKT pathway and explain some of the mechanisms involved in the cytotoxic and cytostatic, we quantified by western blot the activation (A) and expression (B) of AKT in the absence and in the presence of our compounds. Furthermore, AKT pathway has also been shown to be related with the mechanism of all three miRNAs in study. The results are shown in Figure 9.

In the K562 cell line, the exposure to all compounds led to a decrease in p-AKT levels. However, in the K562 RC cell line occurred precisely the opposite (Figure 9A). AKT total levels were slightly elevated in all conditions, being the highest levels noted in cells exposed to EVE. However, these results are not statistically significant.

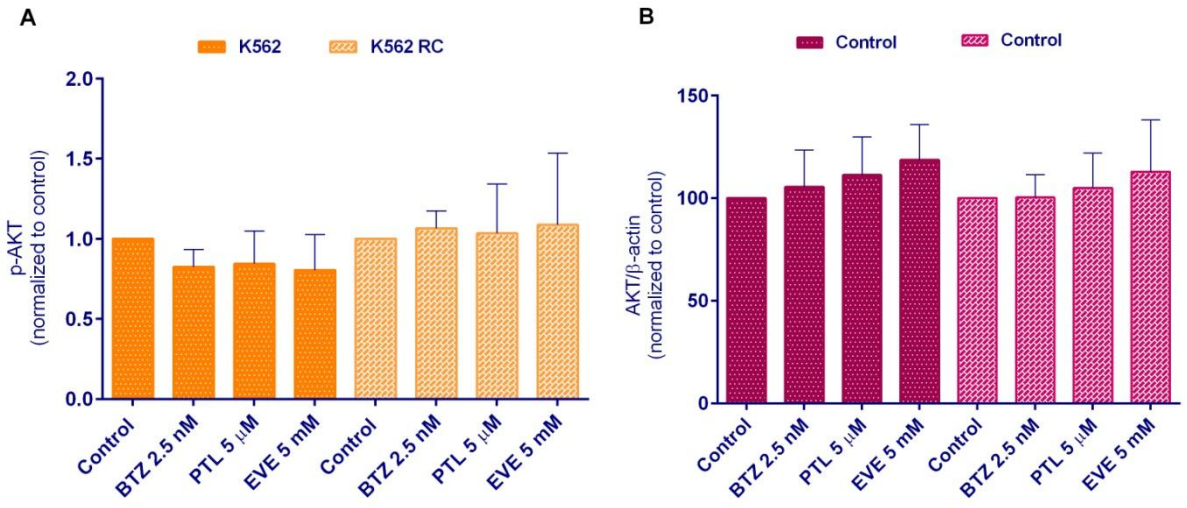

Figure 9. p-AKT and total AKT expression levels in K562 and K562 RC cell lines. p-AKT (A) and total AKT (B) levels were analyzed by western blot in K562 cells and K562 RC cells. Cell lines were incubated during 48h with 5nM of BTZ, 5 µM of PTL and 5 µM of EVE. After this time cells were lysed and prepared as described in Methods and Materials section. Results are expressed as percentage normalized to control and represent mean±SD of three independent experiments.

3.4 MiR-21, miR-125b and miR-155 expression levels in K562 and K562 RC cell lines, treated with Bortezomib, Parthenolide and Everolimus.

The basal levels of miR-21, miR-125b and miR-155 expression are shown in Figure 7. We observed that there was an increase in the expression of miR-21 in K562 RC cells about 0.46-fold more than in K562 cells. On the other hand, a significant decrease of the expression of miR-155 in K562 RC cells is observed when compared with K562 cells(in K562 cells miR-155 expression is 8.17 times higher than in K562 RC cells).In the cell line sensitive to Imatinib we didn´t observe no expression of miR-125b, but in the resistant cell line a considerable expression of miR-125b is noted (Figure 7).

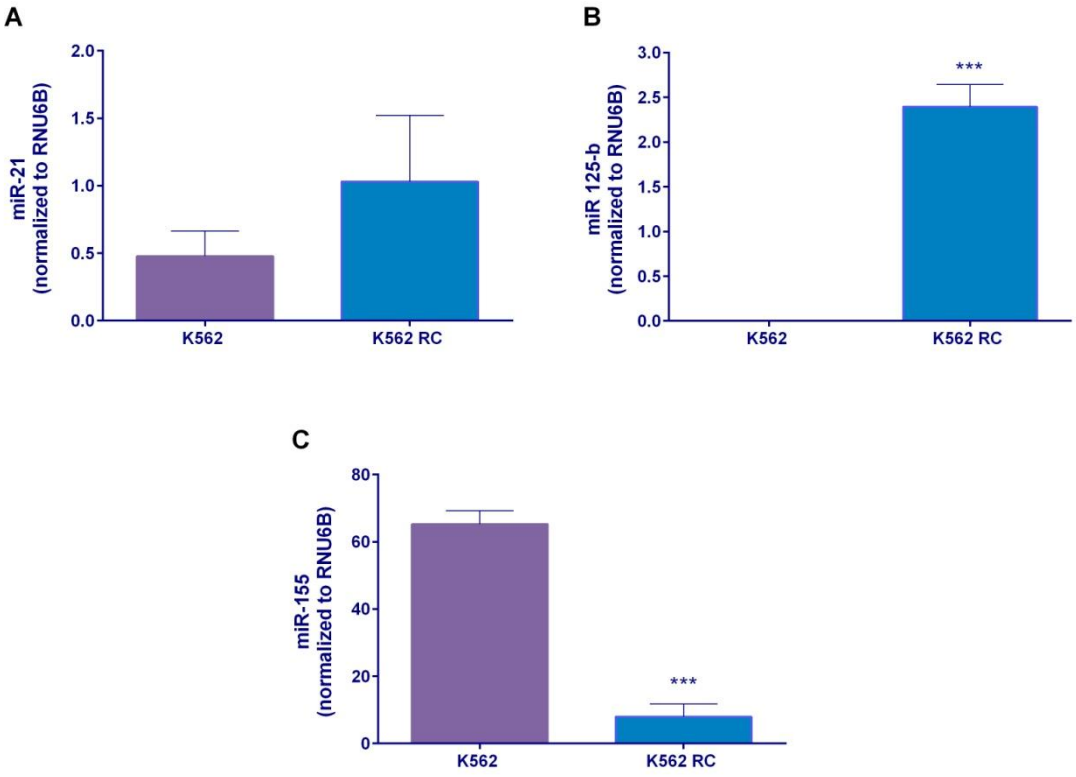

Figure 7. The basal expression levels of miR-21, miR-125b and miR-155 in K562 and K562 RC cells. We evaluated the expression of miR-21, miR-125b and miR-155 in K562 and in K562 RC cells. The results represent the expression levels of miRNAs normalized to nucleolar RNA, RNU6B, and are presented as mean ± SD of three independent experiments. Statistic analysis was performed by comparison with control by Tukey test: *** $p<0.001$.

After treatment of both cell lines with lower concentrations of BTZ (2.5 nM), PTL (5 μM) and EVE (5 μM), expression levels were assessed, as shown in Figure 8.

In K562 cell line (Figure 8A) we observed that EVE was the compound that most increased miR-21 and miR-155 expression. Cells exposed to BTZ present higher levels of miR-155 than miR-21, but the difference was not significant. The opposite happens with PTL exposure, where K562 present higher levels of miR-21 than miR-155. K562 cells have no expression of miR-125b, even upon treatment with these drugs.

In K562 RC cell line (Figure 8B) we could observe that BTZ and PTL promoted the higher expression levels of miR-155. The low expression of miR-21 and miR-155 upon exposure to EVE is in clear contrast with what happen in K562 cells. While in K562 EVE induces an increase in miR-21 and miR-155 expression levels 2 times higher than the control, in K562 RC cells EVE induces a slightly decrease, specially noted in miR-155 which decreases to 0,7 in relation to control (1). BTZ and PTL exposure promoted only an increase of miR-155 expression in K562 RC cells, 1.26 and 1.36, respectively, in relation to control (1) . Although miR-125b expression increased in K562 RC cells in relation to K562 cells, the exposure to the different compounds induce no significant different in the expression levels of this miRNA.

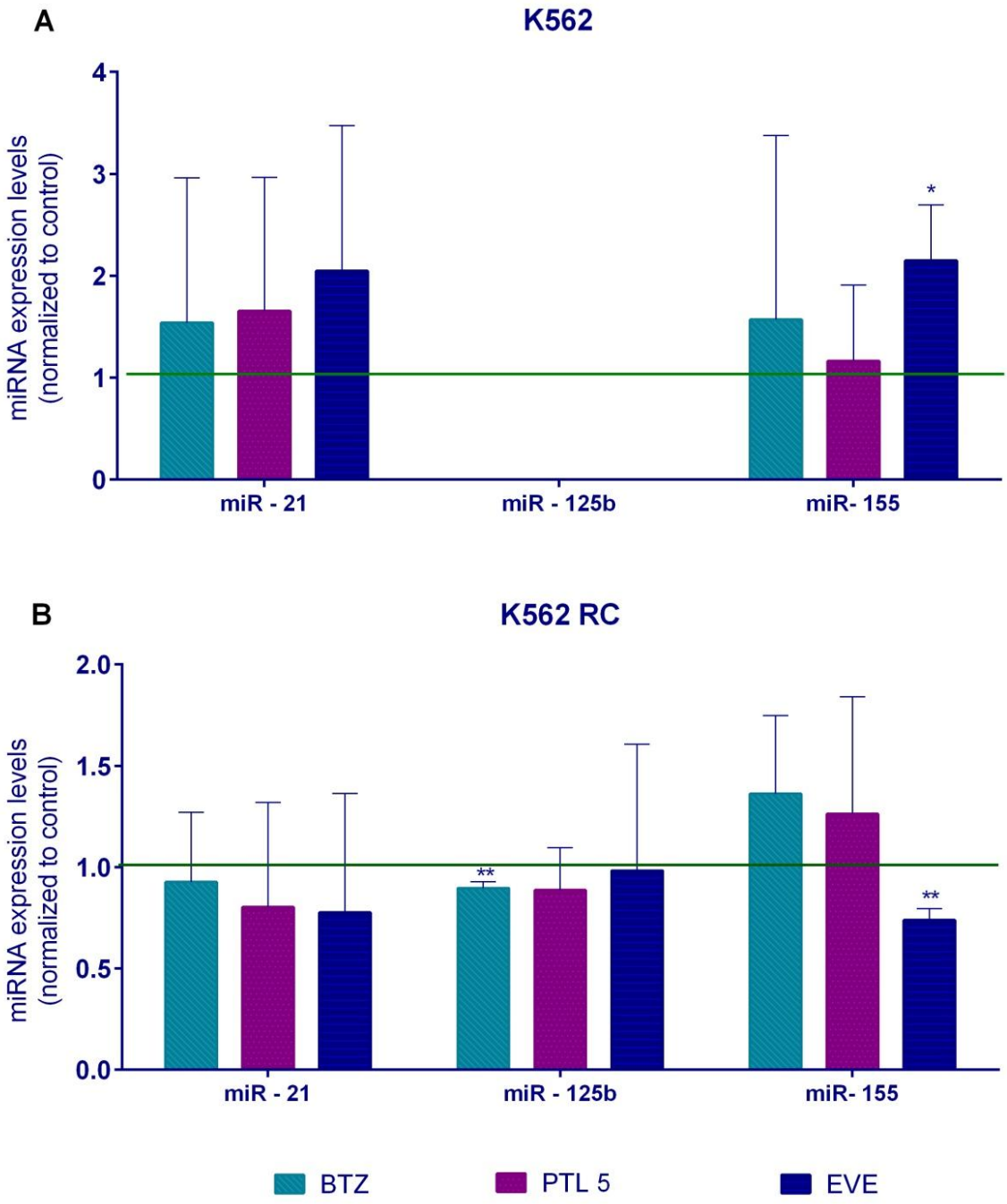

Figure 8. The expression levels of miR-21, miR-125b and miR-155 in K562 and K562 RC cell lines treated with Bortezomib (BTZ), Parthenolide (PTL) and Everolimus (EVE). We evaluated the expression of miR-21, miR-125b and miR-155 in K562 (A) and in K562 RC cells (B), cultured in the absence and presence of BTZ, PTL and EVE, during 48h. The increase or decreased of miRNAs levels is normalized to untreated cells (miRNA expression levels of untreated cells are represented by the green line). The results represent mean±SD of three independent experiments. Statistic analysis was performed by comparison with control by t- test: * $p<0.05$ and ** $p<0.01$.

4. Discussion

This study was initiated by the evaluation of cytotoxic effect of Bortezomib, Parthenolide and Everolimus in two CML cell lines, one sensitive to Imatinib and other resistant to this tyrosine kinase inhibitor (TKI). Analyzing the cytotoxicity curves and the IC_{50} values of the three compounds, the cytotoxic effect of Bortezomib and Parthenolide seems to be more effective in K562 RC pointing out that these compounds can be used in patients that have developed a resistance to Imatinib therapy. In contrast, Everolimus was more effective in K562 cells, suggesting that this compound would have better effects in patients who have no previous resistance to Imatinib, than the ones that already present a resistance to TKI.

Bortezomib is a potent and selective proteasome inhibitor, and in our work we can see that the effect it exerts in the CML cell lines it's through ubiquitin-proteasome pathway, as ubiquitin levels are elevated in K562 and K562 RC cell lines incubated with Bortezomib, during 48h. With proteasome inhibition promoted by Bortezomib, ubiquitinated proteins are all disperse in the intracellular space, making possible to quantify the ubiquitin levels.

Moreover, NF-κB, a transcription factor that has crucial roles in inflammation, immunity, cell proliferation and apoptosis (Viatour P *et al.*, 2005), has been implicated in the mechanism responsible for the antitumor effect of proteasomes inhibitors (Melo and Chuah, 2008). Bortezomib, inhibits proteasome degradation of IκB, the inhibitor of NF-kB, and thereby it binds to NF-κB inhibiting its activation. Activation of NF-kB mainly occurs via IkB kinase (IKK)-mediated phosphorylation of inhibitory molecules, including IkB-α. The inhibition NF-κB activation, in turn, inhibits the transcription of antiapoptotic and antioxidant genes. Moreover, NF-κB has also been related with the promotion of chemoresistance (Ludwig *et al.*, 2005) and our results also show that the basal levels of NF-κB in the Imatinib resistant cell line are higher than in the sensitive line. Therefore, we quantified the activated NF-κB in both cell lines treated with Bortezomib and observed that there is a decrease in the sensitive cell line and in the resistant one. Furthermore, Bortezomib was more potent in inhibiting viability in K562 RC cells than K562 cells, what indicates a direct correlation with decrease in NF-κB activity. For that reason, Bortezomib presents itself as a possible alternative to use in CML patients with resistance to Imatinib.

The tumor suppressor protein p53 is another important substrate for proteasomal degradation. p53 levels and activity are controlled in large part through regulated

ubiquitination and subsequent destruction by proteasome. Inhibition of proteasome leads to p53 stabilization. This protein promotes the induction of the proapoptotic protein BAX that is also a proteasomal substrate. BAX, in turn, inhibits the antiapoptotic protein BCL-2. All this is related with the induction of apoptosis (Ludwig *et al.*, 2005). Our results suggest that in both cell lines there is stabilization in p53, as the levels of the molecule is similar in treated cells and in control. As expected we can observe a significant decrease in BCL-2 and an increase in BAX levels, in the K562 cells both sensitive and resistant to Imatinib when treated with Bortezomib.

Bortezomib has shown the ability to arrest the cells in G2/M phase (Melo and Chuah, 2008), in Bcr/Abl-positive cell lines sensitive or resistant to Imatib, in concentrations similar to ours (Gatto et al., 2003) and in a P-glycoprotein (P-gp) positive leukemia line K562/A02 with 100nM of Bortezomib (LÜ *et al.*, 2010). However our results differ from this.

We could observe that in cells treated with the lower dose of Bortezomib, 5 nM, there is no visible cell cycle arrest. This result might be dependent of the lower dose, which did not have capacity to induce cell arrest. But, in cells treated with the high concentration of Bortezomib, 10 nM, cell cycle arrest occurs at G0/G1. Our results, albeit different from other authors, are in concordance with the study conducted by Albero and colleagues (Albero *et al.*, 2010) that show that in Imatinib sensitive and resistant BCR-ABL1-expressing cells Bortezomib induces cell arrest at G0/G1. These authors explain the results with the observed reduction of Rb phosphorylation and, consequently increase in the activity of Rb, a tumor suppressor protein that regulates transition from G1 into S phase. The cells used in our study are BCR-ABL expressing cells, which allows us to relate our results with these previously described. It is possible that the same mechanism is occurring in our case, and further studies will be necessary to analyze Rb to prove this relation.

Parthenolide is a known NF-κB inhibitor that acts by directly binding to IκK, thus preventing its release from NF-κB subunits and consequent activation of NF-κB. Lack of NF-κB activity render cancer cells prone to undergo apoptosis and become sensitized to cancer drug induced cell death (Guzman *et al.*, 2005). Parthenolide has also show to inhibit STATs mediated apoptotic gene transcription and increase intracellular reactive oxygen species (ROS). The apoptotic mechanism induced by generation of ROS results in a proapoptotic BAX conformational change, release of cytochrome c from mitochondria and caspase activation. Parthenolide also amplifies the apoptotic signal through the sensitization of cancer cells to extrinsic apoptosis, induced by TNF-α (Pajak *et al.*, 2008).

In the studies conducted, our results indicate that in the K562 resistant cells NF-κB decreases, allowing us to presume that in this cell line Parthenolide works through NF-κB pathway, as described in literature. However, in K562 cells sensitive to Imatinib we can observe just a slight decrease of NF-κB in the cells treated with 5 nM of Parthenolide. On the other hand, a significant increase of NF-κB in cells treated with 10 nM of Parthenolide was observed. This last result is quite discrepant from what is found in literature. Guzman and colleagues verified that Parthenolide-mediated apoptosis is strongly associated with inhibition of NF-κB (Guzman *et al.*, 2005).

Observing the dose response curve and cell death type in both cell lines treated with Parthenolide, we can remark that the antiproliferative and cytotoxic effect is dose, time and cell type dependent. In K562 cells, treated with Parthenolide 10 nM during 48h we observe a significant decrease in cell viability about 34.6% higher than the observed in cells incubated with 5 nM of this drug. These results, along with the results obtained by flow cytometry, showing that a large proportion of cells treated with 10 nM of Parthenolide present a high amount of necrosis, allow us to assume that this dose activate different cell death pathways.

These pathways can influence the increase in NF-κB, since it has protective characteristics in the cell, regulating the expression of genes that keep the cell proliferating and protects the cell from conditions that would otherwise cause apoptosis. It is also activated in response to stimuli such as cytokines, LPS and DNA damage. It is known that Parthenolide can act through other pathways besides the NF-κB, such as ROS production. ROS and NF-κB signaling pathways interact in many ways. NF-κB is related to the transcription of genes that influence ROS levels in the cell and in turn ROS levels also regulate the levels of NF-κB activity. Depending on the context, ROS can both activate and inhibit NF-κB signaling (Morgan and Liu, 2011). Having this in mind, we can speculate that with the higher dose, Parthenolide induces high production of ROS, leading to an increase of NF-κB in an attempt to protect the cell from the harms of the drug related with oxidative stress. If the increase in NF-κB is higher than the capacity of Parthnolide to inhibits this transcription factor, these observations could explain our results.

Besides this, Parthenolide was been shown in melanoma cells to have an activity dependent on cell context (Czyz *et al.*, 2010). This is another reason that could explain our results. Another explanation could be that, because there is a great amount of death induced by the higher dose of Parthenolide, only a few viable cells are detected by flow cytometry

and this few viable cells are responsible for the increased NF-κB levels observed. These viable cells, not being affected by Parthenolide in its principal target, the NF-κB pathway, were in some ways stressed enough to start producing NF-κB in a protective way, being responsible for the increase that we detected.

Another interesting fact is that in AML cells, genetic studies using a dominant negative repressor of NF-κB activity have shown that inhibition of NF-κB alone is not sufficient to mediate the robust cell death observed with Parthenolide (Guzman *et al.*, 2005), meaning that other pathways are activated simultaneously. Parthenolide has been shown to suppress STAT3 activity (Pajak *et al.*, 2008). K562 cell line has constitutive activation of STAT3 and a previous study presented that STAT3 plays a critical role in the survival of K562 cells, because knockdown of STAT3 by STAT3 siRNA caused a decrease in STAT3 protein level, inhibition of growth and proliferation, cell cycle arrest, visible morphologic changes, and induction of apoptosis in K562 cells (Ma *et al.*, 2010). STAT3 inhibition could represent a viable strategy to CML therapy, meaning that Parthenolide could be a viable therapeutic option.

The molecular mechanism of Parthenolide mediated apoptosis is also strongly associated with proapoptotic activation of p53. In our studies we could not find a significant difference in p53 expression levels, in both K562 and K562 RC cell lines. Nonetheless, it had been shown that Parthenolide induced cell death in Chronic Lymphocytic Leukemia (CLL) cells in a p53 independent way, being p53 upregulation not so important to Parthenolide induced apoptosis in this cancer cells (Steele *et al.*, 2006).

Our results show that the increase in BCL-2 expression levels in K562 and K562 RC cells, along with the diminished levels of BAX, can point to the involvement of the mitochondrial pathway and activation of ROS as the principal mechanism of action of Parthenolide in these cells. Our results also show that ubiquitin levels are slightly elevated in K562 cells treated with 10 nM of Parthenolide, despite this not being statistically significant. If NF-κB levels are increased this means that IκB is ubiquitinated and therefore not bind to NF-κB subunits, what is in accordance with our results.

In the vast majority of patients with chronic myeloid leukemia that have developed resistance to Imatinib, the resistance is the result of a point mutation in *BCR-ABL* gene. Activation of the tyrosine kinase fusion product BCR-ABL is absolutely required for the development of this type of leukemia, and molecularly defines this disease.

As *BCR-ABL* fusion gene has been shown to activate the PI3K/AKT/mTOR pathway, mTOR inhibitors might have a significant therapeutic effect in leukemias that have acquired BCR-ABL mutations (Easton and Houghton, 2006).

Everolimus is a derivate of rapamycin and an mTOR inhibitor. mTOR is a serine-threonine kinase downstream of PI-3 kinase (PI3K) that is activated upon phosphorylation by AKT (Melo and Chuah, 2008). The late re-activation of mTOR in response to Imatinib (Mancini, M. *et al.*, 2010) is possibly responsible for the development of an Imatinib resistant phenotype (Melo and Chuah, 2008). Everolimus revokes mTOR late re-activation, making this drug very promising in the therapy of CML resistant to Imatinib.

Our results show that Everolimus induces in K562 cells, sensitive and resistant to Imatinib, a cytotoxic effect. Direct inhibitors of mTOR and those pathways activating mTOR, subsequently induce autophagy, being this protein implicated in the inhibition of autophagy (Mancini, M. *et al.*, 2010). Everolimus has shown in our studies to provable induce autophagy, as we can see by the cytoplasmic vacuoles present in the cell smears. Additional studies will be necessary to prove that the cytoplasmatic vacuoles are in fact a result of autophagy. Such studies can comprise the assessment of beclin, LSII and/or LDIII levels by western blot.

Everolimus showed that in both K562 cell lines, besides the cytotoxic effect, observed by the apoptosis triggering, it also has a cytostatic effect on the cells that is confirmed by the cell cycle arrest at G0/G1. The inhibitory effect of rapamycin on the *in vitro* growth of primary CML cells is directly related to induction of G_1 cell cycle arrest and subsequent apoptosis (Melo and Chuah, 2008), what is in concordance with our results.

Everolimus interferes with the assembly of both mTOR complexes: mTORC1 and mTORC2. The inhibition of mTORC2 results in the de-phosphorylation of AKT at Ser^{473} in the hydrophobic motif of C-terminal tail which is required for AKT full activation and prevents AKT re-phosphorylation in response to Imatinib (Mancini, M. *et al.*, 2010, Zeng *et al.*, 2007). We evaluated the levels of phosphorylated AKT in K562 and K562 RC cells treated with Everolimus. K562 cell line sensitive to Imatinib had a decrease in p-AKT, what is in concordance with the mechanism of action of Everolimus. However, K562 cells resistant to Imatinib had an increase in p-AKT, but this effect is not significant.

Various studies have shown that combination of Imatinib and Everolimus acts synergistically in BCR-ABL positive cell lines (Alves, 2011, Mancini, Manuela *et al.*, 2010,

Mohi *et al.*, 2004, Zeng et al., 2007), although this effect is more pronounced in cells with moderate resistance to Imatinib (Dengler *et al.*, 2005).

Additionally, we must have in mind that BCR-ABL kinase mutations are very common in patients with resistance to Imatinib therapy. Previous studies showed that the presence of oncogenic KRAS mutations was associated with lack of benefit after Everolimus therapy (Di Nicolantonio *et al.*, 2010), and also to Imatinib resistance in some CML patients (Agarwal *et al.*, 2008). These alterations in Imatinib resistant cells might have significant influence in the pathways through which Everolimus works, diminishing the cytotoxic effect of Everolimus in K562 resistant cell line.

In this study we observed that miR-21 has higher expression levels in K562 RC cells than in K562 cells, suggesting a possible role of this miRNA in Imatinib resistance. As previously referred, miR-21 up-regulation induces resistance to Daunorubicin in the K562 cell line by regulating the expression of PTEN. Haitao Bai (Bai *et al.*, 2011) demonstrated that miR-21 overexpression down-regulates PTEN activity and thereby increases the activity of AKT. There are other studies that also show that miR-21 overexpression contributes to resistance to therapy in breast cancer (Gong *et al.*, 2011), ovarian cancer (Echevarría-Vargas *et al.*, 2014) and other neoplasias (Pan *et al.*, 2010). In K562 cells exposed to Bortezomib, Parthenolide and Everolimus there is an increase in miR-21 expression. The total AKT levels are elevated in K562 cells in all conditions; however the p-AKT levels are decreased. This could be explained based on the mechanism of action of the drugs, and not by the action of the miRNAs. Everolimus could be an example of this, as it acts trough the dephosphorylation of AKT, what explains the low levels of p-AKT in cells treated with this drug. The increase in p-AKT, caused by miR-21 overexpression, is probably outweighed with the action of Everolimus. MiR-21 overexpression after treatment with rapamycin, from which Everolimus is a derivative, has been previously shown in endothelial cells (Jin *et al.*, 2013) and the same study also showed that inhibition of miR-21 abrogated rapamycin mediated inhibition of endothelial proliferation. The increase in miR-21 expression induced by rapamycin was likely a result in the inhibition of Raptor, a specific component of mTORC1, relating mTORC components with the regulation of the miRNAs expression levels. However, further mechanism of miR-21 mTORC1 regulation should be discussed in later studies.

Knockdown of miR-21 has been presented has a novel therapy in leukemia cells (Feng *et al.*, 2010). Downregulation of miR-21 has shown to enhance chemotherapeutic effect of taxol in breast carcinoma cells (Mei *et al.*, 2010). Our results also show a decrease in mir-21

expression levels in K562 RC cells when exposed to Bortezomib, Parthenolide and Everolimus what can indicate that these compounds have a role in the decrease of miR-21 in K562 RC cells and can be used in patients with resistance to Imatinib therapy, because render the cells more sensitive to chemotherapy.

A study (Talotta *et al.*, 2009) showed that in response to RAS signaling, AP-1 induces miR-21 expression. RAS is the initiating molecule of the RAS/MAPK cascade, one of the known subtracts of *BCR-ABL* fusion gene, leading to decontrolled proliferation of leukemic cells (Alves, 2011). Everolimus has been shown to interfere with the generation of AP-1 (Daniel *et al.*, 2010). Being so, the inhibition of AP-1 by Everolimus could be the reason why miR-21 expression levels are decreased in K562 RC cells treated with this compound.

Another study (Dan *et al.*, 2008) showed that in PTEN-null/inactive prostate cancer cells, mTOR, downstream from AKT, by interaction with and stimulation of IκK, controls NF-κB activity. It seems that the mTOR-associated protein Raptor is required for the ability of AKT to induce NF-κB activity. In the study, rapamycin, an analogue of Everolimus, suppressed the IκK activity in PTEN-deficient prostate cancer cells through a mechanism that may involve dissociation of Raptor from mTOR. We can hypothesize that the same happens in our cells. Our cells express miR-21 that at least inactivates PTEN. In that case, Everolimus exposure would block mTOR and diminish AKT ability to induce NF-κB. On the other hand, NF-κB has been shown to induce miR-21 expression (O'Connell *et al.*, 2011), so the lack of this molecule would probable decrease miR-21 levels. Furthermore, as both Bortezomib and Parthenolide are known for its capacity to inhibit NF-κB, miR-21 could also be decrease by this mechanism in the cells treated with these compounds.

Overexpression of miR-125b has been proposed to contribute to tumor resistance to chemotherapy. MiR-125b has been showed to induce resistance in K562, THP-1 and Jurkat cells to the drug Daunorubicin by reducing apoptosis, while the suppression of miR-125b was found to enhance Daunorubicin cytotoxicity in REH cells (Zhou *et al.*, 2014).

In our studies we did not detect any expression levels of miR-125b in K562 cells but there was a significant expression of miR-125b in K562 RC cells, what is in accordance with the foregoing. The lack of expression of mir-125b in K562 cells, and its expression in resistant ones may mean that this miRNA might have a role in Imatinib resistance. When both cell lines were incubated with Bortezomib, Parthenolide and Everolimus no expression

of miR-125 was observed in the K562 cells, however all compounds contributed to a tenuous decreased of the levels of miR-125b in K562 RC cells.

In K562 cells incubated with Bortezomib, Parthenolide and Everolimus, we noticed an increase in miR-155 expression levels, being the highest increase in cells treated with Everolimus. Nevertheless, in K562 RC cells miR-155 levels increased after incubation with Bortezomib and Parthenolide, but decreased with Everolimus incubation.

Transcription of miR-155 is up-regulated by AP-1 (O'Connell *et al.*, 2011) and as previously stated, Everolimus had been shown to interfere with the generation of AP-1. This influence of Everolimus in AP-1 action can lead to the decrease of miR-155 observed in cells resistant to Imatinib. Being miR-155 an oncomir, the decrease in miR-155 expression levels induced by Everolimus can indicates that Everolimus might be a good therapy to use in CML patients with resistance to Imatinib and miR-155 increased levels.

5. Conclusion

In summary, our results reveal that the compounds use in this study, Bortezomib, Parthenolide and Everolimus, are able to induce a cytotoxic effect in both K562 cells sensitive to Imatinib and in K562 cells resistant to this tyrosine kinase inhibitor. In fact Bortezomib and Parthenolide revealed to be more effective in the K562 resistant cells than in the sensitive ones. These results may indicate that these two compounds could be used as an alternative therapy in CML patients with resistance to Imatinib.

Furthemore, our results also point to an involvement of miR-21, miR-125b and miR-155 in the acquisition of resistance to Imatinib. The exposure to Bortezomib, Parthenolide and Everolimus was able to modulate the expression levels of these miRNAs suggesting that they may provide interesting drug targets for the sensitization of tumor cells to chemotherapy and could be applied to treat chemotherapy resistance in leukaemia patients.

However, these results must be validated using leukemic cells obtained from CML patients with and without resistance to Imatinib.

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