Phosphoinositide 3-kinase inhibition, another step in the quest to cure hematological malignancies

Nuno Simões Costa[1]; Bárbara Almeida Marques[1]; Raquel Alves[2,3,4]; Ana Pires[2]; Joana Jorge[2]; Ana Cristina Gonçalves[2,3,4]; Ana Bela Sarmento Ribeiro[2,3,4,5]

[1]Medical Student, Faculty of Medicine of University of Coimbra (FMUC), Portugal;

[2]Applied Molecular Biology and University Clinic of Hematology, FMUC, Portugal;

[3]Center of Investigation on Environment Genetics and Oncobiology (CIMAGO), FMUC, Portugal;

[4]Center for Neuroscience and Cell Biology (CNC.IBILI), University of Coimbra, Portugal;

[5]Clinical Hematology, Centro Hospitalar e Universitário de Coimbra (CHUC), Portugal.

Table of contents

Abbreviations

AV – Annexin V

AKT – Protein kinase B (PKB)

AML – Acute myelogenous leukemia

APC – Allophycocyanin

APL – Acute promyelocytic leukemia

BKM120 – Buparlisib, NVP-BKM120

CF – Citometria de Fluxo

CLL – Chronic lymphocytic leukemia

CML – Chronic myelogenous leukemia

DLBCL – Diffuse large B-cell lymphoma

DNA – Deoxyribonucleic Acid

FC – Flow cytometry

FDA – Food and Drug Administration

FITC – Fluorescein isothiocyanate

GPCR – G-protein coupled protein receptor

IC_{50} – Half-maximal inhibitory concentration

INPP4B – Inositol polyphosphate 4-phosphatase type II

LMA – Leucemia Mieloide Aguda

MDS – Myelodysplastic syndrome

MGUS – Monoclonal gammopathy of unknown significance

MM – Multiple myeloma, Mieloma Múltiplo

mTOR – Mammalian target of rapamycin

PBS – Phosphate buffered saline

PI – Propidium Iodide

PI3K – Phosphoinositide 3-kinase

PIP2 – Phosphatidylinositol 4,5 bisphosphate [PI(4,5)P2]

PIP3 – Phosphatidylinositol 3,4,5 trisphosphate [PI(3,4,5)P3]

PTEN – Phosphatase and tensin homologue

RAEB – Refractory anemia with excess of blasts

RTK – Receptor of tyrosine kinase

SHIP1 – SH2-containing inositol-5´-phosphatase 1

Abstract

The phosphoinositide 3-kinase (PI3K) is an intermediate signaling molecule that is involved in the activation of multiple effector pathways such as PI3K/AKT/mTOR. PI3K plays a very important role in key cellular processes like cell growth, survival, proliferation, metabolism, motility and DNA transcription. This pathway is deregulated in several types of malignancies including hematological malignancies.

The aim of this study is to evaluate the effect of Buparlisib, NVP-BKM120, hereby called BKM120 (a pan-class I PI3K inhibitor), on *in vitro* models of acute myelogenous leukemia (AML) and multiple myeloma (MM).

For this purpose, we used two *in vitro* models of myeloid malignancies, HEL cells (erythroleukemia) and NB-4 cells (acute promyelocytic leukemia) and an *in vitro* model of lymphoid malignancies, H929 cells (MM). Cell lines were cultured both in absence and in presence of different concentrations of BKM120 administered in single and daily dose schemes. Cell viability was determined at 24, 48 and 72 hours using the resazurin assay. Cell death was analyzed by optical microscopy after May-Grunwald Giemsa staining, and by flow cytometry (FC) using the annexin V and propidium iodide double staining. Caspases activity was evaluated using the ApoStat probe by FC. Cell cycle analysis was also performed by FC with PI/RNAse solution.

BKM120 induces a decrease in cell viability in a dose, time, cell-type and administration scheme dependent manner. In fact, the calculated half maximal inhibitory concentration (IC_{50}) at 48 hours of exposure was 80 nM in the NB-4 cell line, making it the most sensible cell line, approximately 2 µM in the HEL cell line and well beyond 10 µM, the maximal dose tested, in the H929 cell line. Moreover, the daily administration of a small dose of BKM120 reveals a positive effect when compared to the administration of the same dose in the single dose

administration scheme, being this effect more pronounced in the NB-4 cell line. Morphology and FC demonstrated that this compound induced cell death predominantly by apoptosis with an increase of the percentage of cells expressing caspases. The cell cycle analysis showed that BKM120 induces cell cycle arrests in the tested cell lines proving that it also has anti-proliferative properties.

Our results show that BKM120 has the ability to induce cytotoxic and anti-proliferative effects in acute myeloid leukemia and multiple myeloma cell lines, suggesting that PI3K could be a promising therapeutic target for novel anti-cancer therapeutics in patients with these malignancies, mainly with acute myeloid leukemia.

Key-Words: PI3K, BKM120, Acute Myeloid Leukemia, Multiple Myeloma, Apoptosis

Resumo

A fosfatidilinositol 3-quinase (PI3K) é uma proteína que intervém em diversas vias de sinalização intracelular, e uma vez ativada conduz à ativação de vias efetoras como a PI3K/AKT/mTOR. A PI3K tem um papel muito importante em vários processos como crescimento, sobrevivência, proliferação, metabolismo, motilidade e transcrição de ADN. A desregulação desta via está associada à patogénese de processos neoplásicos incluindo neoplasias hematológicas.

O objetivo deste estudo é avaliar o efeito do Buparlisib, NVP-BKM120, de ora em diante designado por BKM120, um inibidor da Classe I do PI3K em modelos *in vitro* de leucemia mieloide aguda (LMA) e mieloma múltiplo (MM).

Para concretizar o nosso objetivo utilizámos dois modelos de LMA, as células HEL (eritroleucemia) e as células NB-4 (leucemia promielocítica aguda) e um modelo de MM, as células H929. As linhas celulares foram cultivadas na ausência e na presença de diferentes concentrações de BKM120 que foi testado em esquemas de administração única e de administração diária. A viabilidade celular foi avaliada às 24, 48 e 72 horas de exposição utilizando um ensaio metabólico da resazurina. A morte celular foi analisada através de microscopia ótica (coloração May-Grunwald Giemsa) e por citometria de fluxo (CF) (anexina V e o iodeto de propídio). Também por CF foi avaliada a actividade das caspases (sonda ApoStat) e o ciclo celular através da marcação com Iodeto de Propídio/RNase.

Os resultados demonstram que o BKM120 induz diminuição da proliferação celular de modo dependente do tempo, da dose, da linha celular e do esquema de administração. De facto, o IC_{50} calculado às 48h de exposição foi de 80 nM para a linha celular NB-4, revelando-se a mais sensível; 2 μM para a linha celular HEL e superior a 10 μM, a dose mais alta testada, para a linha celular H929. A administração diária de BKM120 mostrou-se mais eficaz na redução da proliferação celular que a mesma dose em toma única, particularmente na linha celular NB-

4. A morfologia e a CF mostram que este composto induziu morte celular predominantemente por apoptose, com aumento da percentagem de células que expressavam caspases. A análise do ciclo celular permitiu verificar que o BKM120 induziu bloqueio do ciclo celular em todas as linhas celulares testadas provando que também tem efeitos anti-proliferativos.

Os nossos resultados mostram que o BKM120 induz efeitos citotóxicos e anti-proliferativos em linhas celulares de LMA e de MM sugerindo que o PI3K pode ser um alvo promissor para o desenvolvimento de novos fármacos para o tratamento destas neoplasias particularmente no caso da LMA.

Palavras-chave: PI3K, BKM120, Leucemia Mieloide Aguda, Mieloma Múltiplo, Apoptose

Introduction

Hematological malignancies are a very profound field of study. The urge to find new treatments has enlightened the way to a better understanding of the cellular and molecular pathology leading to the development of more targeted compounds with greater clinical effectiveness and reduced toxicity. In the 1990s, the development of the tyrosine kinase inhibitor imatinib, one of the first specific molecular targeted cancer therapies, revolutionized the treatment of chronic myelogenous leukemia (CML), transforming a highly fatal disease in a manageable chronic condition [1]. Nevertheless, some hematological malignancies still have a very poor prognosis.

Acute myelogenous leukemia (AML) is a heterogeneous group of diseases that share a common characteristic, a clonal proliferation of poorly differentiated hematopoietic cells that infiltrate the bone marrow, blood and other tissues [2]. Comparing to CML, AML continues to have a relatively poor prognosis with an overall 5 years' survival of 25.9% [3]. The annual incidence is 4 cases per 100 000 individuals [3]. It is also important to note that survival improvements over the last decades can be attributed to a better supportive care [4], since AML treatment options have not significantly changed [2].

Multiple myeloma (MM) is characterized by proliferation of malignant monoclonal plasma cells. It is frequently preceded by a monoclonal gammopathy of unknown significance (MGUS) which progresses to a smoldering myeloma and ultimately to multiple myeloma, when the patient becomes symptomatic [5]. MM has an overall 5 years' survival of 48.5% [3]. The annual incidence is 6.3 cases per 100 000 individuals. In the last 10 years, the development of new treatment options like thalidomide, lenalidomide and bortezomib and the autologous stem-cell transplantation significantly improved survival [5].

The phosphoinositide 3-kinase (PI3K) are a group of heterodimer lipid kinases whose main function is to participate in intermediate signaling processes that are involved in various effector and regulatory intracellular pathways. Growth factors induce the dimerization and activation of tyrosine kinase receptors (RTK) or G-protein coupled protein receptors (GPCR) with the possible interaction with RAS proteins, and the recruitment of PI3K to the cell membrane. Here, after being activated, it phosphorylates the substrate phosphatidylinositol 4,5 bisphosphate (PIP2), on the 3-position of the inositol ring, leading to the synthesis of phosphatidylinositol 3,4,5 triphosphate (PIP3). PIP3 binds to proteins that have pleckstrin homology, like protein kinase B (AKT) and PDK1, which activates AKT through phosphorylation. Activated AKT then activates downstream targets as the mammalian target of rapamycin (mTOR). The PI3K/AKT/mTOR pathway, is ubiquitously expressed across all human cells and plays a very important role in the regulation of key cellular events like cellular growth, proliferation, survival, metabolism, motility and adhesion but may also be involved in other cellular processes in specific tissues (Figure 1) [6–8].

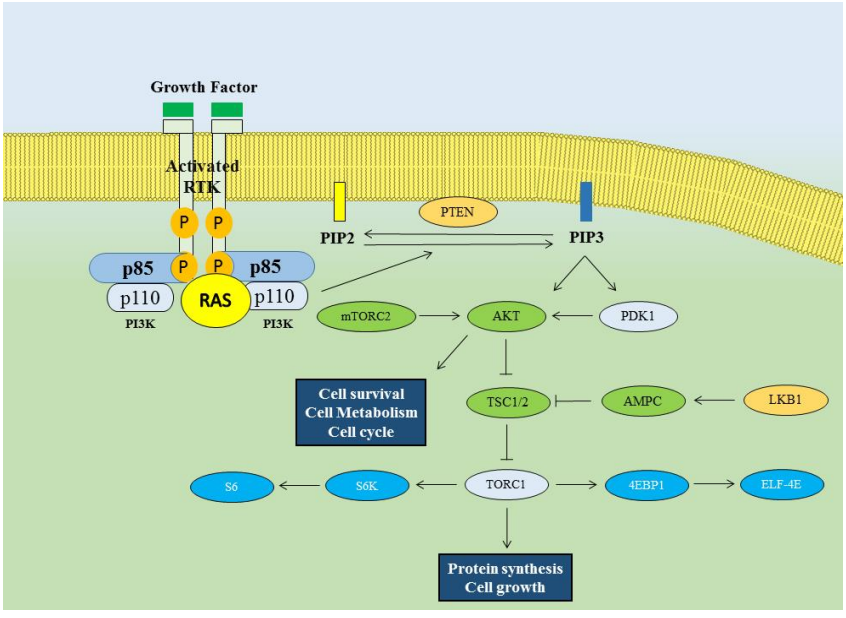

Figure 1. Receptor of tyrosine kinase (RTK) mediated activation of the class IA Phosphoinositide 3-kinase (PI3K) pathway. Growth factor induced dimerization of its receptor and leads to transphosphorylation of the cytoplasmic tail of the RTK. The phosphotyrosine residues act as docking sites for the SH2 domains of the

regulatory subunit p85 of class I PI3K. Simultaneously GTP-RAS also binds to p110 playing an important role in the activation of PI3K. Activated of class I PI3K uses phosphatidylinositol 4,5 bisphosphate (PIP2) as a substrate to generate phosphatidylinositol 3,4,5 triphosphate (PIP3), a lipid that binds to protein kinase B (AKT) and several other effector proteins containing PH domains that play an important role in cell survival, metabolism and cell cycle. These effector proteins are also responsible for the activation of the TSC1/2 complex that regulates protein synthesis. Phosphatase and tensin homologue (*PTEN*) is one of the regulator genes and is responsible for the conversion of PIP3 to PIP2.

There are three classes of PI3K (class I, class II and class III) coded by a vast variety of genes (Table 1) and they are differentiated based on the structure, function, coding genes and substrates. Class I is the most studied regarding its involvement in cancer and is further subdivided based on the type of regulatory subunit expressed [6,9]. Class IA (activated by RTKs) has three types of catalytic subunits (p110α, p110β or p110δ) that form heterodimers with three types of regulatory subunits (p85α, p85β or p85γ) [6,9]. The class IB (activated by GPCR) has one type of catalytic subunit (p110γ) that forms heterodimers with two types of regulatory subunits (p101 or p84) [6,9]. Class II PI3Ks are monomeric enzymes and are divided in three different isoforms (PI3K-C2α, PI3K-C2β and PI3K-C2γ) and its function is not completely understood [6,9]. Class III PI3K has a catalytic subunit (Vps34) and a regulatory subunit (Vps15). Vps34 is ubiquitous and is involved in intracellular trafficking and autophagy [9]. While p110α and p110β are ubiquitously expressed in all cells and tissues, p110δ and p110γ are mainly expressed in leukocytes [9]. In fact, inactivation of class I PI3K leads to a block in B lymphocyte development and conditional deletion of either phosphatase and tensin homologue (*PTEN*) or SH2-containing inositol-5´-phosphatase 1 (*SHIP1*) is associated with an overexpression of the myeloid lineage [10].

Table 1. List of Phosphoinositide 3-kinase (PI3K) genes.

	Gene	Protein	Chromosomal location
Class IA PI3K	PIK3CA	p110α	3q25.3
	PIK3CB	p110β	3q22.3
	PIK3CD	p110δ	1q36.2
	PIK3R1	p85α	5q13.1
	PIK3R2	p85β	19p13.1
	PIK3R3	p85γ	1p34.1
Class IB PI3K	PIK3CG	p110γ	7q22.3
	PIK3R5	p101	17p13.1
	PIK3R6	p84/p87PIKAP	17p13.1
Class II PI3K	PIK3C2A	PIK-C2α	11p15.1
	PIK3C2B	PIK-C2β	1q32.1
	PIK3C2G	PIK-C2γ	12q12.3
Class III PI3K	PIK3C3	Vps34	18q123
	PIK3R4	Vps15	3q22.1

Adapted from Asati *et al* 2016 [11].

Given the physiological importance of this signal transduction pathway, scientific evidence strongly suggests that deregulation in class I PI3K and in its downstream pathways is related with progression to multiple types of cancer. PI3K may be overactivated by gain of function mutations of RTK or GPCR, genetic and epigenetic inactivation in the regulatory genes like *PTEN*, *inositol polyphosphate 4-phosphatase type II* (*INPP4B*) and *SHIP1*, mutations in *RAS* and mutations resulting in overactivation of PI3K subunits or PI3K effectors like AKT or PDK1 [9,12]. Among these mutations, the one in the *PIK3CA* gene, which encodes the p110α, is involved in around 30% of the cases of some of the most prevalent types of cancer (breast, colon, endometrium, ovarium and prostate) [7,9]. PI3K signaling pathway abnormalities are also associated with resistance to conventional therapies [13–15]. Deregulation of the PI3K/AKT/mTOR pathway is also described in hematological malignancies and is detected in a significant number of AML [16–18] and MM [19,20] patients.

Outside the spectrum of cancer, PI3K deregulation is also involved in other pathologies like autoimmune diseases, rheumatic diseases and even mental illnesses like autism and schizophrenia [21–23].

Considering the involvement of PI3K and downstream pathways in oncogenic transformation and in resistance mechanisms to conventional therapies, the development of compounds tailored to target this pathway is a promising field in oncology. Therefore, during the last two decades, various compounds have been developed and six classes are currently on clinical trials: 1. rapamycin analogs; 2. active-site mTOR inhibitors; 3. pan-PI3K/mTOR inhibitors; 4. AKT inhibitors; 5. isoform-selective PI3K inhibitors; and 6. pan-class I PI3K inhibitors [24].

One example of this last class is *Buparlisib* (NVP-BKM120), hereby designated BKM120, which inhibits both class IA PI3K catalytic subunits (p110α, p110β or p110δ) and class IB catalytic subunit (p110γ). BKM120 is more effective against class IA in comparison with class IB [25] and exerts anti-proliferative and cytotoxic effects on solid tumors [13,26] and on hematological malignancies [27–29]. According to the *ClinicalTrials.gov* database, this compound is already undergoing clinical trials in a large spectrum of advanced solid and hematological malignancies.

In this work our aim was to evaluate the effect of BKM120 on cellular *in vitro* models of hematological malignancies. We tested this compound on two models of AML (HEL and NB-4 cells) and one model of MM (H929 cells). BKM120 effect on cell viability, cell death and cell cycle changes was evaluated in single and daily dose administration schemes.

Materials and Methods

Cell lines and cell culture

The HEL cell line is derived from the peripheral blood of a 30-year-old male patient with the diagnosis of erythroleukemia [30]. This cell line has the *JAK2 V617F* mutation [31]. The NB-4 cell line is derived from a bone marrow sample of a 20-year-old female patient with the diagnosis of relapsed acute promyelocytic leukemia. The cell line has the t(15;17) translocation [32]. The H929 cell line is derived from a pleural effusion of a 62-year-old female patient with the diagnosis of MM [33].

Cell lines were routinely grown in RPMI-1640 medium (L-glutamine 2 mM, HEPES-Na 25 mM, penicillin 100U/mL and streptomycin 100 µg/mL) and 10% (HEL and NB-4) or 20% (H929) heat-inactivated fetal bovine serum (FBS) at 37º C in a humidified atmosphere containing 5% CO_2.

Cell viability assays

Resazurin is a cell permeable compound that has redox properties and can be used to study cell viability. Metabolically active viable cells can reduce resazurin into its resorufin product, which is a pink and fluorescent compound. The quantity of resorufin produced can be measured using a spectrophotometer and is proportional to the metabolic activity of the cells in culture [34].

Cells were cultured both in absence and in presence of increasing concentrations of BKM120, ranging from 10 nM to 10 µM, in single dose administration. Depending on cell line, we also tested 25 nM or 250 nM of BKM120 in a daily dose administration scheme. Cell

viability was assessed every 24 hours (at 24, 48 and 72 hours) using the resazurin assay. After treatment, 10 μg/ml of Resazurin solution was added to the cells, which were then incubated at 37°C. Following incubation, the absorbance at 570 nm and 600 nm was measured using a spectrophotometer (*Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments*) and the viability is normalized to the control. The results were expressed in percentage (%) ± SEM of at least 5 independent experiments.

Cell death assessment

- **Annexin V / Propidium Iodide double staining**

During the first stages of apoptosis, a negative phospholipid called phosphatidylserine, moves from the inner to the outer layer of the plasmatic membrane. Since annexin V (AV) has the ability to bind to this phospholipid in the presence of calcium, we can measure its expression using FC by adding a flourochrome that binds AV. On the other hand, in necrosis, the cell membrane becomes increasingly permeable allowing small charged molecules, to which it was impermeable, to enter the cell. Propidium iodide (PI) uses this opportunity to enter the cell and intercalates DNA, leading to an increase in its own fluorescence, which is detected by FC.

In this protocol, cells were simultaneously stained with AV, labeled with the fluorescent probe APC, and PI. This assay allows us to differentiate the cells according to Table 2.

Table 2. Cell death analysis with Annexin V/ Propidium Iodide double staining by Flow Cytometry.

	AV	PI
Viable cells	Negative	Negative
Early apoptotic cells	Positive	Negative
Late apoptotic/Necrotic cells	Positive	Positive
Necrotic cells	Negative	Positive

After 48 hours of exposure to BKM120, cells were co-stained with AV-APC and PI using the using the manufacturer's recommended protocol. Briefly, cells were washed with ice-cold PBS (centrifuged at 500 xg for 5 min), resuspended in 100 µL of binding buffer and incubated with 5 µL of AV-APC solution and 2 µL of PI solution for 15 min at room temperature in the dark. After incubation time, cells were diluted in 400 µL of binding buffer. A six-parameter, four color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used and at least 10.000 events collected by acquisition using CellQuest software (Becton Dickinson, San Jose, CA). The results were analyzed with the Paint-a-Gate software and were expressed in percentage (%) ± SEM of at least 4 independent experiments.

- **Morphological analysis**

Cell morphology is an important method in the evaluation of cell death. At optical microscopy level, multiple morphological features can be used to distinguish between apoptosis and necrosis. In apoptosis we can observe cell shrinkage and nucleus, condensations in nuclear chromatin, karyorrhexis and blebbing of the plasma membrane. In a later stage chromatin condensation leads to the formation of a pyknotic nuclei. In opposition, during necrosis, we can observe a cellular edema where the cell membrane becomes permeable, but nucleus maintains the normal characteristics and organelles start dilating [35].

Smears were made to evaluate morphological characteristics of the treated cell lines

using optical microscopy. After 48 hours of incubation with BKM120, all conditions in study were collected and seeded on slides. Then, cell smears were stained with May-Grünwald-Giemsa method and cell morphology was analysed by light microscopy, using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Camera DXm 1200F.

- **Caspases activity evaluation**

ApoStat, a probe that quantifies caspases activity by FC, acts as a cell permeable, FITC-conjugated pan-caspase inhibitor, which irreversibly labels cells undergoing apoptotic cell death. Increased fluorescence indicates caspases activity [36].

After 48 hours of exposure to BKM120 treatments, $1x10^6$ cells were resuspended in 1 mL of PBS and stained with 2 µL of ApoStat® at 37ºC during 15 min. Then cells were washed and resuspended in 300 µL of PBS being ready to acquisition. A six-parameter, four color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used and at least 10.000 events collected by acquisition using CellQuest software (Becton Dickinson, San Jose, CA). The results were analyzed with the Paint-a-Gate software and were expressed in percentage (%) of cells expressing caspases ± SEM of at least 5 independent experiments.

Cell cycle

PI is a fluorescent dye that has the ability to intercalate DNA. With FC, we can quantify the fluorescence and thus infer the quantity of DNA of each cell. We also know that the DNA quantity varies depending on the cell cycle phase. Therefore, using a specific software and the raw data obtained in the FC, we can determine the proportion of cells in each different phase of the cell cycle [37].

After 48 hours of BKM120 treatment, cell cycle was analyzed by FC using PI/RNAse

solution (Immunostep). Briefly, after 48 hours of incubation, cells were collected and washed with PBS for 5 min at 1000 xg. The pellet was resuspended in 200 µL of ice cold 70% ethanol solution, during vortex agitation, and incubated during 30 min at 4°C. Then, cells were washed with PBS, resuspended in 400 µL of PI/RNase solution and incubated for 15 minutes at room temperature. Cell cycle distribution was analysed using the ModFit LT software (Verity Software House). Results were expressed in percentage (%) of cells in the different cell cycle phases (G_0/G_1, S, and G_2/M) ± SEM of at least 4 independent experiments, according with the PI intensity. When present, a population with lower quantity of DNA then G_0/G_1 was identified and called apoptotic peak, corresponding to apoptotic cells.

Statistical analysis

We used GraphPad Prism 6 software (version 6.00 for Windows; GraphPad Software, Inc., San Diego, CA, USA). All data was expressed as mean ± SEM of the number of independent experiments (indicated in the figure legends). One way and two-way ANOVA were used to determine the statistical significance, considering a *p* value of <0.05. We used the results from dose-response curves to create a nonlinear regression and then calculate the half maximal inhibitory concentration (IC_{50}) for each tested cell line.

Results

BKM120 reduces cell viability in AML and MM cell lines

We verified that BKM120 reduced cell viability in a time, dose and cell line dependent manner (Figure 2). During the 72 hours of exposure, cell viability assays were conducted every 24 hours and the viability progressively decreased. In fact, the IC_{50} for the HEL cell line was over 10 µM at 24 hours, 2 µM at 48 hours and 350 nM at 72 hours. For the NB-4 cell line was 2 µM at 24 hours, 80 nM at 48 hours and 40 nM at 72 hours. In the H929 cell line, the IC_{50} was over 10 µM during the 72 hours of exposure but a cell viability decrease was also evident and happened every 24 hours. These results prove that the time of exposure to the compound influenced cell viability. The dose also played an important role in cell viability since greater doses of BKM120 led to faster cell viability decreases. Regarding the type of cell line, for the same dose of compound and time frame, the NB-4 cell line had the greatest reduction in cell viability.

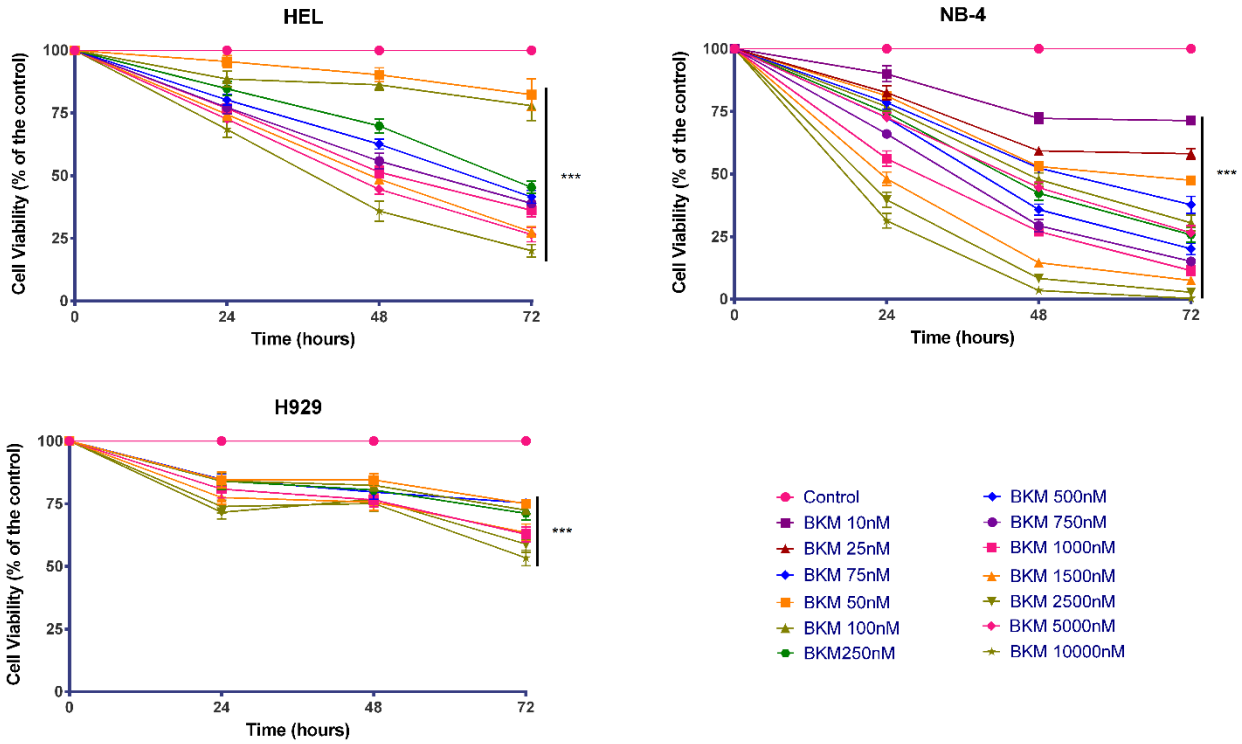

Figure 2. Dose-response curves of the single dose administration scheme of BKM120 in HEL, NB-4 and H929 cell lines. Cells were incubated with BKM120 for 72 hours, in a density of 0.3 x 10^6 cells/mL for NB-4, 0.4 x 10^6 cells/mL for HEL and 0.5 x 10^6 cells/mL for H929, as indicated in the figure. Viability dose response curves were established by resazurin assay each 24 hours, as described in the methods section. Cell viability is expressed in cell percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 5 independent experiments. * $p < 0.05$; ** $p<0.01$; *** $p<0.001$

A daily dose administration scheme was also evaluated in order to access if the frequency of administration influenced the reduction on cell viability, when compared to the single dose administration scheme. Results are represented in Figure 3 and demonstrate that the administration of a small cumulative dose of BKM120 induced a more pronounced reduction in cell viability when compared to the total dose in the single administration. The daily administration scheme led to a statistically significant additional reduction in cell viability after 72 hours of exposure was 20% in de HEL cell line, 28% in the NB-4 cell line and about 24%

in the H929 cell line. The NB-4 cell line was the one that benefited the most from this administration scheme. Using the daily dose administration scheme, we also verified that, in order to obtain the same effect, the dose needed was 10 times smaller than in single administration scheme.

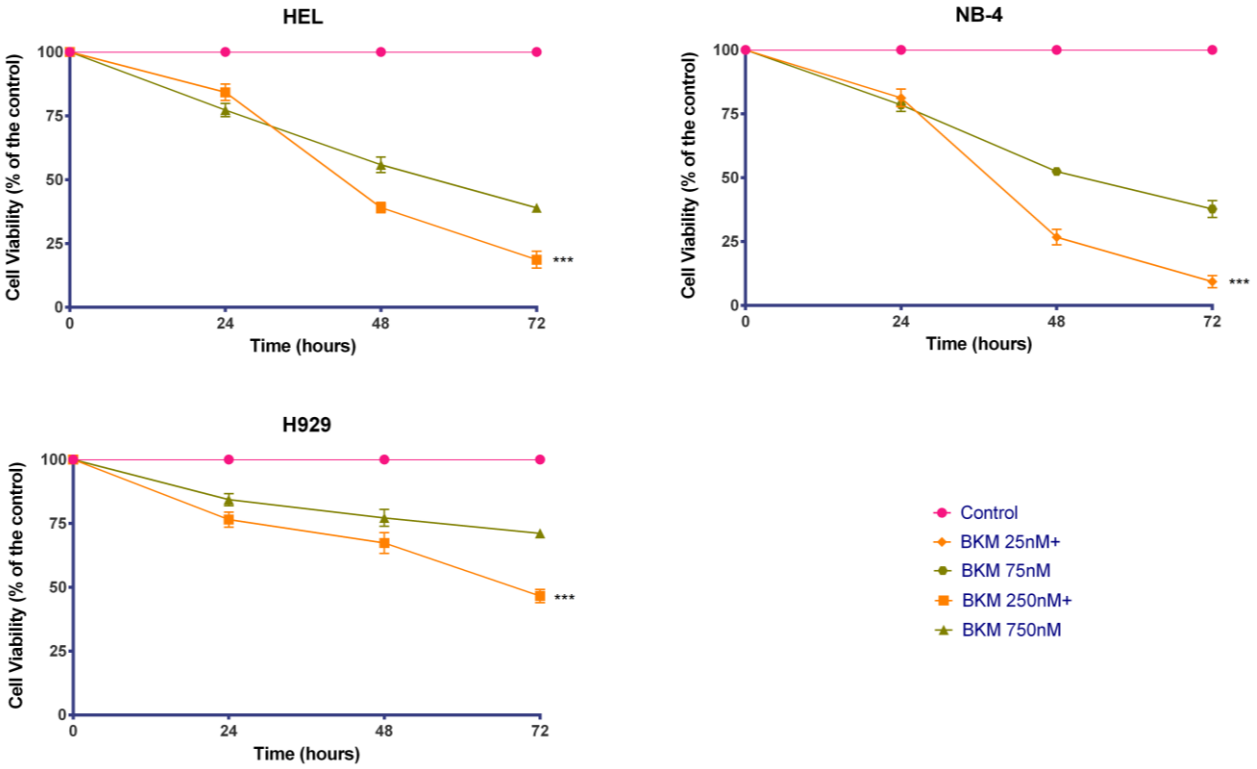

Figure 3. Dose-response curves of the daily dose administration scheme of BKM120 in HEL, NB-4 and H929 cell lines. Cells were incubated for 72 hours, in a density of 0.3 x 10^6 cells/mL for NB-4, 0.4 x 10^6 cells/mL for HEL and 0.5 x 10^6 cells/mL for H929, in the absence or the presence of the indicated concentrations of BKM120. The dose that represents the daily dose administration scheme is identified by (+). Viability dose response curves were established by resazurin assay each 24 hours, as described in the methods section. Cell viability is expressed in cell percentage (%) normalized to control. Data are expressed as mean ± SEM obtained from at least 5 independent experiments. Statistical analysis results from a comparison with the single dose administration of the same dose * $p < 0.05$; ** $p<0.01$; *** $p<0.001$

BKM120 induces caspase mediated apoptotic cell death in AML and MM cell lines

We analyzed the cell death induced by BKM120 using FC, through the AV/ PI double staining and also using optical microscopy with the May-Grünwald-Giemsa staining. A test to evaluate the percentage of cells expressing Caspases was also conducted.

- **Annexin V / Propidium Iodide double staining**

Our results revealed that exposure to BKM120 leads to a decrease in the number of live cells and to a parallel increase in cell death (Figure 4). Apoptosis was the cell mechanism activated by this inhibitor and the results were statistically significant for early apoptosis populations in all cell lines. Differences in necrotic cells were not statistically significant. Variations in early apoptosis were observed in the three cell lines and are dose dependent. The increase in early apoptosis was more pronounced in the NB-4 cell line when exposed to a BKM120 dose of 1000 nM.

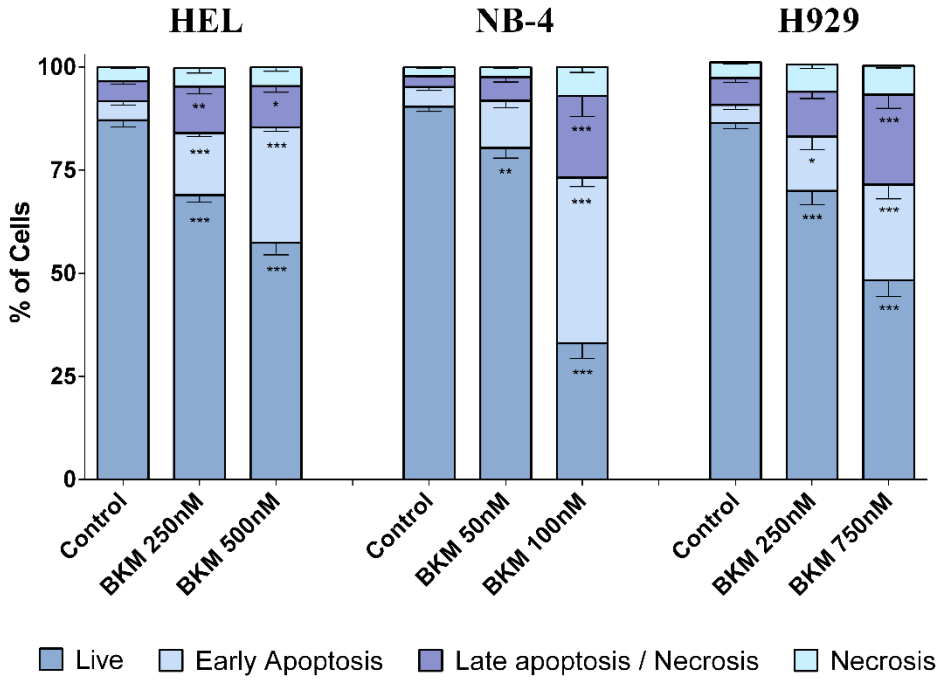

Figure 4. Evaluation of BKM120 induced cell death by flow cytometry in HEL, NB-4 and H929 cell lines. Cells were incubated for 48 hours, in a density of 0.3 x 10^6 cells/mL for NB-4, 0.4 x 10^6 cells/mL for HEL and 0.5

x 10^6 cells/mL for H929, in the absence or presence of different concentrations of BKM120 as indicated in the figure. Cell death was detected by annexin V/propidium iodide double staining and analysed by flow cytometry as described in the methods section. Data are expressed in percentage (%) and represent mean ± SEM of at least 4 independent determinations. * $p < 0.05$; ** $p<0.01$; *** $p<0.001$

- **Morphological analysis**

Cell morphology supports the results obtained in the AV/PI studies. Cells treated with BKM120 presented multiple morphological features that suggest apoptotic cell death (Figure 5). The main features identified are 'blebbing', chromatin condensation, nuclear fragmentation and the presence of apoptotic bodies. The relative number of cells presenting features of necrosis was not significant, further confirming that the main mechanism of cell death was apoptosis.

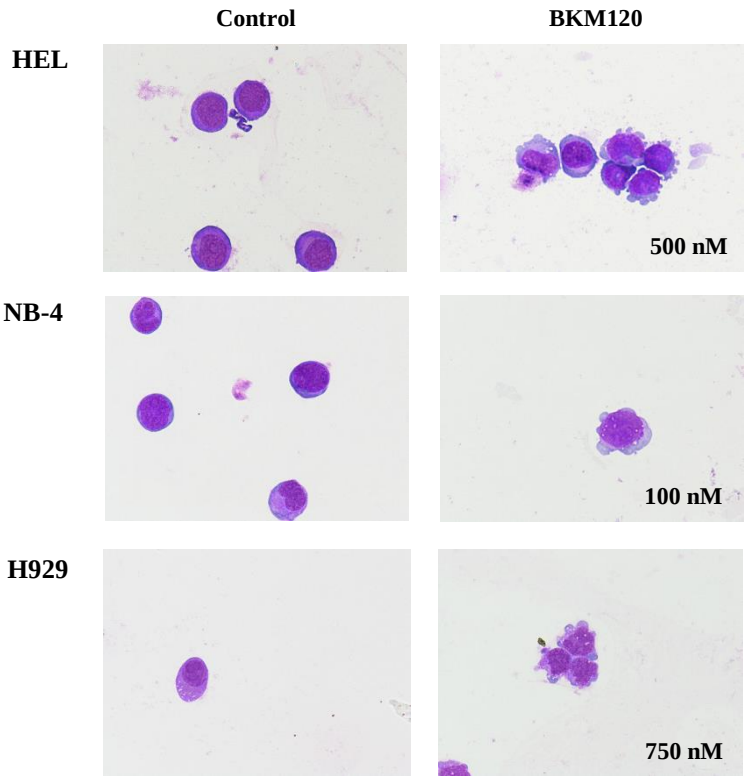

Figure 5. **Cell morphology analysis by optical microscopy in HEL, NB-4 and H929 cell lines.** Cells were incubated for 48 hours, in a density of 0.3 x 10^6 cells/mL for NB-4, 0.4 x 10^6 cells/mL for HEL and 0.5 x 10^6 cells/mL for H929, in the absence or presence of BKM120 and stained using a May-Grünwald-Giemsa method as

described in the methods section. The cells were analyzed by light microscopy (amplification 500x).

- **Caspase activity evaluation**

In order to further confirm the cell death mechanisms, we also evaluated caspases activity (Figure 6). Results showed that increasing concentrations of BKM120 lead to an increase in the percentage of cells expressing activated caspases. This increase was dose dependent and statistically significant, when compared to control in the three tested cell lines. After exposure to 100 nM of BKM120 almost 50% of NB-4 cells presented activated caspases.

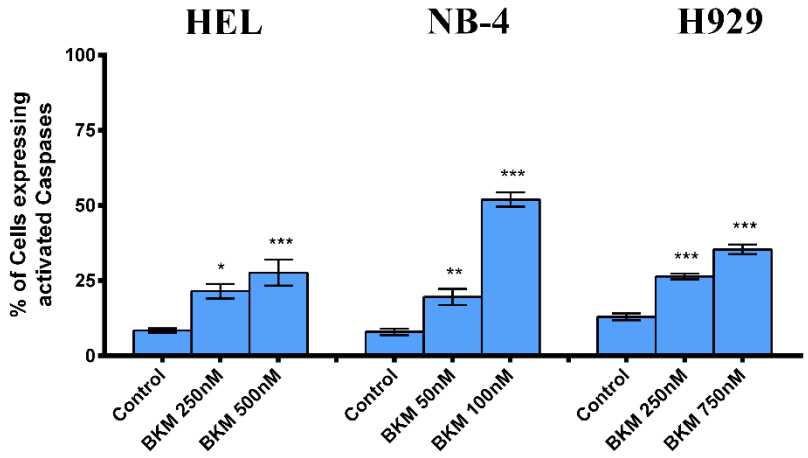

Figure 6. Flow cytometry studies of Caspases expression levels in HEL, NB-4 and H929 cells treated with BKM120. Cells were incubated with BKM120 for 48 hours, in a density of 0.3 x 10^6 cells/mL for NB-4, 0.4 x 10^6 cells/mL for HEL and 0.5 x 10^6 cells/mL for H929. Caspases expression levels were analyzed using the ApoStat® probe according to the protocol described in the methods section. Results are expressed in percentage of cells expressing activated Caspases and represent the mean ± SEM of at least 5 independent experiments.

* $p < 0.05$; ** $p<0.01$; *** $p<0.001$

BKM120 induces cell cycle arrest in AML and MM cell lines

In order to access if PI3K inhibition mediated by BKM120 interferes with the cell cycle progression, we conducted the PI/RNAse test as described in the methods section. As demonstrated in Figure 7, the effect produced by BKM120 on cell cycle was cell line dependent. For HEL (500 nM) and H929 (750 nM) cell lines, we observed a significant increase in percentage of cells on the G_0/G_1 phase. In the NB-4 (100 nM) cells the significant increase was observed in cells in S phase. In summary, this results show that BKM120 also induces dose and cell line dependent anti-proliferative effects by inducing a cell cycle arrest. Additionally, with this method we can also see the apoptotic peak, which represents DNA fragmentation, a typical process in apoptotic cell death. In our results, we observed an increased in apoptotic peak been the effect dose dependent. The differences were statistical significant in the NB-4 (100 nM) and H929 (750 nM) cell lines.

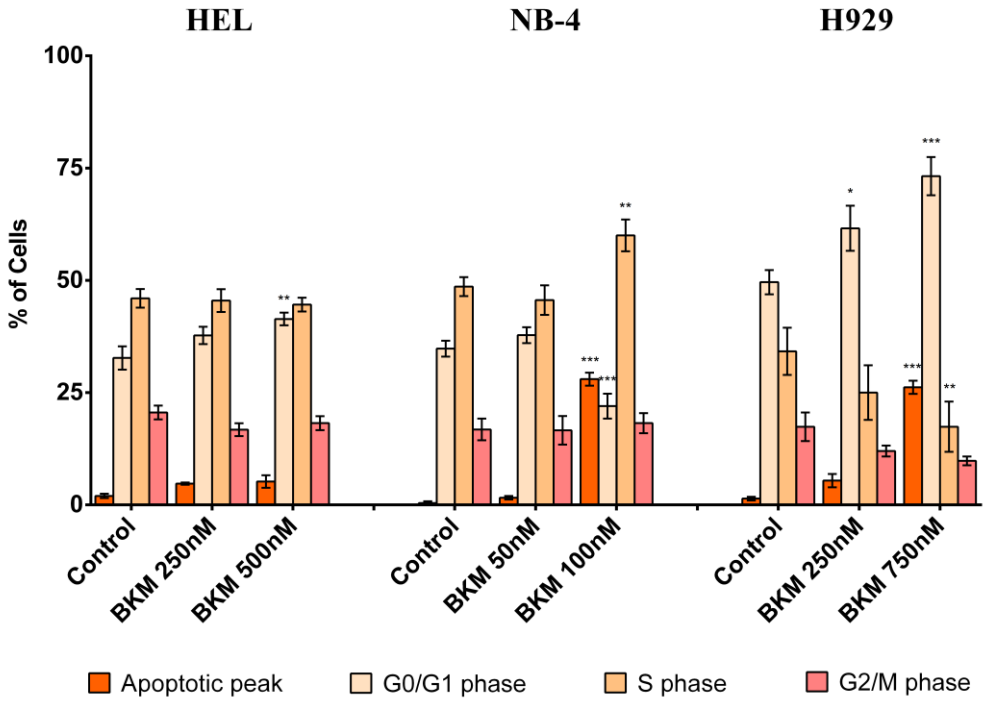

Figure 7. Effect of BKM120 on the cell cycle distribution of HEL, NB-4 and H929 cells by flow cytometry. Cells were incubated with BKM120 for 48 hours, in a density of 0.3 x 10^6 cells/mL for NB-4, 0.4 x 10^6 cells/mL for HEL and 0.5 x 10^6 cells/mL for H929. Cell cycle distribution was detected using Propidium iodide / RNAse

by flow cytometry as described in the methods section. Data are expressed as percentage of cells in Apoptotic peak, G_0/G_1 phase, S phase, G_2/M phase and represent mean ± SEM obtained from at least 4 independent experiments. * $p < 0.05$; ** $p<0.01$; *** $p<0.001$

Discussion and Conclusion

PI3K is responsible for the activation of a very complex set of downstream pathways which are involved in various key cellular processes. Deregulation of this protein is present in several types of cancer, including many types of hematological malignancies. The role of PI3K is related not only with tumorigenesis, but also with resistance to conventional chemotherapy, making its inhibition a promising path for the development of new treatments. In this study we evaluated the therapeutic potential of the pan-class I PI3K inhibitor – BKM120 – using *in vitro* cellular models of hematological malignancies.

Our results show that BKM120 has a cytotoxic effect in AML and MM cell lines both in single and daily dose administration schemes. The cytotoxic effect is time, dose, cell line and scheme of administration dependent being the AML cell line the most sensible with an IC_{50} of 80 nM (NB-4) and 2 µM (HEL) at 48 hours of exposure. The MM cell line was the less sensitive with an IC_{50} of over 10 µM even after 72 hours of exposure. The HEL cell line has the *JAK2 V617F* mutation [31], a *TP53* mutation [38] and a PIK3R3 (p85γ) substitution missense mutation [39]. Since p85γ is a class I PI3K isoform, its over-activation may contribute to tumorigenesis. A recent study suggested that PI3K inhibitors can be combined with *JAK2* inhibitors in *JAK2 V617F* mutated cells [40]. The NB-4 cell line has the t(15;17) [32] and there are no documented PI3K mutations. A study with this cell line suggested that PI3K inhibitors can potentiate apoptosis when combined with As_2O_3 in acute promyelocytic leukemia cell lines [41]. The H929 cell line has a rearrangement of the cellular *c-myc* proto-oncogene [33], a PIK3C2B (PIK-C2β) substitution missense mutation [39] but, since this gene codes a class II PI3K isoform, its role in tumorigenesis may not be as important as a gain of function mutation in a class I PI3K isoform [12].

Lymphoid cells, such as MM cells, were expected to be more sensitive to PI3K inhibition because of the role of class I PI3K in B-cells development. Results from the cell viability rezasurin assay show that the tested lymphoid cell line is considerably more resistant than myeloid counterparts since its IC_{50} at 48 hours of exposure is greater than 10 µM. The FC AV/PI studies also show that after exposure to a dose of 750 nM during 48 hours, 48% of the H929 cells were marked as live, also suggesting that this cell line was the most resistant. Nevertheless, it would have been interesting to test other lymphoid cell lines in order do access if there is a difference between the lymphoid and myeloid lineages.

Pan-class I PI3K inhibitors are effective because they inhibit class I isoforms and because it is believed that class I isoforms have redundant function being able to sustain PI3K activity and activation of downstream effectors [42]. However, in order to achieve a significant effect, they need to be administered in high doses that tend to limit clinical tolerance [24]. Other problems with pan-class I inhibitors are their off-target effects, as they are not fully selective to PI3K sub-units and may also bind to other kinases, leading to toxicity [24]. BKM120 adverse effects have already been described in several phase I clinical trials and include decreased appetite, diarrhea, nausea, hyperglycemia and rash [26]. Regarding these effects, a daily dose administration scheme was designed in order to test if a fractionated administration of a small cumulative dose could maintain the therapeutic effectiveness. Our results revealed that daily dose administration led to a significant additional reduction in cell viability when compared to the administration of the same dose at once. This type of administration scheme may have important consequences in the clinical setting since it has the potential to minimize dose related adverse effects.

Concerning cell death mechanisms that BKM120 activates, our results showed that it was mediated by apoptosis, which was dose dependent and confirmed by the multiple techniques used including a marked increase in the percentage of cells expressing caspases.

These results were concordant with other studies that demonstrated that BKM120 was able to induce apoptotic cell death in the MM H929 cell line [43] and in other AML cell lines [27,28].

PI3K is not only important in cell survival regulation but also in other key cellular processes like cell cycle regulation. We also tested BKM120 anti-proliferative effects in these cell lines and noted cell cycle arrests, specifically in the G_1 phase of NB-4 and H929 cells and in the S phase of HEL cells. These results were dose dependent, statistically significant and had already been demonstrated in the MM H929 cell line [43] and in other AML cell lines [27]. Another study has also described that BKM120 has the ability to interfere with microtubule dynamics [44].

In response to the low specificity and toxicity of pan-class I inhibitors, the isoform selective PI3K inhibitors allow a more precise inhibition, minimizing the effects on other cells and tissues [24]. The most targeted isoform is p110δ because of its role in B-cell development; p110α use as a target is related with the frequent mutation in its regulator gene *PIK3CA* and p110β is also used since it has an important role in tumors lacking PTEN [24]. Inhibition of p110δ isoform is already showing promising results in the treatment of some B-cell neoplasms like chronic lymphocytic leukemia (CLL) and some non-Hodgkin lymphomas [45]. In fact, the Food and Drug Administration (FDA), already approved the use of a p110δ isoform-selective PI3K inhibitor, called idelalisib, in the treatment of relapsed CLL in combination with rituximab. It would have been interesting to compare BKM120 effects with isoform selective inhibitors in the present study.

Associations of BKM120 with conventional chemotherapy drugs used in the treatment of hematological malignancies are also showing promising results. BKM120 has been showed to induce apoptosis in MM cells resistant to conventional therapies [28]. PI3K inhibition has also shown promising results in resistant myeloid leukemia since one of the mechanisms of resistance to RTK inhibitors is overexpression of the PI3K signaling pathway. These studies

show that targeting this pathway may help overcome or even prevent resistance mechanisms to conventional treatments [24].

Despite being based on a cell line culture study, our results show that BKM120 could be explored as a therapeutic approach in the treatment of hematological malignancies. Though this being a promising conclusion, further studies are warranted to validate this compound as an effective treatment for AML and MM. It is important to test BKM120 on three dimensional cell cultures, on *in vivo* animal models and in advanced stage tumors since other factors, not represented on cell cultures, like the microenvironment, also play a central role in the pathogenesis of these malignancies. We also want to evaluate the molecular effects of the compound, accessing the expression of PI3K isoforms and phosphorylation status of downstream effector proteins like AKT and mTOR, using techniques like real time polymerase chain reaction and western blot.

In conclusion, our study supports PI3K as a promising target in hematological neoplasms and BKM120 as a possible therapeutic approach in AML and MM. The exploration of cellular pathways as potential targets for cancer treatment expands our knowledge about cell biology, therefore improving patient survival and quality of life, the ultimate goal of biomedical research.

Acknowledgments

Este projecto de investigação não teria sido idealizado e concretizado sem o apoio das seguintes pessoas a quem quero deixar um sincero agradecimento.

À minha orientadora, a Professora Doutora Ana Bela Sarmento Ribeiro por me ter acolhido neste projecto, por estimular o meu espírito crítico, por ter estado sempre disponível para esclarecer as dúvidas que foram surgindo e por ter encorajado também a minha participação em congressos científicos que enriqueceram e potenciaram a minha aprendizagem e permitiram desenvolver capacidades, nomeadamente no que diz respeito à comunicação oral, que serão muito úteis no meu futuro profissional.

À minha coorientadora, a Dra. Raquel Silva Alves, por ter sido incansável na forma como coordenou a componente laboratorial do trabalho, pela forma como esteve atenta às minhas dificuldades estando sempre disponível para responder às minhas questões, mostrando não só sólidos conhecimentos científicos, mas também grandes virtudes no que diz respeito ao trabalho em equipa.

À Dra. Ana Cristina Gonçalves, por ter tido também um papel muito importante na componente laboratorial e por ter estado sempre disponível para responder às minhas dúvidas e para me aconselhar na elaboração do trabalho.

À Dra. Ana Pires, à Dra. Joana Jorge e a toda a restante equipa do Laboratório de Biologia Molecular Aplicada por também terem colaborado na componente laboratorial do projeto e por terem estado sempre disponíveis para me ajudar.

À minha colega e amiga Bárbara Almeida Marques por ter iniciado comigo esta caminhada e por ter mantido um espírito de cooperação que foi muito produtivo.

Por último, agradeço à minha família e aos meus amigos, que caminharam comigo nesta viagem de 6 anos, estando sempre ao meu lado, mesmo nos momentos mais difíceis.

References

1. Mbbs PAT, Kantarjian HM, Cortes JE. Diagnosis and Treatment of Chronic Myeloid. Mayo Clin Proc [Internet]. Mayo Foundation for Medical Education and Research; 2015;90(10):1440–54. Available from: http://dx.doi.org/10.1016/j.mayocp.2015.08.010

2. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med [Internet]. Massachusetts Medical Society; 2015 Sep 16;373(12):1136–52. Available from: http://dx.doi.org/10.1056/NEJMra1406184

3. Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse SF, et all. SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Natl Cancer Inst [Internet]. 2015; Available from: http://seer.cancer.gov/csr/

4. Ferrara F, Schiff CA. Acute myeloid leukaemia in adults. Lancet. 2013;381(381):484–95.

5. Palumbo A, Anderson K. Multiple Myeloma. N Engl J Med [Internet]. Massachusetts Medical Society; 2011 Mar 16;364(11):1046–60. Available from: http://dx.doi.org/10.1056/NEJMra1011442

6. Hawkins PT, Anderson KE, Davidson K, Stephens LR. Signalling through Class I PI3Ks in mammalian cells. Biochem Soc Trans. 2006;34(Pt 5):647–62.

7. Zhao L, Vogt P. Class I PI3K in oncogenic cellular transformation. Oncogene. 2008;18(9):1199–216.

8. Xia P, Xu X. PI3K / Akt / mTOR signaling pathway in cancer stem cells : from basic research to clinical application. 2015;5(5):1602–9.

9. Wang X, Ding J, Meng L. PI3K isoform-selective inhibitors: next-generation targeted

cancer therapies. Acta Pharmacol Sin [Internet]. Nature Publishing Group; 2015;36(10):1170–6.

10. Polak R, Buitenhuis M. The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. Blood [Internet]. 2012;119(4):911–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22065598

11. Asati V, Mahapatra DK, Bharti SK. PI3K / Akt / mTOR and Ras / Raf / MEK / ERK signaling pathways inhibitors as anticancer agents : Structural and pharmacological perspectives. Eur J Med Chem [Internet]. Elsevier Masson SAS; 2016;109:314–41. Available from: http://dx.doi.org/10.1016/j.ejmech.2016.01.012

12. Mayer IA, Arteaga CL. The PI3K / AKT Pathway as a Target for Cancer Treatment. 2015;(September 2015):1–18.

13. Hu Y, Guo R, Wei J, Zhou Y, Ji W, Liu J, et al. Effects of PI3K inhibitor NVP-BKM120 on overcoming drug resistance and eliminating cancer stem cells in human breast cancer cells. Cell Death Dis [Internet]. 2015;6(12):e2020. Available from: http://www.nature.com/doifinder/10.1038/cddis.2015.363

14. O'Brien NA, McDonald K, Tong L, Von Euw E, Kalous O, Conklin D, et al. Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT. Clin Cancer Res [Internet]. 2014;20(13):3507–20. Available from: http://clincancerres.aacrjournals.org/content/20/13/3507.full

15. Martelli AM, Evangelisti C, Chappell W, Abrams SL, Bäsecke J, Stivala F, et al. Targeting the translational apparatus to improve leukemia therapy: roles of the PI3K/PTEN/Akt/mTOR pathway. Leuk Off J Leuk Soc Am Leuk Res Fund, UK. 2011;25(December 2015):1064–79.

16. Kubota Y, Ohnishi H, Kitanaka A, Ishida T, Tanaka T. Constitutive activation of PI3K is involved in the spontaneous proliferation of primary acute myeloid leukemia cells: direct evidence of PI3K activation. Leuk Off J Leuk Soc Am Leuk Res Fund, UK. 2004;18(8):1438–40.

17. Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kB, MAPkinase and p53 pathways. Leukemia [Internet]. 2005;27:586–94. Available from: http://www.nature.com/doifinder/10.1038/sj.leu.2403653

18. Sujobert P, Bardet V, Cornillet-lefebvre P, Hayflick JS, Prie N, Verdier F, et al. Essential role for the p110δ isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. Control. 2005;106(3):1063–6.

19. Harvey RD, Lonial S. PI3 kinase/AKT pathway as a therapeutic target in multiple myeloma. Future Oncol. England; 2007 Dec;3(6):639–47.

20. To L, Editor THE. Distinct roles of class I PI3K isoforms in multiple myeloma cell survival and dissemination. 2014;1–5.

21. Ball J, Archer S, Ward S. PI3K inhibitors as potential therapeutics for autoimmune disease. Drug Discov Today [Internet]. Elsevier Ltd; 2014;19(8):1195–9. Available from: http://dx.doi.org/10.1016/j.drudis.2014.04.002

22. Banham-hall E, Clatworthy MR, Okkenhaug K. The Therapeutic Potential for PI3K Inhibitors in Autoimmune Rheumatic Diseases. Open Rheumatol J. 2012;34:245–58.

23. Enriquez-barreto L, Morales M. The PI3K signaling pathway as a pharmacological target in Autism related disorders and Schizophrenia. Mol Cell Ther [Internet]. Molecular and Cellular Therapies; 2016;9:203–10. Available from: http://dx.doi.org/10.1186/s40591-

016-0047-9

24. Fruman D, Rommel C. PI3K and Cancer: Lessons, Challenges and Opportunities David. Nat Rev Drug Discov. 2013;18(9):1199–216.

25. Maira S-M, Pecchi S, Huang A, Burger M, Knapp M, Sterker D, et al. Identification and Characterization of NVP-BKM120, an Orally Available Pan-Class I PI3-Kinase Inhibitor. Mol Cancer Ther [Internet]. 2012;11(2):317–28. Available from: http://mct.aacrjournals.org/cgi/doi/10.1158/1535-7163.MCT-11-0474

26. Bendell JC, Rodon J, Burris H A, De Jonge M, Verweij J, Birle D, et al. Phase I, dose-escalation study of BKM120, an oral pan-class I PI3K inhibitor, in patients with advanced solid tumors. J Clin Oncol. 2012;30(3):282–90.

27. Allegretti M, Ricciardi MR, Licchetta R, Mirabilii S, Orecchioni S, Reggiani F, et al. The pan-class I phosphatidyl-inositol-3 kinase inhibitor NVP-BKM120 demonstrates anti-leukemic activity in acute myeloid leukemia. Sci Rep. 2015;5:1–11.

28. Zheng Y, Yang J, Qian J, Zhang L, Lu Y, Li H, et al. Novel phosphatidylinositol 3-kinase inhibitor NVP-BKM120 induces apoptosis in myeloma cells and shows synergistic anti-myeloma activity with dexamethasone. J Mol Med. 2014;90(6):695–706.

29. Amrein L, Shawi M, Grenier J, Aloyz R, Panasci L. The phosphatidylinositol-3 kinase I inhibitor BKM120 induces cell death in B-chronic lymphocytic leukemia cells in vitro. Internatonal J Cancer. 2013;133(1):1–9.

30. Martin P, Papayannopoulou T. HEL cells: a new human erythroleukemia cell line with spontaneous and induced globin expression. Science (80-) [Internet]. 1982 Jun 11;216(4551):1233–5.

31. Quentmeier H, Macleod RAF, Zaborski M, Drexler HG. JAK2 V617F tyrosine kinase

mutation in cell lines derived from myeloproliferative disorders. Leukemia. 2006;2:471–6.

32. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood. 1991;77(5):1080–6.

33. Gazdar A, Ole K, Kirsch IR, Hollis F. Establishment and characterization of a human plasma cell myeloma culture having a rearranged cellular myc proto-oncogene. Blood. 1986;67(6):1542–9.

34. Riss TL, Niles AL, Minor L. Cell Viability Assays Guidance Manual. 2013;

35. Rello S, Stockert JC, Moreno V, Gámez A, Pacheco M, Juarranz A, et al. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. Apoptosis. 2005;10:201–8.

36. R&D Systems Apoptosis Catalog.

37. Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol. 1975;66.

38. Zhao W, Du Y, Ho WT, Fu X, Zhao ZJ. JAK2V617F and p53 mutations coexist in erythroleukemia and megakaryoblastic leukemic cell lines. Exp Hematol Oncol [Internet]. 2012;1:15.

39. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, et al. COSMIC : exploring the world ' s knowledge of somatic mutations in human cancer. Nucleic Acids Res. 2015;43(October 2014):805–11.

40. Szymańska J, Smolewski P, Majchrzak A, Cebula-Obrzut B, Chojnowski K, Treliński J. Pro-Apoptotic Activity of Ruxolitinib Alone and in Combination with Hydroxyurea,

Busulphan, and PI3K/mTOR Inhibitors in JAK2-Positive Human Cell Lines. Adv Clin Exp Med. 2015;(503):195–202.

41. Tabellini G, Tazzari PL, Bortul R, Evangelisti C, Billi AM, Grafone T, et al. Phosphoinositide 3-kinase / Akt inhibition increases arsenic trioxide-induced apoptosis of acute promyelocytic and T-cell leukaemias. 2005;2:716–25.

42. Foukas LC, Berenjeno IM, Gray A, Khwaja A, Vanhaesebroeck B. Activity of any class IA PI3K isoform can sustain cell proliferation and survival. 2010;107(25):11381–6.

43. Sahin I, Azab F, Mishima Y, Moschetta M, Tsang B, Glavey S V., et al. Targeting survival and cell trafficking in multiple myeloma and Waldenstrom macroglobulinemia using pan-class I PI3K inhibitor, buparlisib. Am J Hematol [Internet]. 2014;89(11):1030–6. Available from: http://doi.wiley.com/10.1002/ajh.23814

44. Brachmann SM, Kleylein-Sohn J, Gaulis S, Kauffmann a., Blommers MJJ, Kazic-Legueux M, et al. Characterization of the Mechanism of Action of the Pan Class I PI3K Inhibitor NVP-BKM120 across a Broad Range of Concentrations. Mol Cancer Ther. 2012;11(8):1747–57.

45. Forcello N, Saraiya N. Idelalisib: The First-in-Class Phosphatidylinositol 3-Kinase Inhibitor for Relapsed CLL, SLL, and Indolent NHL. J Adv Pract Oncol. 2014;5(6):455–9.