SARA LOPES PETRONILHO

ANTITUMOR ACTIVITY OF SPLICING INHIBITOR
PLADIENOLIDE B IN ERYTHROLEUKEMIA – A
STUDY IN CELL LINES
ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE HEMATOLOGIA/BIOLOGIA MOLECULAR
APLICADA

TRABALHO REALIZADO SOB A ORIENTAÇÃO DE:
PROF. DOUTORA ANA BELA SARMENTO ANTUNES CRUZ RIBEIRO
DRA. ANA CRISTINA PEREIRA GONÇALVES

MARÇO/2015
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

Sara Petronilho¹, Raquel Alves²³⁴, Ana Cristina Gonçalves²³⁴, Ana Bela Sarmento-Ribeiro²³⁴⁵

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

4- Center of Investigation in Environment, Genetics and Oncobiology (CIMAGO), FMUC, Portugal;

5- Hematology Department, Centro Hospitalar Universitário de Coimbra (CHUC), Portugal
Table of Contents

Abstract/Resumo 3
Abbreviations 7
Introduction 9
Materials and Methods 13
  Cell culture 13
  Cell density and viability analysis 13
  Assessment of cell death 14
  Cell cycle analysis 15
  Spliceosome mutation detection 16
  Statistical analysis 17
Results 18
  Antitumoral activity of Pladienolide B 18
  Cell death analysis 20
  Cell cycle analysis 23
  SF3B1 mutation analysis 24
Discussion and Conclusion 25
Acknowledgements 29
References 30
Abstract

The splicing of pre-mRNA into functional mRNA, carried out by the spliceosome, represents a crucial step for the cell's genetic expression. Mutations in some of the spliceosome’s components have been identified in several hematological malignancies, including myelodysplastic syndromes and acute myeloid leukemia (AML), which could constitute a potential therapeutic target to be explored. In this context, we evaluated the therapeutic potential of a splicing inhibitor, Pladienolide B (Pla-B), in two erythroleukemia cell-lines.

K562 and HEL cells were incubated in the absence or presence of increasing concentrations of Pla-B in single dose (from 0.25 to 100 nM) and in daily administration (for 0.5 nM). Cell viability and density were evaluated using the trypan blue method. Cell death was determined by optical microscopy (May-Grunwald Giemsa staining) and flow cytometry (FC). Cell cycle analysis was evaluated by FC, using a PI/RNAse solution. DNA sequencing was performed to assess the presence of SF3B1 mutations in exons 14 and 15.

Treatment with Pla-B significantly decreased the viability and proliferation of the K562 and HEL cells in a time, concentration and administration schedule dependent manner. HEL cells were more sensible to Pla-B than K562 cells (after 72 hours of incubation the IC₅₀ was 1.5 nM and 25 nM, respectively), which may be due to different cell genetic backgrounds. In fact, K562 cells present the BCR-ABL fusion gene and HEL cells the JAK2 V617F mutation. However, SF3B1 mutations in exons 14 or 15 were not detected in any cell model used, suggesting that the observed cytotoxic effect is not dependent on this spliceosome mutation. Pla-B induced cell death preferentially by apoptosis and induced also an accumulation of cells in the G0/G1 phase of the cell cycle.
Our results show that Pla-B induces a cytostatic and cytotoxic effect in K562 and HEL cells, suggesting that Pla-B could represent a new therapeutic approach in the treatment of erythroleukemia.

**Keywords:** Pladienolide B, Splicing inhibitor, $SF3B1$, AML, Erythroleukemia
Resumo

O splicing de pre-mRNA em mRNA funcional, mediado pelo spliceossoma, representa uma etapa fundamental na expressão genética da célula. Nos últimos anos, mutações em alguns dos componentes do spliceossoma foram identificadas em várias neoplasias hematológicas, incluindo síndromes mielodisplásicos e leucemia mielóide aguda, representando alvos terapêuticos por explorar. Neste contexto, avaliámos o potencial terapêutico de um inibidor do splicing, Pladienolide B (Pla-B), em duas linhas celulares de eritroleucemia.

As células K562 e HEL foram incubadas na presença ou ausência de concentrações crescentes de Pla-B, em dose única (0.25 nM a 100 nM) e dose diária (0.5 nM). A viabilidade e a densidade celulares foram avaliadas pelo método de azul tripano. A morte celular foi determinada por microscopia óptica (coloração de May-Grunwald Giemsa) e citometria de fluxo (FC). A análise do ciclo celular foi realizada por FC, usando uma solução de PI/RNase. Mutações nos exões 14 ou 15 do gene \textit{SF3B1} foram pesquisadas através de sequenciação do DNA.

O tratamento das células K562 e HEL com Pla-B reduziu significativamente a viabilidade e proliferação celulares, de um modo dependente de tempo, concentração e modo de administração do fármaco. As células HEL mostraram-se mais sensíveis ao fármaco do que as células K562 (apo\textit{s} 72 horas de incubação, o IC50 foi de 1.5 nM e 25 nM, respectivamente), o que pode ser devido a diferenças genéticas. De facto, as células K562 apresentam o gene de fusão \textit{BCR-ABL}, enquanto as HEL apresentam a mutação do \textit{JAK2 V617F}. No entanto, não foram encontradas mutações, em nenhum dos modelos, nos exões 14 ou 15 do gene \textit{SF3B1}, o que sugere que o efeito citotóxico observado não é dependente desta mutação. Verificámos que o Pla-B induz morte celular preferencialmente por apoptose, bem como induz uma
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

acumulação das células na fase G0/G1 do ciclo celular. Mutações nos exões 14 e 15 do gene SF3B1 foram excluídas.

Os nossos resultados sugerem que o Pla-B apresenta um efeito anti-proliferativo e citotóxico em ambas as linhas celulares, e que poderá representar uma nova abordagem terapêutica no tratamento da eritroleucemia.

**Palavras-Chaves:** Pladienolide B, inibidor do splicing, SF3B1, LMA, eritroleucemia
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

**Abbreviations**

AEL: acute erythroleukemia

AML: acute myeloid leukemia

AML-MRC: acute myeloid leukemia with myelodysplasia-related changes

APC: allophycocyanin

ATCC: american type culture collection

ATP: adenosine triphosphate

AV: annexin V

CML: chronic myelogenous leukemia

ddNTP: dideoxy nucleoside triphosphates

DMSO: Dimethyl sulfoxide

FAB: french-american-british

FBS: fetal bovine serum

FC: flow cytometry

IC\textsubscript{50}: half maximal inhibitory concentration

MDS: myelodysplastic syndrome

MDS/MPN: myelodysplastic syndrome/myeloproliferative syndrome

MIF: mean intensity fluorescence
mRNA: messenger ribonucleic acid

PBS: phosphate buffer solution

PI: propidium iodide

Pla-B: pladienolide B

*PRPF40B*: Pre-mRNA processing factor 40 homolog B

RNA: ribonucleic acid

RPMI: roswell park memorial institute

*RUNX1*: runt related transcription factor 1

PCR: polymerase chain reaction

SEM: standard error of the mean

*SF1*: splicing factor 1

SF3B: splicing factor subunit 3

SRSF2: serine/arginine-rich splicing factor 2

snRNA: small nuclear ribonucleic acids

*TET2*: ten eleven translocation 2 gene

U2AF: U2-associated factor

WHO: world health organization

*ZRSR2*: zinc finger, RNA-binding motif and serine/arginine rich 2
Introduction

Splicing is a fundamental cellular process by which the non-coding sequences known as introns are removed from pre-mRNA and the flanking exons are ligated to form functional mRNA. This complex multistep process is carried out by the spliceosome, a macromolecule composed of five small nuclear ribonucleic acids (snRNA) and numerous associated proteins (Figure 1) [1]. It starts with the spliceosome assembly phase, with formation of the E (Early) complex, which results from the ligation of the U1 snRNP to the 5’ splice site and from the U2AF (U2-Associated Factor) to the 3’ splice site and adjacent polypyrimidine tract (mediated by the subunits U2AF1 and U2AF2, respectively). This step is followed by an ATP dependent alteration in the transcript positioning which brings together the two exons and consequently facilitates the ligation of the U2 snRNP to the branch point sequencing, generating the A complex. The SF3B1 is the part of this U2 snRNP which mediates the binding to the intronic branch point sequence. The B complex is later formed by addition of the U4/U5/U6, leading to an ATP dependent conformational rearrangement which constitutes a catalytically active C complex, with the resultant release of U2AF, U1 and U4 snRNPs. Finally, two transesterifications occur and the intronic sequence is removed, with consequent connection of the contiguous exons [2].

Somatic mutations interfering with the splicing process have been identified in several malignancies, mostly in hematological neoplasms [2]. They were found to be especially prevalent in patients presenting with myelodysplastic syndromes (MDS), a heterogeneous group of diseases characterized by cell line dysplasia, ineffective hematopoiesis, peripheral cytopenias and higher predisposition to acute myeloid leukemia (AML) [2]. The most common mutations were found in U2AF1, SRSF2 and SF3B1, the latter being associated with the specific phenotype of ringed sideroblasts. These mutations were heterozygous, mutually
exclusive and often missense, occurring in specific hotspots for SF3B1 (in exons 14 and 15) and SRSF2. U2AF1 mutations affected exons 2 and 6, which correspond to the 2 zinc finger domains of the protein. Interestingly, evidence shows that the mutations did not result in a widespread splicing dysfunction, but rather in alterations of the splicing pattern of specific genes. For instance, U2AF1 mutations were associated with defective splicing of intron 5 of TET2 gene, whereas SF3B1 and SRSF2 mutations affected splicing of RUNX1 gene [3].

Figure 1. Schematic representation of the pre-mRNA splicing process, from J. Boulwood et al. in [1]. The E complex is formed by binding of the U1 snRNP to the 5’ splice site and SF1, SRSF2, ZRSRS2, U2AF35 and U2AF65 to the 3’ splice site. The U2 snRNP replaces SF1 and binds to the branch site, forming complex A, and aids the binding of U4/U5/U6 snRNP, to form complex B. This brings the two adjacent splice sites together and forms complex C, with subsequent intron removal and ligation of the contiguous exons.
Given this new genetic insight, over the past few years several substances that target the splicing process have been tested as antineoplastic agents in different malignancies, including breast, brain, colon, lung, ovarian, renal, gastric and prostate neoplasias in vitro [4,5]. Some in vivo studies have also been conducted, both in animal models [4,5] and in patients with advanced solid tumors [6], in the context of a phase I study. Between these new drugs is Pladienolide B (Pla-B), a twelve-membered macrolide ring, which inhibits the splicing process through direct targeting of the SF3B complex [7]. Various studies have shown its strong antitumor activity against the previously stated human cancer cell lines, as well as in primary cultures, with an IC$_{50}$ value in a low nanomolar range. Moreover, it has been proven to inhibit tumor growth and even induce complete regression in colon and gastric human cancer xenograph models in mice [4,5]. Furthermore, since Pla-B has shown a potent effect in cell lines resistant to conventional drugs [4] it could be a viable option in the treatment of refractory disease. However, to our knowledge, no data are available regarding the antineoplastic effect of this drug against hematological malignancies. The fact that spliceosome mutations are present in a variety of myeloid malignancies suggests that these could play an important role in defining a malignant phenotype.

Acute erythroleukemia (AEL) is a rare type of AML which consists of a clonal proliferation of erythroid precursors and usually also other myeloid precursors in bone marrow. Until recently, it was classified as an M6 AML, according to the French-American-British (FAB) classification system [8]. In 2008, the WHO proposed a new classification, where the category of AML with myelodysplasia-related changes (AML-MRC) was proposed. This category includes all cases of AML with presence of either morphologic evidence of significant multilineage dysplasia, specific MDS–related cytogenetic abnormalities, or a history of MDS or a MDS/MPN. Therefore, some AEL cases are now classified as AML-MRC. Cases with blasts comprising less than 20% of all cells but more
than 50% of erythroid precursors are classified as AEL, if blasts constitute 20% or more of the nonerythroid cells. If blasts constitute less than 20% of nonerythroid cells, the cases are classified into various categories of myelodysplastic syndromes (MDS). In a retrospective, multi-institutional study of patients with AEL conducted in 2010 [9], the majority of patients presented a secondary leukemia, whether with a previous history of MDS, chronic cytopenia or therapy-related disease. Furthermore, AEL is frequently accompanied by unfavorable cytogenetics. Another study [10] indicated that the outcome is better for patients treated with hypomethylating agents when compared to standard cytarabine based chemotherapy. However, the prognosis was still poor, with a median overall survival time of 15.4 months in the patients who received azacytidine. Thus, the development of novel drugs for the better management of this disease is imperative.

In this context, we studied the antineoplastic effect of Pladienolide B in two erythroleukemia cell lines, the K562 and HEL cells. We evaluated the effect of this new drug in cell growth and viability, and performed a cell death and cell cycle analysis, in order to better elucidate the mechanism of action of this drug. In addition, both cell lines were screened for the presence of mutations in the SF3B1 gene, to correlate mutation status with Pla-B efficacy.
Materials and Methods

Cell culture: In this study we used two different cell lines, K562 and HEL, which were purchased from the American Type Culture Collection (ATCC). The K562 cells were originally obtained from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia (CML) in blast crisis (characterized as erythroleukemia) and bear the translocation (9; 22), with consequent presence of the Philadelphia Chromosome [11]. The HEL cells were originally obtained from the bone marrow of a 30-year-old caucasian male with erythroleukemia and are JAK2-V617F mutated [12]. All cell lines were routinely grown in an advanced RPMI-1640 medium (L-glutamine 2 mM, HEPES-Na 25 mM, penicillin 100 U/mL and Streptomycin 100 µg/mL), supplemented with 5% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. Cells were cultured at initial density of $0.5 \times 10^6$ cells/mL for the K562 and $0.4 \times 10^6$ cells/mL for the HEL cell line.

Pladienolide B (Pla-B) was purchased from Santa Cruz Biotechnology (UK) and dissolved in DMSO. Both the HEL and K652 cell lines were incubated for 72 hours in the absence and in the presence of increasing concentrations of Pla-B, ranging from 0.25 to 100 nM. The drug was administered in single dose and for 0.5 nM of Pla-B was also tested a daily administration scheme. For flow cytometry and morphological studies was used 2.5 nM of Pla-B.

Cell viability and density analysis: The K652 and HEL cells viability and density was accessed every 24 hours during 72 hours by the trypan blue exclusion method. This method is based on the principle that live/viable cells possess intact cell membranes that exclude the dye, whereas dead/non-viable cells do not. Briefly, equal volumes of cell suspension and trypan blue (Sigma Aldrich) were added, loaded in a hemacytometer and counted under a microscope. Cell viability was calculated as the percentage of viable cells whereas cell
density was determined by the number of viable cells. The IC$_{50}$ (drug concentration that reduces the viability in 50%) was evaluated by a dose response curve.

**Assessment of cell death:** Cell death was evaluated by flow cytometry (FC), using anexin V (AV) and propidium iodide (PI) double staining. In apoptotic cells, phosphatidylserine migrates from the inner to the outer layer of the cell membrane. In the presence of calcium, AV exhibits an affinity for this phospholipid and when associated with a fluorochrome detects its redistribution in the cell membrane. In necrotic cells, the membrane becomes permeable and molecules such as PI enter the cell and binds to the DNA. Therefore, this assay discriminates among viable cells (AV-/PI-), early apoptotic (AV+/PI-), late apoptotic/necrotic (AV+/PI+), and necrotic cells (AV-/PI+), as represented in **Figure 2.** Succinctly, after 48 hours of incubation, the cells were co-stained with AV-APC (BD Pharmingen) and PI (BioLegend) using the manufacturer’s recommendations. The cells were washed with PBS, centrifuged at 500g for 5 minutes, resuspended in 100 µL of binding buffer and incubated with 5 µL of AV-APC solution and 2.5 µL of PI for 15 minutes in the absence of light. After incubation time, cells were diluted in 400 µL of binding buffer and analyzed by FC. Results are expressed in % ± SEM of at least three independent experiments.

![Figure 2](image.png)

**Figure 2.** Example of a dot plot obtained from a flow cytometry assay. Blue dots represent viable cells, green dots represent necrotic cells, red dots represent apoptotic cells and yellow are late apoptotic/necrotic cells.
The ApoStat Probe is designed to identify and quantify caspase activity in cells by FC. About 1x10^6 cells were resuspended in 1000 µL of PBS and incubated with 1µg of ApopStat. After a 15 min incubation period at 37°C, the cells were washed and resuspended in 400 µL of PBS for analyzed by FC. Results are represented in % ± SEM and in MIF (mean fluorescence intensity) ± SEM, which represents the mean fluorescence intensity detected in cells and is proportional to the number of molecules labeled with the antibody.

Cell death essays were conducted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and at least 10,000 events were collected by acquisition using CellQuest software (Becton Dickinson, San Jose, CA) and analyzed using Paint-a-Gate (Becton Dickinson, San Jose, CA).

Morphological studies were conducted to evaluate the alterations induce by exposure to Pla-B. After 48 hours of incubation, the cells were centrifuged at 1000xg for 5 minutes, being the supernatant excluded. The pellet was resuspended in 10 µL of FBS. Smears were stained for 3 minutes with May-Grünwald solution, and then for 15 minutes with Giemsa solution. After rinsing with distilled water, cell morphology was analyzed by light microscopy (Nikon Eclipse 80i microscope equipped with a Nikon digital camera DXm 1200F).

**Cell cycle analysis:** Cell cycle analysis was performed by FC, using PI/RNase solution (Immunostep). As previously said, PI is a fluorescent dye that stains DNA in permeable cells. The fluorescence intensity, read by FC, is proportional to the DNA quantity of each cell, allowing us to determine the relative proportion of cells in the G0/G1 phase (fewer amount of DNA), S phase (coincident with DNA replication) and G2/M phase (double DNA of the G0/G1 phase). Given that apoptotic cells undergo the process of DNA fragmentation, these cells are represented as an apoptotic peak pre-G1, with the fewest DNA quantity. 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, being incubated during 30 min on cold. Then, cells were washed with PBS, resuspended in 400 µL of PI/RNase solution and analyzed by FC. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used and at least 10,000 events were collected by acquisition using CellQuest software (Becton Dickinson, San Jose, CA). Results were analyzed using Modfit software (Becton Dickinson, San Jose, CA) and are expressed in % ± SEM of at least 3 independent experiments.

**Spliceosome mutation detection:** The presence or exclusion of mutations on exons 14 and 15 of the *SF3B1* gene was analyzed using the Sanger sequencing method. This method allows DNA sequencing by selective incorporation of chain terminating ddNTP by a DNA polymerase during *in vitro* DNA replication. Briefly, the *SF3B1* fragment was amplified using PCR primers flanking exons 14 and 15 (**Table 1**).

### Table 1. Sequences of primers used in the present study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Fragment Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF3B1 exon 14F</td>
<td>CCAACTCATGACTGTCTTTTCTT</td>
<td>362</td>
</tr>
<tr>
<td>SF3B1 exon 14R</td>
<td>GGGCAACATAGTAAGACCCCTGT</td>
<td></td>
</tr>
<tr>
<td>SF3B1 exon 15F</td>
<td>TTGGGGCATTAAACCTG</td>
<td>261</td>
</tr>
<tr>
<td>SF3B1 exon 15R</td>
<td>AATCAAAAGGTAATTTGTTGGA</td>
<td></td>
</tr>
</tbody>
</table>

PCR was performed in a reaction volume of 24 µl containing 2 µl of genomic DNA, 0.2 µl of Taq polymerase 5U/µl, 2.5 µl of Taq PCR Buffer, 0.5 µl of dNTP (10 mM each) and 0.5 µl of each of the primers. The PCR program was as follows: initial denaturation at 95 °C for 3 minutes and 34 cycles of amplification at 95 °C for 45 seconds, 60°C or 54°C (for exon 14 or
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

15, respectively) for 45 seconds and 72 °C for 45 seconds. A final extension step at 72 °C for 10 minutes was performed. After purification of the amplicons using the ExoSAP-IT enzyme, according to the manufacturer’s protocol, cycle sequencing was performed using a Big Dye Terminator v3.1 Cycle Sequencing kit, following the manufacturer’s instructions. The thermal cycling profile was as follows: initial denaturation at 94 °C for 3 minutes and 24 rounds of amplification at 96 °C for 10 seconds, 50°C for 5 seconds and 60 °C for 1 minute and 45 seconds. The products of the sequencing reactions were purified using DyeEx 2.0 columns and read in an optic microplate. Sequencing data were analyzed using Chromas software.

4.6 Statistical analysis: Statistical analysis was performed using the GraphPad Prism software. Student’s t test, analysis of variance, Dunnett’s test and Tuckey test were used to compare the different groups. A significance level of p < 0.05 was considered statistically significant. Results are expressed in mean ± SEM of the number of independent experiments indicated in the figure legend.
Results

Anti-tumoral activity of Pladienolide-B

We studied the influence of Pla-B in cell density and viability in two erythroleukemia cell lines, K562 and HEL, in single dose administration (Figure 3). Pla-B induces a decrease of cell density and cell viability in a time- and dose dependent manner, for both cell lines. For the K562 cell line, the exposure to Pla-B in concentrations higher than 2.5 nM induces a significant decrease of cell density when comparing with untreated cells. The same significant decrease was observed for HEL cells (Figure 3A), revealing the cytostatic effect of Pla-B. In terms of cell viability, we also observed a significant reduction following Pla-B treatment. However, this effect is cell line dependent, being the half maximal inhibitory concentration (IC$_{50}$) after 72 hours of incubation of 25 nM and 1.5 nM for K562 and HEL cells, respectively. Comparing the cytotoxic effect between cell lines, the HEL cell line revealed an IC$_{50}$ 15 times lower than K562 cells.
Figure 3. Dose-response curves of Pla-B in K562 and HEL cell lines. Cells were incubated in a density of 0.5 and 0.4 x 10^6 cells/mL, respectively, for 72 hours, in the absence and presence of increasing concentrations of Pla-B. Cell density (A) and viability (B) were established by the trypan blue method, as previously described in material and methods. 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

To assess if the frequency of administration influences the cytostatic and cytotoxic effect of Pla-B, a daily administration scheme was tested using the concentration of 0.5 nM (Figure 4). A daily administration of 0.5 nM was compared with 1.5 nM of Pla-B at single dose, since this corresponds to the cumulative dose at the end of 72 hours. The daily administration of Pla-B reveals to be more effective in the reduction of cell density and cell viability than the administration of 1.5 nM at single dose, especially in the HEL cells. After 72 hours of exposure, the daily dose induced an additional reduction of 15% and 45% in K562 and HEL cells viability, respectively, when compared to the single dose. The differences in cell viability induced by the different schemes of drug administration were statistical significant.
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

Figure 4. Dose response curves for daily administration of Pla-B in K562 and HEL cell lines. Cells were incubated in a density of 0.5 and 0.4 x 10^6 cells/mL, respectively, for 72 hours, in the absence (control) and presence Pla-B, in single or daily administration (#). Cell density (A) and viability (B) were established by the trypan blue method, as previously described. 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 comparing with 1.5nM of Pla-B at single dose.

Cell death analysis

We analyzed the cell death induce by Pla-B using Annexin V/PI double staining and by optical microscopy. As represented in figure 4, the exposure to 2.5 nM of Pla-B results in a significant reduction of viable cells with a significant increase of cells in early apoptosis. The cell death induced was mainly mediated by apoptosis, but in case of HEL cells, we also observed significant differences in percentage of cells in necrosis.
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

Figure 5. Cell death analysis induced by Pla-B in K562 and HEL cell lines using flow cytometry. Cells were incubated in a density of 0.5 and 0.4 x 10^6 cells/mL, respectively, for 48 hours, in the absence (control) or presence of 2.5nM of Pla-B. Cell death was detected by annexin V/propidium iodide staining, analyzed by flow cytometry. Data are expressed in percentage (%) and represent mean ± SEM of at least 3 independent determinations. * p<0.05, ** p<0.01, *** p<0.001.

In agreement with flow cytometry analysis, the morphological evaluation showed typical alterations of cell death mediated by apoptosis in both cell lines. After treatment with Pla-B it was possible to observe blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation, confirming apoptosis (Figure 6). In the HEL cell line, we also observed cells with permeable membrane and intact nucleus characteristic of necrosis, revealing the activation of both cell death mechanisms.
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

Figure 6. Cell morphological analysis by optical microscopy. K562 and HEL cells were incubated in a density of 0.5 and 0.4 x 10^6 cells/mL, respectively for the K562 (A) and HEL (B) cells, for 48 hours, in the absence (control) or presence of 2.5 nM of Pla-B and stained using a May-Grünwald-Giemsa method. The cells were analyzed by light microscopy (amplification 100x).

We also analyzed if cell death induced by Pla-B was mediated by caspases activation (Figure 7). Treatment with Pla-B induced an increase in percentage of cells with activated caspases (of 13% for the K562 cells and 30% for the HEL cells, represented in Figure 7A) and in the expression level of caspases (of 5% in the HEL cells, represented in Figure 7B), being more pronounced in HEL cells. The differences in percentage of cells were statistical significant for HEL cell line.

Figure 7. Evaluation of caspases expression levels in K562 and HEL cells treated with Pla-B by flow cytometry. Cells were incubated in a density of 0.5 and 0.4 x 10^6 cells/mL, respectively, for 48 hours, in the absence (control) or presence of 2.5nM of Pla-B. Caspases expression levels were analyzed using the ApoStat probe. Results are expressed in percentage of cells positive (A) and in MIF (B) and represent the mean ± SEM of at least 3 independent experiments. ** p<0.01
Cell cycle analysis

Besides the confirmed cytotoxic effect induced by Pla-B, to confirm the cytostatic effect, we analyzed the effect on cell cycle progression by FC using a PI/RNase. As represented in Figure 8, Pla-B induced a significant arrest in the G0/G1 phase in both cell lines, when compared with untreated cells (respectively, 44±1.9% vs 37±1% in K562 cells and 63±0.9% vs 35±2.1% in HEL cells). Additionally, by the same technique it was detected the presence of an apoptotic peak (pre-G1), corresponding to DNA fragmentation typical of apoptotic cells.

![Graph showing cell cycle distribution of K562 and HEL cells after Pla-B treatment.](image)

**Figure 8.** Cell cycle distribution of K562 and HEL cells after Pla-B treatment. Cells were incubated in a density of 0.5 and 0.4 x 10^6 cells/mL, respectively, for 48 hours, in the absence (control) or presence of 2.5 nM of Pla-B. Cell cycle distribution was detected using PI/RNase by flow cytometry. Data are expressed as percentage of cells in G0/G1 phase, S phase, G2/M phase and apoptotic peak and represent mean ± SEM obtained from at least 3 independent experiments. * p<0.05, ** p<0.01, *** p<0.001.
**SF3B1 mutation analysis:**

The mutational status of *SF3B1* could influence the response levels of cells to Pla-B. Thus, using the previously described DNA sequencing method, we searched for mutations in exons 14 and exon 15 of the *SF3B1* gene in both cell lines. For our in vitro models, we did not find any mutations in these two exons. Particularly, no substitutions were revealed in positions H662D or K700E, respectively.
Antitumor activity of splicing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

Discussion and Conclusion

In this study, we evaluated the therapeutic efficacy of Pladienolide B (Pla-B), a splicing inhibitor that specifically targets the ribonucleoprotein complex SF3B. By impairing the binding of the spliceosome to the branch sequence, it leads to a splicing dysfunction that results in an accumulation of un-spliced mRNA in the cell and consequently in a reduction of cell viability [7, 13]. Nevertheless, its mechanism of action is still not fully understood. As mutations in spliceosome components are extremely prevalent in myeloid neoplasias [3], this drug could represent a new therapeutic target in the treatment of such diseases.

Our study shows that Pla-B presents a cytotoxic and a cytostatic effect against erythroleukemia cell lines in a time, dose and administration scheme dependent manner. Induction of cell death was mainly mediated by apoptosis, with activation of caspases. These results were in accordance with the experimental study conducted by Sato et al. in gastric cancer cell lines, which reveal the activation of apoptotic cell death [5]. We verified that Pla-B induces an accumulation of cells in the G0/G1 phase, reflecting the anti-proliferative action of this drug. A previous study conducted by Mizui et al. in WiDr cells had shown a preferential arrest in G1 phase after treatment with Pla-B, but also in the G2/M-phase, in a time dependent manner [4].

The administration scheme has been explored with attempt to minimize the side effects of anti-cancer agents. We analyzed two different administration schemes, namely the single dose administration and the small daily dose administration. Our results show that the daily dose administration of 0.5 nM of Pla-B appears to have more efficacy in both cell lines when compared with 1.5 nM at single dose, since it induces a higher cytotoxicity. Daily administration scheme could constitute a valid option to reduce the potential side effects of Pla-B.
Interestingly, Pladienolide B also presented a cell type dependent effect. In fact, HEL cells were much more sensitive to Pla-B than K562 cells, with an IC\textsubscript{50} at 72 hours of exposure of 1.5 nM vs 25 nM, respectively. Further studies are needed to disclose the exact reason by which this phenomenon occurs; however, several differences between these two cell lines can potentially contribute to the differential response.

Firstly, although both K562 and HEL are erythroleukemia cell lines, they have a different origin and genetic background. As previously described, K562 cells were first obtained from a patient with a CML in blast crisis, and present the translocation between chromosomes 9 and 21, that originates Philadelphia chromosome and the fusion gene \textit{BCR-ABL} \cite{11}. The HEL cell line, on the other hand, was obtained from a patient with an erythroleukemia, who had previously received treatment for a Hodgkin Lymphoma, and present the \textit{JAK2-V617F} mutation \cite{12}. These genetic differences may contribute to the different drug-responses. In fact, the presence of the \textit{BCR-ABL} fusion gene has been associated with changes in the expression of proteins involved in alternative pre-mRNA splicing, a process which greatly diversifies the transcriptome and which is thought to contribute to the oncogenic processes. \cite{14} Additionally, the \textit{BRC-ABL} \textsuperscript{p210} oncoprotein has been shown to increase the expression of multiple genes involved in pre-mRNA splicing \cite{14}. These aberrances in the splicing process can lead to important cell dysfunction, with a considerable effect in the regulation of cell proliferation and apoptosis \cite{15}. Thus, it would be necessary to study the splicing function of the K562 cells in order to better understand the lower drug-sensitivity and if response is correlated with previous splicing dysfunction.

Several authors have also hypothesized that cells may react differently to Pla-B according to the presence or absence of different splicing factor mutations \cite{1,2,16,18}. In the present study, only exons 14 and 15 of gene \textit{SF3B1} were screened for mutations. As stated in
the results section, no mutations were found in the K562 or the HEL cell lines in neither exon. However, we did not exclude other mutations in the same or in a different gene. The possible mutation in other point may also contribute to the different response levels. H662D (C>G) and K700E (A>G) mutations in exons 14 and 15, respectively, have been described as hotspots in SF3B1 gene. Even though these mutations are between the most frequent in hematological neoplasias [16], numerous other mutations have been identified, both in SF3B1 and in other splicing related genes. Particularly, the mutation R1074H in SF3B1 gene has been associated with resistance to Pladienolide B in colorectal carcinoma cells in vitro [7]. The mutation confers resistance since it impairs the binding of Pla-B to the target [7]. However, a mutation in this residue has so far not been detected in patients [2].

Other mutated genes which affect splicing that have been identified in myeloid neoplasias may also influence the efficacy of drugs that target this process. These include mutations in genes SRSF2, U2AF1, ZRSR2, SF3A1, U2AF2, SF1 and PRPF40B [16]. Therefore, and given the high occurrence of mutations affecting these genes in myeloid neoplasias [1,17], we cannot exclude their existence in our models. Nevertheless, it is important to realize that even though the presence of a mutation may explain a different response to the drug, the cause-effect mechanism is not linear or well understood. In fact, it is not yet well established whether splicing mutations represent a gain or loss of function, although the presence of hot spots and the absence of nonsense or frameshift changes suggest that the vast majority are gain of function/neomorphic mutations [1]. Hypothetically, in the presence of a gain of function/neomorphic mutation, the use of specific spliceosome inhibitors may allow a preferential targeting of the mutant phenotype. Moreover, since splicing factor mutations appear to be mostly heterozygous [3], disruption of the remaining allele using splicing inhibitors may induce preferential killing of the mutant cells and, eventually, spare normal cells. Additionally, it has been proposed that these mutations may result in aberrant
alternative splicing, in which case the splicing inhibitor may inhibit the gain of function effect of the mutation and restore a normal phenotype [18]. On the other hand, in loss of function mutations/dominant-negative effect, the use of splicing inhibitors may result in dysfunction of the normal allele, with worsening of the phenotype [2]. Therefore, not only is it important to identify the mutations, but also to better characterize their effect, in order to improve our understanding and prediction of drug response.

Moreover, a study conducted in gastric cell lines using a Pla-B derivative associated a higher expression of cell cycle proteins, such as cyclin E and p16, with higher sensitivity to this drug [5]. The authors suggest that these molecules could be used as a biological biomarkers in the future, although a more profound analysis is needed [5]. Therefore, differences in the expression of these molecules in our cell lines should be tested in a posterior study to clarify the different sensitivity to Pla-B.

Undoubtedly, further studies are needed to clarify the mechanism of action of Pla-B, as well as its potential role in the treatment of hematological malignancies. In particular, understanding the reason why these two cell lines respond differently in the presence of the drug may help to understand the mechanism of action of Pla-B, as well as the profile of patients which may benefit from this treatment.

In conclusion, Pladienolide-B shows a high antitumor activity against HEL and K562 cell lines, inducing cell death preferentially by apoptosis and a G0/G1 cell cycle arrest. Even though further elucidations are needed, Pla-B most certainly represents a promising new approach in the treatment of erythroleukemia.
Acknowledgements

This project was supported by Center of Investigation in Environment, Genetics and Oncobiology (CIMAGO).

I thank Doutora Letícia Ribeiro and Dra. Margarida Coucelo for their support in DNA analysis (Hemato Oncology Laboratory of the Pediatric Hospital, Coimbra).

I thank Professor Ana Bela Sarmento, as well as the whole laboratory team, for their support in numerous occasions, without which this work would not have been possible.
Antitumor activity of splici

ing inhibitor Pladienolide B in erythroleukemia – a study in cell lines

References


