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DIOGO PIRES DOS SANTOS

THE POTENTIAL THERAPEUTIC EFFECT OF HEAT SHOCK PROTEIN 90 INHIBITION IN CHRONIC MYELOID LEUKEMIA

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Trabalho realizado sob a orientação de:
PROF. DOUTORA ANA BELA SARMENTO ANTUNES CRUZ RIBEIRO
PROF. DOUTORA MARIA JOANA LIMA BARBOSA DE MELO

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THE POTENTIAL THERAPEUTIC EFFECT OF HEAT SHOCK PROTEIN 90

INHIBITION IN CHRONIC MYELOID LEUKEMIA

Santos D.[1], Alves R.[1,2], Gonçalves A.C.[1,2], Jorge J.[1,2], Catarino S.[3], Girão H.[3], Melo J.B.[2,4] Sarmento-Ribeiro A.B.[1,2,5,6]

[1] Laboratory of Oncobiology and Hematology (LOH) and University Clinic of Hematology – Faculty of Medicine, University of Coimbra (FMUC), 3000-354 Coimbra, Portugal;

[2] Coimbra Institute for Clinical and Biomedical Research (iCBR) – Group of Environment Genetics and Oncobiology (CIMAGO) – FMUC, 3000-354 Coimbra, Portugal;

[3] Coimbra Institute for Clinical and Biomedical Research (iCBR) – FMUC, 3000-354 Coimbra, Portugal;

[4] Cytogenetics and Genomics Laboratory, Faculty of Medicine, University of Coimbra, Portugal.

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

[6] Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Coimbra, Portugal

Abstract

Heat Shock Protein 90 (HSP90) facilitates the maturation, stability, activity and intracellular folding of more than 200 proteins, called 'client proteins'. In cancer cells, HSP90 helps to overcome multiple environmental stresses, including genomic instability/aneuploidy, proteotoxic stress, increased nutrients demand, reduced oxygen levels, and to prevent destruction by the immune system. One of these client proteins of HSP90 is BCR-ABL, the oncoprotein responsible for Chronic Myeloid Leukemia (CML). Alvespimycin (17-DMAG) is a HSP90 inhibitor that has better pharmacokinetic properties and fewer side-effects compared to others benzoquinone ansamycins.

This work aims to study the potential therapeutic effect of 17-DMAG in CML cell lines (sensitive and resistant to imatinib) and to explore the role of HSP family in the sensitivity to Imatinib.

In this context, we used three CML cell lines: the K562 cells, sensitive to Imatinib, and the K562-RC and K562-RD cells, resistant to Imatinib. Cells were incubated in the absence and presence of increasing concentrations of 17-DMAG (from 1 to 1000 nM) in single dose. The dose-response curves were determined by the resazurin assay. Cell death was determined through microscopy (May-Grunwald Giemsa staining) and flow cytometry (FC), using Annexin V and Propidium Iodide (PI) double staining. The Apostat Probe was used to evaluate caspase expression levels and the JC-1 probe to determine the mitochondrial membrane potential, through FC. Cell cycle was evaluated with FC, using PI/RNase assay. The protein expression levels of HSP family were assessed with western blot.

Our results showed that 17-DMAG induces a reduction in cell lines metabolic activity in a time, dose and cell type dependent manner, with an IC_{50} of 50 nM for K562 and less than 50 nM for the K562-RC and K562-RD cell lines, after 48 hours of treatment. This compound induces cell death predominantly by apoptosis, confirmed through morphological analysis, FC

and by the increase in JC-1 Monomers/Aggregates ratio. Apart from the cytotoxic effect, the cell cycle analysis showed that 17-DMAG induces cell cycle arrest in G_0/G_1 for K562 and K562-RC, and in G_2/M for K562-RD. The HSP protein analysis showed that K562 have slightly more expression of HSP90 than K562-RC and K562-RD cells and the treatment with 17-DMAG induced a significant increase in HSP70 expression. In conclusion, our results suggest that inhibition of HSP90 by 17-DMAG could be used as a new potential approach in the treatment of CML, even in cases of Imatinib resistance.

Resumo

A Proteína de Choque Térmico 90 (HSP90) facilita a maturação, estabilidade, atividade e enrolamento intracelular de mais de 200 proteínas, designadas "proteínas clientes". Nas células cancerígenas, a HSP90 ajuda a superar múltiplos stresses ambientais, incluindo instabilidade genómica/aneuploidia, stress proteotóxico, necessidades nutricionais aumentadas, níveis de oxigénio reduzidos, e ajudam na evasão ao sistema imune. Uma das proteínas clientes da HSP90 é a proteína BCR-ABL, uma oncoproteína responsável pela Leucemia Mieloide Crónica (LMC). A Alvespimicina (17-DMAG) é um inibidor da HSP90 que tem melhores propriedades farmocinéticas e menos efeitos secundários comparando com outras ansamicinas benzoquinonas.

Este trabalho tem por objetivo o estudo dos efeitos do 17-DMAG em linhas celulares de LMC (sensível e resistentes ao Imatinib) e explorar o papel da família das HSP na sensibilidade ao Imatinib.

Neste contexto, utilizámos três linhas celulares de LMC: a linha K562, sensível ao Imatinib, e as linhas K562-RC e K562-RD, resistentes ao Imatinib. As células foram incubadas na ausência e na presença de concentrações crescentes de 17-DMAG (de 1 a 1000 nM) em administração única. As curvas dose-resposta foram determinadas pelo teste da resazurina. A morte celular foi determinada por microscopia (coloração May-Grunwald Giemsa) e por citometria de fluxo (CF), usando dupla fixação de Anexina V e Iodeto de Propídeo (IP). A sonda Apostat foi usada para avaliar os níveis de expressão das caspases e a sonda JC-1 para determinar o potencial de membrana mitocondrial por CF. O ciclo celular foi avaliado por CF, usando o teste PI/RNase. Os níveis de expressão proteica da família das HSP foram analisados por western blot.

Os nossos resultados mostraram que o 17-DMAG induz uma redução da atividade metabólica das linhas celulares, dependente do tempo, da dose e da linha celular, com um IC_{50}

de 50 nM para as células K562 e inferior a 50 nM para as células K562-RC e K562-RD, após 48 horas de tratamento. Este composto induz morte celular predominantemente por apoptose, confirmado pela análise morfológica, CF, e pelo aumento da razão JC-1 Monómeros/Agregados. A análise do ciclo celular mostrou que o 17-DMAG induz bloqueio do ciclo celular em G_0/G_1 nas células K562 e K562-RC e em G_2/M nas células K562-RD. A análise proteica das HSP mostrou que as células K562 têm ligeiramente mais expressão de HSP90 que K562-RC e K562-RD e o tratamento de 17-DMAG induziu um aumento significativo da expressão de HSP70. Em conclusão, os nossos resultados sugerem que a inibição de HSP90 pelo 17-DMAG poderá constituir uma potencial nova abordagem terapêutica da LMC, mesmo em casos de resistência ao Imatinib.

Palavras-chave: LEUCEMIA MIELOIDE CRÓNICA, BCR-ABL, 17-(DIMETILAMINOETILAMINO)-17-DEMETOXIGELDANAMICINA, RESISTÊNCIA AO IMATINIB, PROTEÍNAS DE CHOQUE TÉRMICO

Abbreviations and Acronyms

17-AAG: 17 allylamino-17 demethoxygeldanamycin; tanespimycin

17-DMAG: 17 dimethylaminoethylamino-17-demethoxygeldanamycin; alvespimycin

A: Aggregates

AML: Acute Myeloid Leukemia

AV: Annexin-V

BCRP: Breast Cancer Related Protein

CML: Chronic Myeloid Leukemia

c-Myc: Myelocytomatosis oncogene

CP: Chronic Phase

CTD: C-terminal domain

EDTA: Ethylenediamine tetraacetic acid

EGFR: Epidermal growth factor receptor

FC: Flow cytometry

GA: Geldanamycin

HIF-1α: Hypoxia inducible factor 1α

HSC: Hematopoietic stem cell

HSF-1: Heat shock factor 1

HSP: Heat Shock Protein

IC$_{50}$: Half-maximal inhibitory concentration

IM: Imatinib mesylate

JC-1: 5,5',6,6'-tethrachloro-1,1'3,3'-tetraethylbenzimidazolcarbocyanine iodide

JNK/SPAK: c-jun-N-terminal kinase

LSC: Leukemia Stem Cell

M: Monomers

MAPK: Mitogen-activated protein kinase

MD: Middle-domain

MIF: Mean intensity fluorescence

NF-kβ: Nuclear factor kappa B

NTD: N-terminal domain

OCT1: Organic cation transporter 1

OCTN2: Organic cation/carnitine transporter

PBS: Phosphate-buffered saline

Ph: Philadelphia Chromosome

PI: Propidium iodide

PI$_3$K: Phosphoinositide 3-kinases

RIPA: Radioimmunoprecipitation assay

STAT: Signal transducers and activator of transcription

TK: Tyrosine kinase

TKI: Tyrosine-kinase inhibitor

VHL: von Hippel-Lindau

Ψmit: Mitochondrial membrane potential

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Introduction

Chronic myelogenous leukemia (CML) is a pluripotent hematopoietic stem cell (HSC) disorder characterized by the Philadelphia chromosome (Ph) translocation.[1] It has an incidence of 1-2 cases per 100.000 adults and it accounts for approximately 15% of newly diagnosed cases of leukemia in adults.[2] The human Ph arises from a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)][3], that originates the chimeric oncogene *BCR-ABL* with the protein product BCR-ABL, a tyrosine kinase (TK) with constitutive activity.[4] This constitutively active TK enhances proliferation and viability of myeloid cells lineage.[5] BCR-ABL activates many signaling pathways, including RAS small GTPase, mitogen-activated protein kinase (MAPK), signal transducers and activator of transcription (STAT), c-jun-N-terminal kinase (JNK/SPAK), phosphoinositide 3-kinases (PI$_3$K), nuclear factor kappa B (NF-kβ) and myelocytomatosis oncogene (c-Myc), through which BCR-ABL promotes survival and proliferation and reduces apoptosis of leukemia cells.[6] Many of these signaling pathways are normally regulated by hematopoietic growth factors, such as stem cell factor, thrombopoietin, interleukin-3, or granulocyte/macrophage colony-stimulating factor.[5] In many model systems, BCR-ABL completely abrogates growth factor dependence and has been associated with reduced requirement for growth factors in primary hematopoietic cells.[5]

BCR-ABL forms a complex with heat shock protein 90 (HSP90) and their interaction is necessary for the cellular stability and the aberrant kinase activity of BCR-ABL.[7] Heat shock proteins (HSPs) are highly conserved molecular chaperones that are synthesized and expressed by the cell in response to stress conditions[8], as elevated temperature, hypoxia, non-physiologic pH, nutrient deprivation, and also cancer.[9] Recent studies have shown that HSPs are often highly expressed in various types of cancer.[8]

HSP90 is one of the most abundant proteins in eukaryotic cells, comprising 1-2% of cellular proteins under non-stress conditions.[10] There are two major cytoplasmic isoforms of HSP90, HSP90α (inducible form/major form) and HSP90β (constitutive form/minor form).[10] It is an ATP-dependent molecular chaperone that is essential for the maintenance, activation or maturation of a wide range of proteins (known as clients).[7] Apart from the conformational changes induced by ATP hydrolysis, the function of HSP90 is also regulated by co-chaperones (Cdc37, p23, ubiquitin ligase-associated protein SGT1, Aha1, HOP, TAH1, CHIP) and post-translational modifications.[3] It mediates diverse fundamental cellular processes/functions including cell cycle control, cell survival, hormone signaling, trafficking, and response to cellular stress.[7] HSP90 helps cancer cells overcome multiple environment stresses, including genomic instability/aneuploidy, proteotoxic stress, increased nutrients demand, reduced oxygen levels and evasion from destruction by the immune system.[11] The HSP90 expression levels in cancer cells is 2- to 10-fold higher than the expression in normal cells.[12] HSP90 forms a dimer at physiological temperatures and each protomer consists of three domains: N-terminal domain (NTD), that possesses an ATP binding site; Middle-domain (MD), that contains a binding site for client proteins and co-chaperones; and C-terminal domain (CTD), that is essential for HSP90 dimerization.[7] Signaling oncoproteins that are also HSP90 clients include mutant cKIT, HER2, mutant epidermal growth factor receptor (EGFR), BCR-ABL, and BRAF, that are also involved in other cancer hallmarks, including induction of angiogenesis, resistance to cell death (apoptosis evasion), and metastasis promotion.[11]

The current recommended first line therapy for patients with chronic phase (CP) CML is Imatinib Mesylate (IM), a rationally designed TK inhibitor with impressive efficacy.[4] It has been shown to induce a complete hematologic and cytogenetic response in most of CP CML patients.[6] Since the introduction of imatinib, the annual mortality in CML has decreased from 10%-20% down to 1%-2%.[2] Although it is very effective in treating CP CML patients, IM will

unlikely provide a cure to these patients for two obvious reasons.[6] One reason is resistance (especially in patients with advanced-stage disease[3]) which mechanisms include mutations in the kinase domain of BCR-ABL (especially the well-known T315I mutation)[3], amplification of the *BCR-ABL* oncogene and reduction in effective intracellular concentrations of the drug by altered drug efflux or influx.[4] Another reason for IM failure is the fact that CML leukemia stem cells (LSCs) are insensitive to IM treatment.[6] It is important to point out that insensitivity of LSCs to IM is not associated with the development of IM-resistant mutations in BCR-ABL.[6] It is widely accepted that targeting CML stem cells is essential for curing CML, because these cells survive and prevail with IM treatment and are responsible for disease relapse.[3] Like normal HSCs, LSCs can be defined as a specific cell population that can self-renew and has the ability to initiate cancer development.[3]

To reach a goal of curing CML, therapeutic strategies targeting LSCs must be developed.[6] Strategies to reach a cure will possibly require targeting other molecular features of the BCR-ABL protein.

Geldanamycin (GA) and its synthetic derivatives are HSP90 inhibitors that bind to the N-terminal ATP-binding pocket of HSP90 with an affinity much higher than either ATP or ADP therefore effectively short-circuiting the chaperone cycle.[12,13] Consequently, GA dissociates mature multi-chaperone complexes by inhibiting HSP90 ATPase activity and the released client proteins are subsequently degraded by the ubiquitin-proteasome pathway.[12] GA, however, was not clinically developed because of its poor solubility and substantial hepatotoxicity.[14] The production of two geldanamycin analogues, 17 allylamino-17 demethoxygeldanamycin (17-AAG; tanespimycin) and 17 dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; alvespimycin) improved upon the toxicity associated with GA and resulted in significantly improved chemical synthesis processes.[11] Alvespimycin is less sensitive to and less dependent on NQO1, has better pharmacokinetic properties and

fewer side effects.[11] It appears to be only minimally metabolized by CYP3A4 and therefore, intestinal CYP3A4 should not interfere with 17-DMAG's oral activity.[15] In conclusion, 17-DMAG is associated with a longer plasma half-life, greater oral bioavailability, and less extensive metabolism.[15]

This work aims to study the potential therapeutic effect of 17-DMAG in CML cell lines (sensitive and resistant to imatinib) and to explore the role of HSP family in the sensitivity to Imatinib.

In this context, we studied the effect of Alvespymicin, a HSP90 inhibitor, in three CML cell lines, one sensitive (K562) and two resistant to IM (K562-RC and K562-RD). We evaluated the effect of this 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, we assessed the protein expression levels of HSP family with western blot.

Materials and Methods

Cell Culture

In this study we used three different CML cell lines: one sensitive to IM, K562 which was purchased from American Type Culture Collection (ATCC) and two resistant to IM, K562-RC and K562-RD, which were developed in the Applied Molecular Biology and University Clinic of Hematology of the Faculty of Medicine of the University of Coimbra.

K562 cell line was established by *Lozzio and Lozzio*, from the pleural fluid of a patient with chronic myeloid leukemia in blast crisis which carried the Philadelphia chromosome (translocation 9;22).[16] The resistant line K562-RC was obtained by continuous exposure to IM and the resistant line K562-RD was obtained by discontinuous exposure to IM.[17]

Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Gibco, Invitrogen) at 37º C in a humidified atmosphere containing 5% CO_2. K562-RC and K562-RD were also cultured with 250 nM of IM.

Cell Proliferation and Viability Studies

To evaluate the effect of 17-DMAG on cell viability, all cell lines were cultured in absence and in presence of increased concentrations of HSP90 inhibitor and analyzed with the resazurin assay. Alvespimycin (17-DMAG) was purchased from Selleckchem and dissolved in Dimethyl sulfoxide (DMSO). All cell lines were incubated for 72 hours in absence and in presence of increasing concentrations of 17-DMAG, ranging from 1 nM to 1000 nM.

Rezasurin is a blue and weakly fluorescent compound, reduced by dehydrogenases enzymes in metabolically active cells in resorufin, a red and highly fluorescent compound. The

optical density was measured at 570-600 nm using a Bio-Rad microplate reader. The metabolic activity was calculated in percentage and compared to control cells.

The cells were plated at a cell density of 0.5×10^6 cells/mL and the resazurin was added to a final concentration of 10 µg/mL (Sigma-Aldrich) in 24h intervals during 72h.

Analysis of Heat Shock Protein Expression

The cell lines were cultured in absence and in presence of 10 nM and 100 nM of 17-DMAG, during 48 hours. Next, the cells were washed with PBS and resuspended in RIPA buffer (10 mM Tris base, 0.25 M sucrose and 1 mM EDTA, in the presence of protease inhibitors). The lysates were sonicated and then centrifuged at 10,000 xg, at 4 °C, for 10 min. The supernatants were analyzed for protein content using a bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Inc.). For the immunodetection of proteins, 30 µg of total cell protein was separated by electrophoresis on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Next, the membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h and incubated with specific primary antibodies overnight at 4 °C. At the end of the incubation time, cells were washed with TBS-T and incubated with peroxidase-conjugated antibodies. HSP/Chaperone antibody sample kit was purchased from Cell Signaling Technology. The HSP40, HSP60, HSP70, HSP90 (1:1000) and α-Tubulin (1:10000, Sigma) were detected using ECL and ImageQuant imaging system (GE Healthcare). The densitometric analysis was performed using ImageJ software, and the values obtained represent the ratio between the immunodetected protein and the loading control (α-Tubulin).

Assessment of Cell Death

Cell death was determined through optical microscopy, using the May-Grunwald Giemsa staining and through flow cytometry (FC), using the Annexin-V (AV) and propidium iodide (PI) double staining. For this analysis, the cells were treated with 10 nM and 100 nM of 17-DMAG and incubated during 48h. In apoptotic cells, phosphatidilserine migrates from the inner to the outer layer of the cell membrane. In the presence of calcium, AV exhibits 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 bind to the DNA. Therefore, this assay discriminates between viable cells (AV-/PI-), early apoptotic (AV+/PI-), late apoptotic/necrotic (AV+/PI+), and necrotic cells (AV-/PI+). 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 the incubation time, cells were diluted in 400 µL of binding buffer and analyzed with FC. Flow cytometry analysis was performed using a six-parameter, four-colour FACSCalibur™ flow cytometer (Becton Dickinson, USA). For each assay, 1 x 10^6 cells were used and at least 25,000 events were collected by acquisition using CellQuest software (Becton Dickinson, USA) and analyzed using Paint-a-gate software (Becton Dickinson, USA).[18] Results are expressed in % ± standard error of the mean (SEM) of at least five independent experiments.

For optical microscopy assays, cells were transferred to slides, fixed, stained and evaluated under light microscopy, using a Nikon Eclipse 80i equipped with a Nikon Digital Camera DXm 1200F for morphological analysis (amplification 500x).[18]

Activation of Caspases

The Apostat Probe is designed to identify and quantify caspase activity in cells by FC. Cells were incubated for 48h in absence and in presence of 10 nM and 100 nM of 17-DMAG. About 1×10^6 cells were resuspended in 1000 µL of PBS and incubated with 1 µg of Apostat. After a 15 min incubation period at 37°C, the cells were washed with PBS, centrifuged at 500g for 5 minutes, resuspended in 400 µL of PBS and analyzed with FC, using the equipment and the programs described in the previous section. Results are represented in % $\pm$ SEM of at least three independent experiments.

Cell Cycle Assessment

Cell cycle analysis was performed with FC, using PI/RNAse solution (Immunostep). As mentioned before, 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 G_0/G_1 phase (fewer amount of DNA), S phase (coincident with DNA replication) and G_2/M phase (double DNA of the G_0/G_1 phase). Given that apoptotic cells undergo the process of DNA fragmentation, these cells are represented as an apoptotic peak, with the fewest quantity of DNA. Cells were incubated as indicated above. At 48h, cells were washed with PBS, resuspended in 200 µL of ice ethanol, and incubated for 30 min in the dark at 4°C. Then, cells were washed with PBS, resuspended in 300 µL of PI/RNase solution and analyzed with FC, with the equipment already described. The results were analyzed using Modfit software (Becton Dickinson, USA) and expressed by % $\pm$ SEM of cells at Sub-G_1, G_0/G_1, S and G_2/M of at least five independent experiments.

Mitochondrial Membrane Potential Assessment

Mitochondrial membrane potential (Ψmit) was evaluated using 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide dye (JC-1). This lipophilic cationic probe exists in a monomeric form (M) emitting at 527 nm (green fluorescence) and is able to reversibly form aggregates (A), which emits 590 nm, greenish-orange fluorescence, as the mitochondrial membrane becomes increasingly polarized. In apoptotic cells, mitochondrial membrane potential collapses, and JC-1 cannot accumulate within the mitochondria, remaining in its monomeric form in the cytosol. These cells, exhibit a higher Monomer/Aggregate ratio of JC-1 (M/A) than viable cells.[19] Cells were incubated for 48h in absence and in presence of 10 nM and 100 nM of 17-DMAG. Briefly, the cells were resuspended in 1000 µL of PBS and were incubated with JC-1 at final concentration of 5 µg/ml for 15 min at 37 ºC, in the dark. At the end of the incubation period, the cells were washed twice in cold PBS, resuspended in a total volume of 300 µl, and analyzed with FC, with the equipment previously described. Results are expressed in mean ± SEM of Monomer/Aggregate ratio of JC-1 of at least three independent experiments and this ratio was calculated as the fraction of mean intensity fluorescence (MIF) observed for each molecule.

Statistical Analysis

Data are expressed in mean ± SEM of the number of independent experiments indicated in the figure legend. Statistical analysis was performed using the GraphPad Prism software. Kruskal-Wallis test and Ordinary one-way ANOVA were used to determine the statistical significance. Dunn's and Holm-Sidak's multiple comparison test were used to compare the different groups. A significance level of $p < 0.05$ was considered statistically significant.

Results

Effect of Alvespimycin (17-DMAG) in Cells Metabolic Activity

The effect of the 17-DMAG in the metabolic activity of the IM-sensitive line K562 and in the IM-resistant lines, K562-RC and K562-RD, is illustrated in figure 1. Our results show that all conditions decrease the metabolic activity, in a time, dose and cell type dependent manner. As represented in fig.1A, the 17-DMAG in a single dose administration induces a decrease on metabolic activity of K562 cell line, with an IC_{50} between 100 nM and 250 nM after 24h. The IC_{50} reduces, after 48h, to approximately 50 nM showing the importance of incubating time. There is a slightly reversion of effect in some concentrations between 48h and 72h. Analyzing the dose-response curve of the IM-resistant cell line K562-RC in fig.1B, there is also a decrease on metabolic activity with a single dose administration of 17-DMAG. The IC_{50} after 24h is slightly lower, with a value between 50 nM and 100nM, which shows that this cell line is more sensitive. The IC_{50} reduces to a value between 25 nM and 50nM after 48h, showing once more, the importance of incubating time. Similarly to what occurred with the K562 cell line, there is a slight reversion of effect in some concentrations between 48h and 72h. The dose-response curve of the IM-resistant cell line K562-RD represented in fig.1C, shows a decrease in the metabolic activity with a single dose administration of 17-DMAG. However, these cells require a lower dose in comparison with the two previous cell lines, with a IC_{50}, after 24h, between 50 nM and 100 nM. After 48h, the IC_{50} reduces to a value between 25 nM and 50 nM, being however slightly higher in comparison to the K562-RC. There is a regression in some concentrations between 24h and 48h and also between 48h and 72h (as observed in the two previous lines).
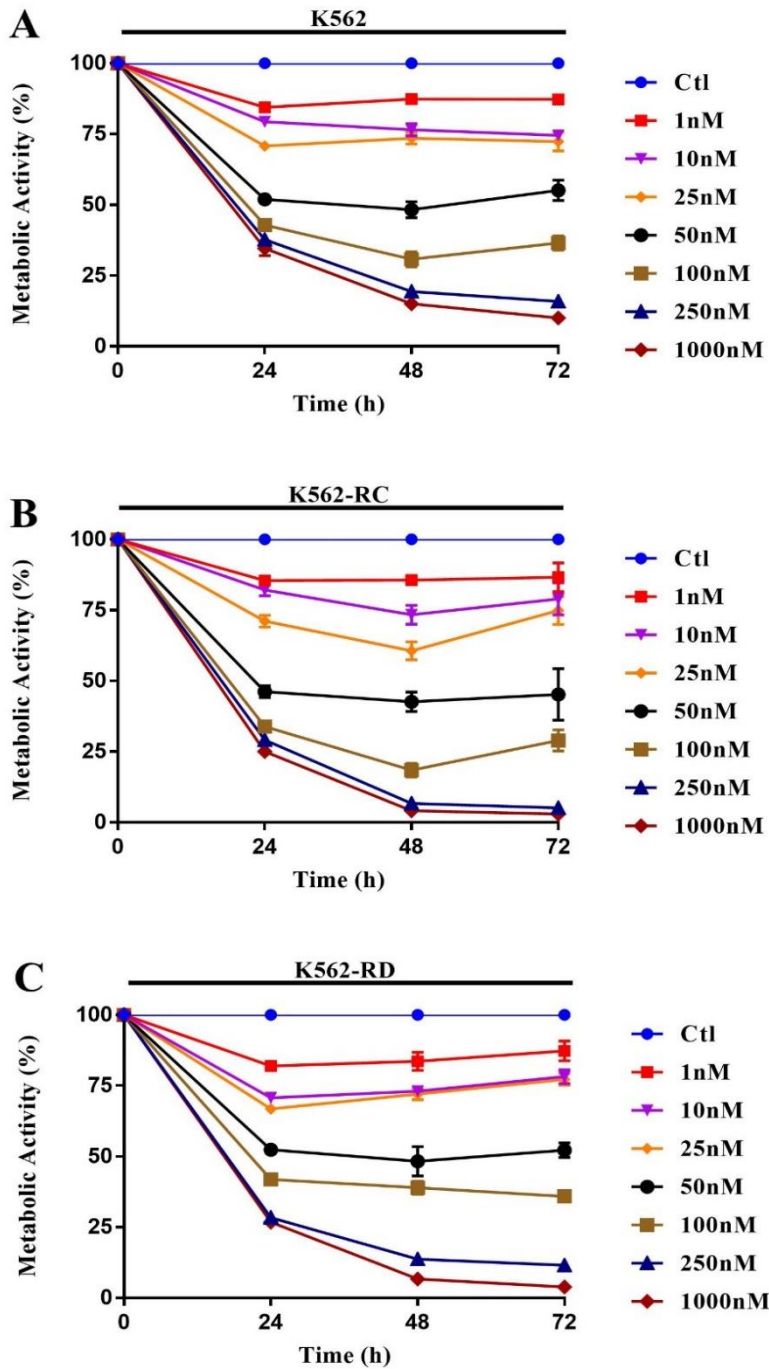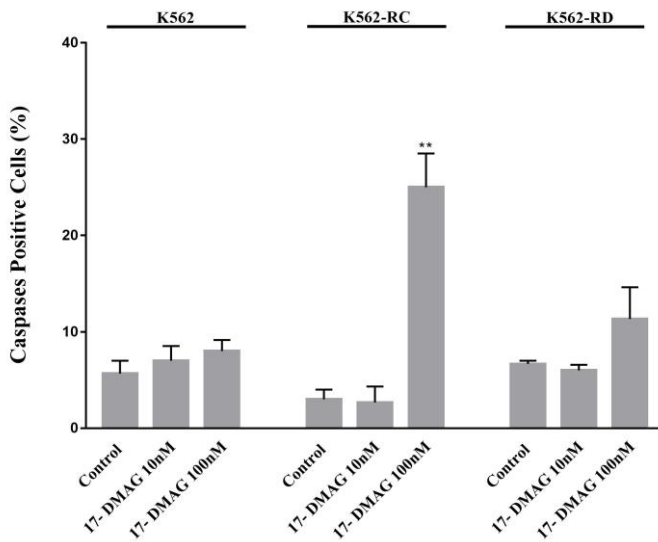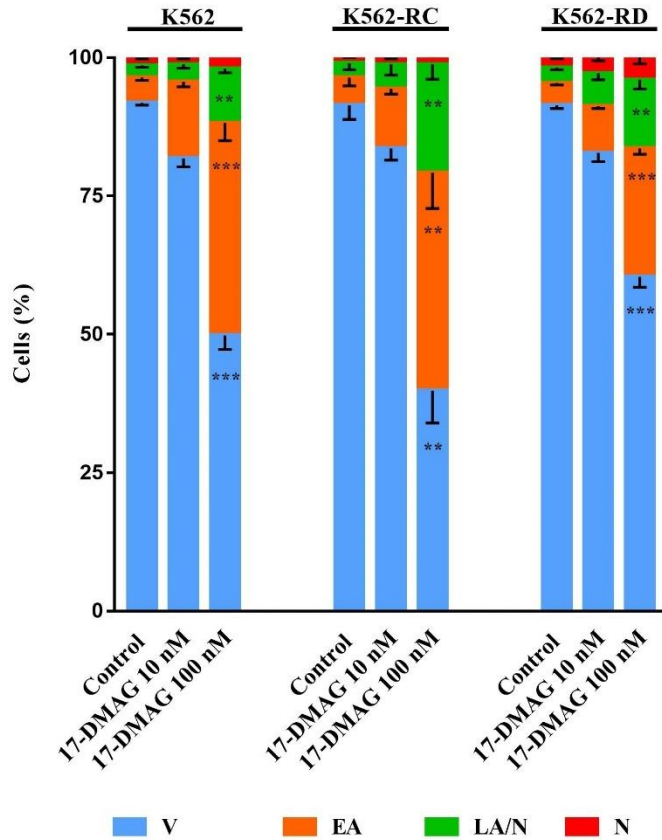
Fig. 1 Dose-response curves of Alvespimycin (17-DMAG) in single dose administration in imabitinib-sensitive and imatinib-resistant chronic myeloid leukemia cell lines. K562, K562-RC and K562-RD cells were incubated during 72h, in the absence (control; Ctl) and in the presence of increasing concentrations of 17-DMAG in single dose (A, B, C). Dose response curves were established by rezasurin assay each 24 hours, as described in Materials and Methods section. The results are expressed in percentage (%) normalized to control and represent the mean ± SEM of at least 6 independent experiments.

Alvespimycin (17-DMAG) Induces Cell Death by Caspases-Dependent Apoptosis

We analyzed the effect of 17-DMAG in cell death using Annexin V/PI double staining and Apostat probe alongside with morphological analysis. As represented in fig. 2, 17-DMAG induced a dose-dependent decrease in the percentage of viable cells (V) in all cell lines, with statistically significant differences when comparing the treatment with 100 nM of 17-DMAG (higher dose) to the control ($p<0.01$ for K562-RC and $p<0.001$ for K562 and K562-RD). Furthermore, we observed a significant increase of apoptosis, mainly in early apoptosis (EA) but also in late apoptosis/necrosis (LA/N), being the differences statistically significant when comparing the higher dose of 17-DMAG to the control (EA: $p<0.001$ for K562 and K562-RD and $p<0.01$ for K562-RC; LA/N: $p<0.01$ for all cell lines). This effect was also dose-dependent.

Additionally, to confirm the apoptosis induction, we analyzed the caspases expression level using Apostat probe. As illustrated in fig. 3, there is an increase of caspases positive cells, in all cell lines, being the effect dose-dependent. In K562 cells (fig. 3), we observed a slight increase in caspases positive cells in a dose-dependent manner, while in K562-RC and K562-RD it was only detected an increase of caspases positive cells with the higher dose of 17-DMAG (K562-RC: $p<0.01$; K562-RD: $p>0.05$), having K562-RC 2.21-fold higher caspases positive cells than K562-RD. The morphological analysis (fig. 4) confirms the previous results showing cellular contraction, nuclear fragmentation, and blebbing, all morphological aspects of apoptosis.
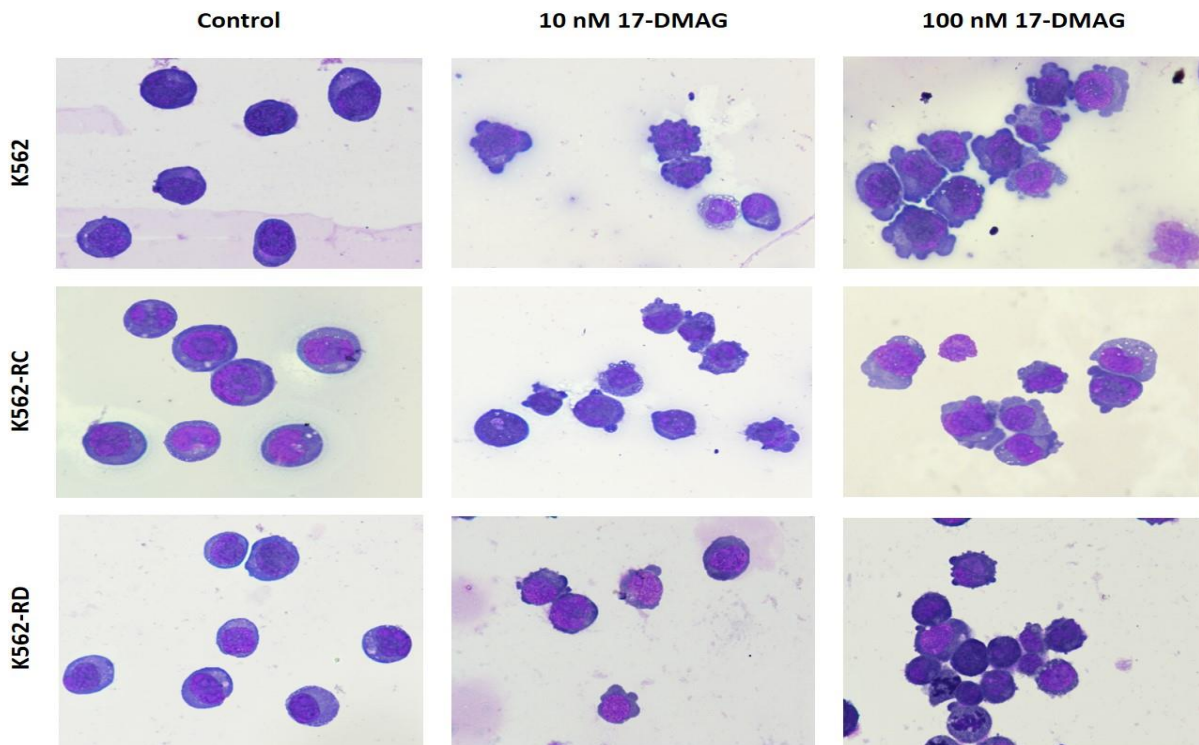
Fig. 2 Analysis of cell death induced by 17-DMAG in K562, K562-RC and K562-RD. Cell death was determined with annexin V and propidium iodide and analyzed by flow cytometry after 48h of incubation; data is expressed in percentage (%) of viable cells (V), early apoptotic cells (EA), late apoptotic/necrotic cells (LA/N) and necrosis (N) and represents the mean ± SEM of at least 5 independent experiments. Alvespimycin, 17-DMAG; *$p<0.05$;**$p<0.01$;***$p<0.001$ (comparison to control)

Fig. 3 Analysis of caspase activity induced by 17-DMAG in K562, K562-RC and K562-RD. The activation of caspases was evaluated by flow cytometry using the Apostat probe and is represented in percentage (%) of cells with activated caspases. Data is expressed in percentage (%) caspases positive cells. Results were obtained after 48h of incubation and represent the mean ± SEM of at least 3 independent experiments. Alvespimycin, 17-DMAG; *$p<0.05$;**$p<0.01$;***$p<0.001$ (comparison to control)

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Fig. 4 Morphological aspects of K562, K562-RC and K562-RD after 48h of treatment with 17-DMAG. Cell smears were stained with May-Grunwald-Giemsa (amplification: 500x)

Alvespimycin (17-DMAG) Promotes Cell Cycle Arrest

In order to study if 17-DMAG has a cytostatic effect in addition to its cytotoxic potential, we performed cell cycle analysis to study the distribution of cells through cell cycle phases in all cell lines. In K562 and K562-RC cells (fig. 5), we observed a significant cell cycle arrest in G_0/G_1 phase with the administration of the higher dose (100 nM) of 17-DMAG, being the differences statistically significant when compared to the control ($p<0.01$) in K562 cells. In contrast, in K562-RD cells (fig. 5) we detected an increase of cells in G_2/M with the higher dose of 17-DMAG.

Furthermore, 17-DMAG induced a dose-dependent sub-G_1 peak, corresponding to DNA fragmentation typical of apoptotic cells, being the differences statistically significant when comparing the higher dose (100 nM) to the control in all cell lines ($p<0.01$), confirming the

cytotoxic effect of the compound (fig. 5). K562, K562-RC and K562-RD had an increase of 8.4%, 19.4% and 2.8% of cells in sub-G_1, respectively, comparing the higher dose to the control.
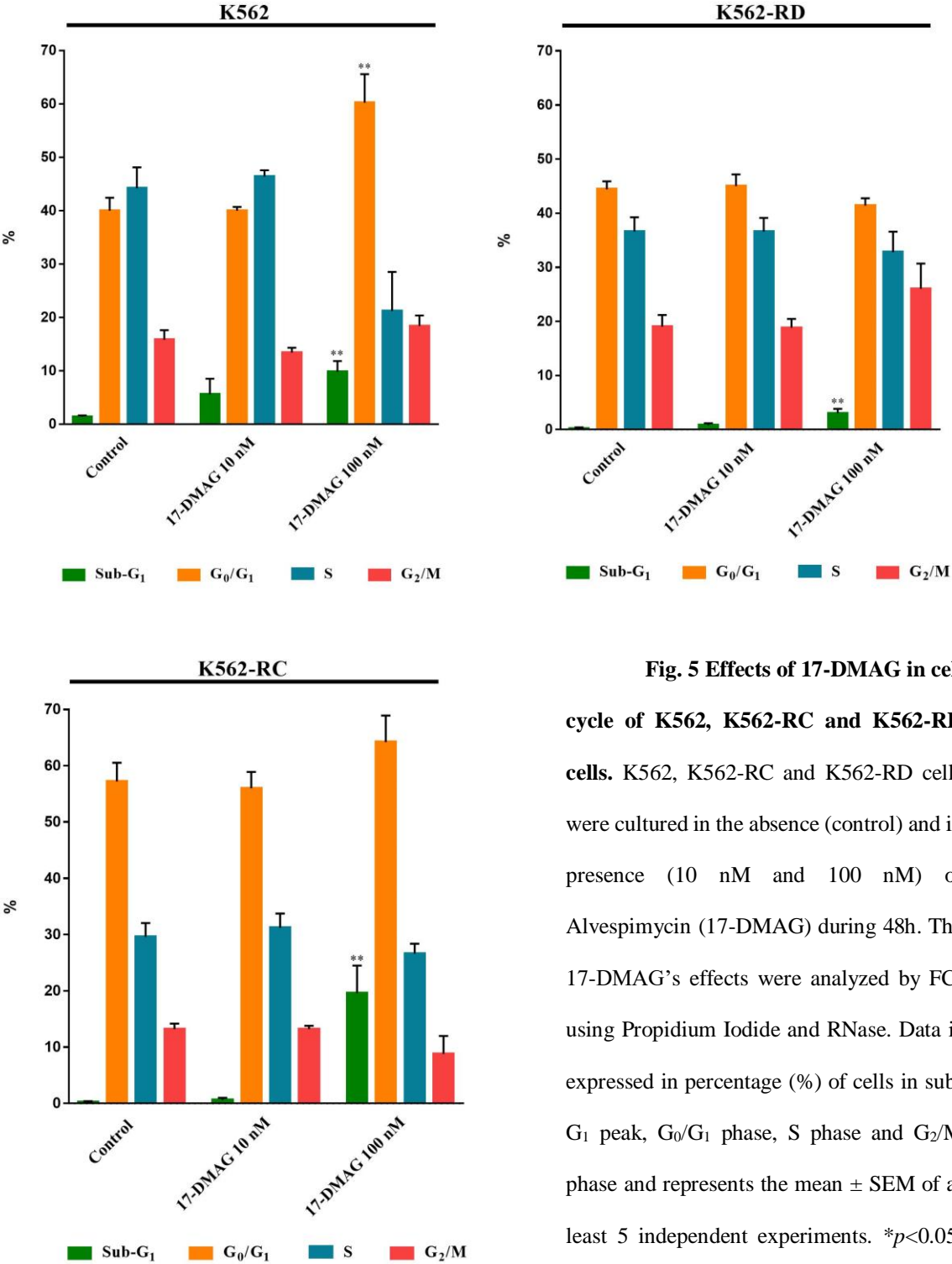

Fig. 5 Effects of 17-DMAG in cell cycle of K562, K562-RC and K562-RD cells. K562, K562-RC and K562-RD cells were cultured in the absence (control) and in presence (10 nM and 100 nM) of Alvespimycin (17-DMAG) during 48h. The 17-DMAG's effects were analyzed by FC, using Propidium Iodide and RNase. Data is expressed in percentage (%) of cells in sub-G_1 peak, G_0/G_1 phase, S phase and G_2/M phase and represents the mean ± SEM of at least 5 independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ (comparison to control)

Alvespimycin (17-DMAG) Decreases Mitochondrial Membrane Potential

Since mitochondria can be involved in apoptosis, we analyzed the mitochondrial membrane potential using JC-1 dye. Apoptotic cells exhibit a higher Monomer/Aggregate ratio of JC-1 (M/A) than viable cells. As represented in fig. 6, 17-DMAG induces a dose-dependent increase in M/A JC-1 ratio in all cell lines, showing that 17-DMAG decreases mitochondrial membrane potential. In K562 (fig. 6), we observed a more significant increase of M/A JC-1 ratio when the higher dose (100 nM) of 17-DMAG was administrated (3.43-fold higher M/A ratio when compared to the control; $p<0.05$). Likewise, the higher dose (100 nM) of 17-DMAG in K562-RC cells increased approximately 12.57-fold higher M/A ratio when compared to the control ($p<0.05$; fig. 6). In contrast, we only observed a slight increase of M/A JC-1 ratio when both doses were administered in K562-RD cells (fig. 6).
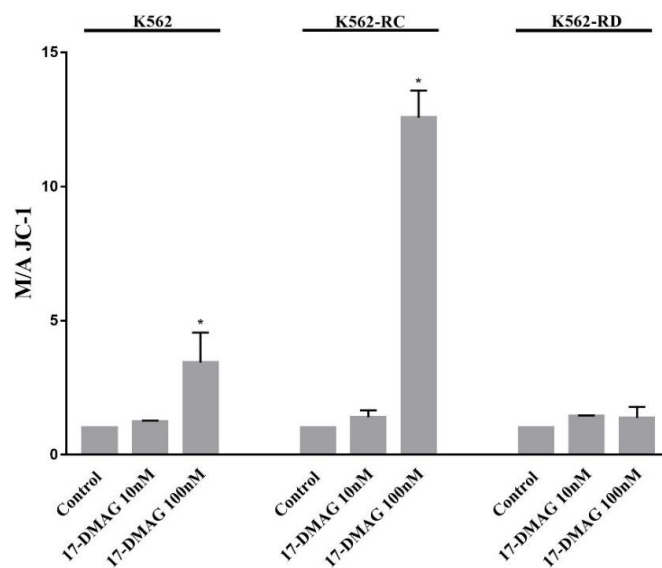

Fig. 6 Mitochondrial membrane potential (ψ_{mit}) **evaluation in K562, K562-RC and K562-RD cells.** Cells were cultured in the absence (control) and in presence of Alvespimycin (17-DMAG) during 48h. All cell lines were treated with 10 nM and 100 nM. The ψ_{mit} was analyzed by flow cytometry using JC-1 fluorescent probes, as describe in Materials and Methods section. JC-1 probe coexists in Monomeric (M) or Aggregate (A) forms depending on the mitochondrial membrane potential. An increase in the M/A ratio indicates a decrease in ψ_{mit}. Results are expressed in mean ± SEM of M/A ratio of JC-1, of at least 3 independent experiments, and this ratio was calculated as the fraction of fluorescence observed for each JC-1 form. *$p<0.05$; **$p<0.01$; ***$p<0.001$ (comparison to control)

Heat Shock Proteins Expression

Alvespimycin is a HSP90 inhibitor and the heat shock proteins expression, HSP90, HSP70, HSP60 and HSP40, was analyzed in all cell lines. As represented in fig. 7, all cell lines presented similar expression levels of HSP90, being slightly higher in K562 cells. Moreover, these cells have 2.36-fold higher HSP70 expression than K562-RC and 2.60-fold higher than K562-RD. In contrast, K562-RC and K562-RD have 1.35-fold and 1.59-fold higher HSP60 expression, respectively in comparison to K562 cells. Finally, K562-RC have 1.29-fold higher HSP40 expression than K562 and 1.85-fold higher than K562-RD ($p<0.05$ comparing to K562-RD). We also analyzed the protein expression after the treatment with 17-DMAG (fig. 8). In general, this drug induced a dose-dependent increase of HSPs expression in all cell lines. In K562 cells the higher dose induced 2.1 times more expression of HSP60 ($p<0.05$) (fig. 8B), 5.6 times more expression of HSP70 ($p<0.05$) (fig. 8C) and 2.1 times more expression of HSP90 ($p<0.05$) (fig. 8D), being the differences statistically significant when compared to the controls. In K562-RC cells, the higher dose induced 6 times more expression of HSP70 (fig. 8C), being the differences statistically significant when compared to the control ($p<0.05$). In K562-RD the higher dose induced 2.29 times more expression of HSP40 (fig. 8A) and 7.5 times more expression of HSP70 (fig. 8C), being the differences of HSP70 expression statistically significant when compared to the control ($p<0.05$).
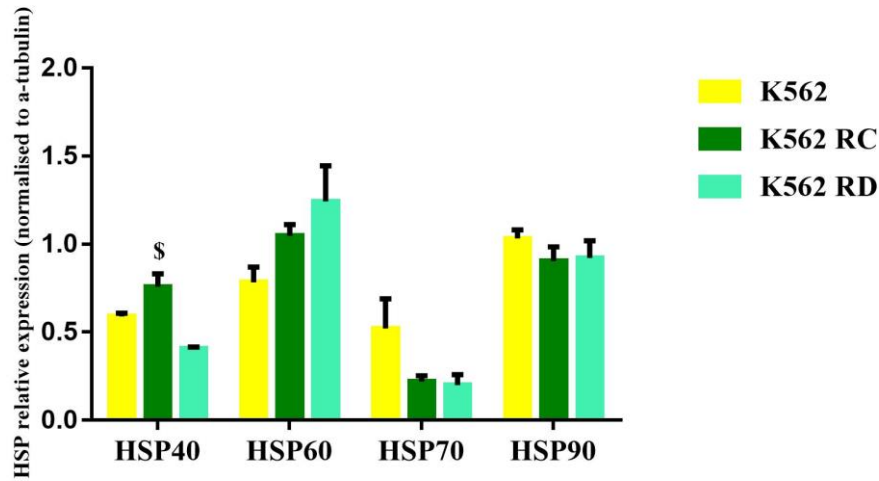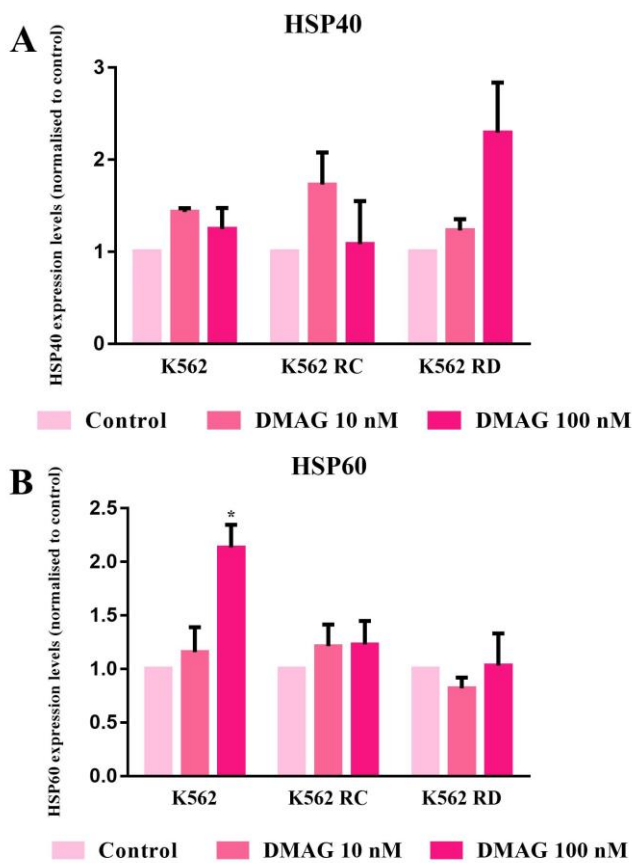
Fig. 7 HSP expression in K562, K562-RC and K562-RD cells. The densitometric analyses were performed using ImageJ software, and the values obtained represent the ratio between the immunodetected protein and the loading control (α-Tubulin) and are expressed as the mean ± SEM of at least 3 independent experiments. $p<0.05$; $$p<0.01$; $$$p<0.001$ (comparison to K562-RD)
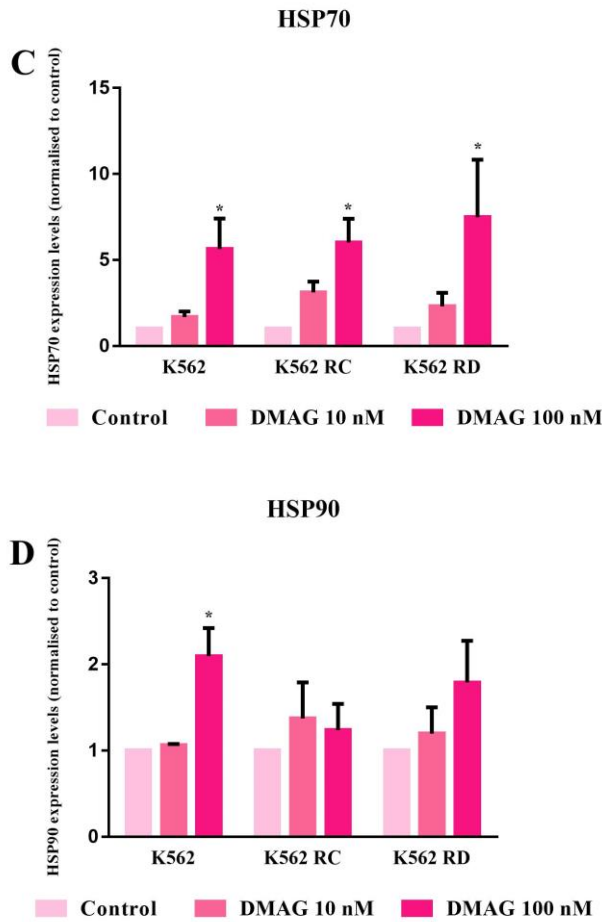
Fig. 8 HSP expression in K562, K562-RC and K562-RD cells treated with 17-DMAG (A, B, C, D). In E, representative Western blots identifying expression of HSPs in K562, K562-RC and K562-RD in the absence (control) and in presence of 17-DMAG (10 nM and 100nM). Cells were cultured in the absence (control) and in presence of Alvespimycin (17-DMAG) during 48h. All cell lines were treated with 10 nM and 100 nM. The densitometric analysis was performed using ImageJ software, and the values obtained represent the ratio between the immunodetected protein and the loading control (α-Tubulin) and are expressed as the mean ± SEM of at least 3 independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ (comparison to control)
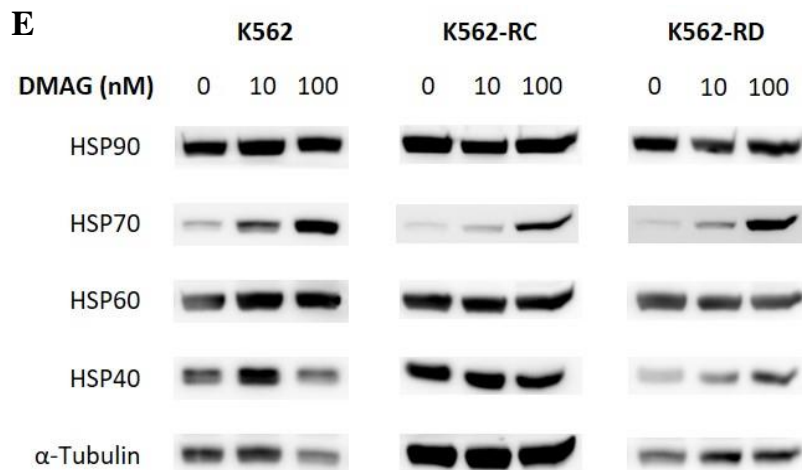
Discussion

Although the treatment with tyrosine-kinase inhibitors (TKI) has revolutionized the management, natural history and prognosis of CML, due to the resistance to TKI that has been frequently observed, especially in patients with advanced-stage disease, and to the impossibility to attain a molecular cure with the TKI, strategies targeting other molecular features of the BCR-ABL protein have been developed.[1,3,4,6,12,20]

The HSP90 inhibitors, by preventing nucleotide-dependent cycling, interfere with the chaperone activity of HSP90, resulting in targeting of client proteins to the proteasome, the cell's garbage disposal, through which they are degraded.[21] Even if the proteasome is inhibited, client proteins are not rescued from HSP90 inhibition, but instead accumulate in a misfolded, inactive form in detergent-insoluble subcellular complexes.[21] The ability of HSP90 inhibitors to affect multiple oncogenic pathways simultaneously is a unique and therapeutically attractive feature of these compounds.[22] HSP90 inhibitors are found to concentrate in tumor tissue, while being rapidly cleared from normal tissue with a half-life similar to that of the drug in plasma.[21] A study conducted by Kamal *et al* (2003) reported that HSP90 derived from tumor cells has a 100-fold higher binding affinity for 17-AAG than does HSP90 from normal cells.[23] They also reported that this therapeutic selectivity of HSP90 inhibitors is due to the presence of a high-affinity activated form of HSP90 in malignant cells that is in multi-chaperone complex with high ATPase activity, whereas the HSP90 in normal tissues is in a latent, uncomplexed form that is of low-affinity.[23]

A considerable amount of research has been carried out to understand the role of HSP90 in hematological malignancies.[3] Since HSP90 has a pivotal role in malignant transformation, it has been considered a promising target for cancer therapy and several HSP90 inhibitors are under investigation for cancer treatment.[8] In our study, we evaluated the therapeutic potential

of 17-DMAG (HSP90 inhibitor) in monotherapy. We demonstrated that 17-DMAG has a cytotoxic effect in IM-sensitive and IM-resistant cell lines in a time, dose and cell type dependent manner. Nevertheless, K562-RC seems to be more sensitive to this drug, since it requires smaller doses to achieve the IC_{50}, followed by K562-RD and K562. Gorre *et al.* (2002) reported similar results.[1] In their study, they suggested that the stability of BCR-ABL *in vitro* is more dependent on HSP90 when it carries imatinib-resistant mutations.[1] Although, K562-RC and K562-RD cells exhibit imatinib resistance through a different mechanism, i.e. overexpression of P-glycoprotein and BCRP and underexpression of OCT1 and OCTN2 instead of BCR-ABL mutations[17], these resistant cell lines were also more sensitive to the HSP90 inhibitor.

Furthermore, we showed that 17-DMAG induced cell death by apoptosis in all cell lines. This result was confirmed by cell death analysis, morphological studies, caspases studies and mitochondrial membrane potential. This result is supported by the appearance of a sub-G_1 peak in the cell cycle analysis and by the morphological aspects typical of apoptosis observed in cell smears. Moreover, 17-DMAG also increased caspases positive cells. The activation of this mechanism of death was in concordance with previous studies that have shown that benzoquinone ansamycins inhibit growth and induce apoptosis of BCR-ABL positive leukemic cell lines.[24,25] These studies demonstrate that treatment with GA, or its less toxic analogue 17-AAG, induces cytosolic accumulation of cytochrome c and both cleavage and activities of caspase-9 and caspase-3, triggering apoptosis of HL-60/BCR-ABL and K562 cells.[25] GA or 17-AAG down-regulate intracellular BCR-ABL and c-RAF protein levels, as well as reduce AKT kinase activity.[25] Another study conducted by Rao *et al.* (2010) demonstrated that 17-DMAG treatment inhibited activated TRKA and its downstream signaling through p-AKT and p-ERK1/2, resulting in apoptosis of cell lines and primary human Acute Myeloid Leukemia (AML) and CML cells.[26] A number of reports have also shown that GA and 17-AAG induced

cell cycle arrest in G_0/G_1 and G_2/M phase as well as apoptosis due to proteasomal degradation of mitogenic signaling proteins including the PI3 kinase/AKT, IKK, NFκB, RIP, and RAS/RAF/MEK/ERK MAPK pathways.[12] In the intrinsic pathway (also called "mitochondrial pathway"), apoptosis results from an intracellular cascade of events in which mitochondrial permeabilization plays a crucial role.[27] Having this in consideration we evaluated Ψmit and our results indicated a higher M/A ratio of JC-1, that confirm the contribution of intrinsic pathway in the apoptosis process. A study conducted by Li *et al.* (2017), revealed that treatment with 17-DMAG decreased mitochondrial membrane potential in SU-DHL-4 diffuse large B-cell lymphoma cells.[28] Georgakis *et al.* (2006) demonstrated that cell death in mantle cell lymphoma cell lines with treatment of 17-AAG was associated with the activation of the intrinsic/mitochondrial caspase pathway.[29]

Additionally, our study showed an antiproliferative effect of 17-DMAG sustained by the increase of cells in G_0/G_1 phase in K562 and K562-RC. We also detected an increase of cells in G_2/M phase in K562-RD. Inhibition of HSP90 most commonly leads to arrest at the G_1 to S transition.[30] In addition to influencing cyclin D levels, HSP90 also binds and stabilizes the cognate partners of cyclin D, like CDK4 and CDK6.[30] GA and 17AAG have been shown, in a panel of breast cancer cell lines, to induce G_1 arrest.[31] Additionally, depletion of the HSP90 client kinases CHK1, WEE1 and MYT1 that participate in the G_2/M DNA damage checkpoints, as well as CDC25C phosphatase, was observed in human colon cancer and glioblastoma cell lines exposed to GA.[12]

HSPs participate in tumorigenesis through specific changes in HSP expression levels, involving, in most cases, up-regulation of HSP that make cancer cells more resistant to adverse growing conditions, including anticancer therapies.[32] Having this in consideration, we evaluated the expression of HSP40, HSP60, HSP70 and HSP90 in our CML cell lines. We found that the greatest difference between sensitive and resistant cell lines was the expression

of HSP70, which was overexpressed in the IM-sensitive cell line. A study conducted by Lee *et al.* (2007) showed that the antiapoptotic proteins HSP70 and STAT5 were significantly downregulated in IM-resistant variants.[33] On the other hand, a study conducted by Pocaly *et al.* (2007) demonstrated that IM-resistant K562 had a threefold increase in HSP70 expression compared to their IM-sensitive counterparts.[34] Not all of the molecular chaperones are over-expressed during tumor development/formation, suggesting that there are specific molecular changes that lead to up- or downregulation of specific HSPs.[32] Possibly, these differences could be explained by the fact that the resistance to IM has different molecular mechanisms, such as BCR-ABL dependent mechanism (e.g. BCR-ABL mutations and BCR-ABL amplifications) and BCR-ABL independent mechanism (e.g. overexpression of efflux transporters like P-glycoprotein). In our study, 17-DMAG induced a significant increase in the expression of HSP70 in sensitive and resistant cell lines. Several studies demonstrated that HSP70 induction results from HSP90 inhibition by 17-DMAG.[35-37] According to these studies, HSP70 is considered the marker of HSP90 inhibition since HSP90 inhibitors leads, through a negative feedback loop, to activation of the heat shock transcription factor (HSF1), which causes transcriptional induction of HSP70, HSP27, and to a lesser degree, HSP90, all of which protect cancer cells from apoptosis.[11]

Preclinical and clinical studies during this past decade have uncovered the central role of HSP90 in diverse cellular processes and provided encouraging results for HSP90 inhibitors in myeloid leukemia, multiple myeloma, lymphoma and also in solid cancers.[3] A study conducted by Zhang *et al* (2012) has implied that hypoxia inducible factor 1α (HIF1α) plays a crucial role in survival and maintenance of LSCs.[38] Deletion of HIF1α impairs the propagation of CML through impaired cell cycle progression and apoptosis induction of LSCs.[38] Compared to normal HSCs, LSCs appear to be more dependent on the HIF1α pathway.[38] Interestingly, HSP90 is critical for stabilizing HIF1α.[3] Another study conducted by Mo *et al* (2012) has

demonstrated that the inhibition of HSP90 by Tanespimycin (17-AAG) impaired HIF1α stability in a von Hippel-Lindau (VHL) independent manner, and blocked cancer cell invasiveness.[39] Together, these studies demonstrated that inhibition of HSP90 can effectively inhibit the survival and proliferation of LSCs and provide a therapeutic strategy for eradicating LSCs in CML.[3]

However, resistance is often a problem correlated to cancer therapy. Preclinical evidence indicates that silencing the cochaperones HSP70, HSP27, or HSF-1 has been associated with increased sensitivity to HSP90 inhibition.[14] HSP90 inhibitors have shown either additive or synergistic activity in combination with a variety of chemotherapeutic agents, including gemcitabine, carboplatin, docetaxel and irinotecan, and a significant increase in the activity of HSP90 inhibitors was also shown when combined with radiotherapy.[14] These approaches could help to overcome the problem of resistance.

In conclusion, 17-DMAG might be a new potential therapeutic approach for CML, since this drug reduces the metabolic activity, induces cell death by apoptosis and cell cycle arrest in G_0/G_1 and in G_2/M in a dose and cell type dependent manner. Additional studies are needed to clarify some molecular mechanisms of 17-DMAG, to improve the toxicity concerns of HSP90 inhibitors, to evaluate the potential benefit of combined therapy and to explore the HSP90 structure.

Acknowledgements

References

1.   Gorre ME, Ellwood-Yen K, Chiosis G, Rosen N, Sawyers CL. BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90. *Blood.* 2002;100(8):3041-3044.

2.   Jabbour E, Kantarjian H. Chronic myeloid leukemia: 2018 update on diagnosis, therapy and monitoring. *Am J Hematol.* 2018;93(3):442-459.

3.   Ho N, Li A, Li S, Zhang H. Heat shock protein 90 and role of its chemical inhibitors in treatment of hematologic malignancies. *Pharmaceuticals (Basel).* 2012;5(8):779-801.

4.   Heaney NB, Holyoake TL. Therapeutic targets in chronic myeloid leukaemia. *Hematol Oncol.* 2007;25(2):66-75.

5.   Sattler M, Griffin JD. Molecular mechanisms of transformation by the BCR-ABL oncogene. *Semin Hematol.* 2003;40(2 Suppl 2):4-10.

6.   Chen Y, Peng C, Sullivan C, Li D, Li S. Novel therapeutic agents against cancer stem cells of chronic myeloid leukemia. *Anticancer Agents Med Chem.* 2010;10(2):111-115.

7.   Miyata Y, Nakamoto H, Neckers L. The therapeutic target Hsp90 and cancer hallmarks. *Curr Pharm Des.* 2013;19(3):347-365.

8.   Lianos GD, Alexiou GA, Mangano A, et al. The role of heat shock proteins in cancer. *Cancer Lett.* 2015;360(2):114-118.

9.   Holzbeierlein JM, Windsperger A, Vielhauer G. Hsp90: a drug target? *Curr Oncol Rep.* 2010;12(2):95-101.

10.   Sreedhar AS, Kalmar E, Csermely P, Shen YF. Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett.* 2004;562(1-3):11-15.

11.   Hong DS, Banerji U, Tavana B, George GC, Aaron J, Kurzrock R. Targeting the molecular chaperone heat shock protein 90 (HSP90): lessons learned and future directions. *Cancer Treat Rev.* 2013;39(4):375-387.

12.   Fukuyo Y, Hunt CR, Horikoshi N. Geldanamycin and its anti-cancer activities. *Cancer Lett.* 2010;290(1):24-35.

13.   Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell.* 2003;3(3):213-217.

14.   Scaltriti M, Dawood S, Cortes J. Molecular pathways: targeting hsp90--who benefits and who does not. *Clin Cancer Res.* 2012;18(17):4508-4513.

15.   Jhaveri K, Miller K, Rosen L, et al. A phase I dose-escalation trial of trastuzumab and alvespimycin hydrochloride (KOS-1022; 17 DMAG) in the treatment of advanced solid tumors. *Clin Cancer Res.* 2012;18(18):5090-5098.

16.   Koeffler HP, Golde DW. Human myeloid leukemia cell lines: a review. *Blood.* 1980;56(3):344-350.

17.   Alves R, Fonseca AR, Goncalves AC, et al. Drug transporters play a key role in the complex process of Imatinib resistance in vitro. *Leuk Res.* 2015;39(3):355-360.

18.   Goncalves AC, Barbosa-Ribeiro A, Alves V, Silva T, Sarmento-Ribeiro AB. Selenium compounds induced ROS-dependent apoptosis in myelodysplasia cells. *Biol Trace Elem Res.* 2013;154(3):440-447.

19.   Almeida S, Sarmento-Ribeiro AB, Januario C, Rego AC, Oliveira CR. Evidence of apoptosis and mitochondrial abnormalities in peripheral blood cells of Huntington's disease patients. *Biochem Biophys Res Commun.* 2008;374(4):599-603.

20.   O'Hare T, Eide CA, Deininger MW. New Bcr-Abl inhibitors in chronic myeloid leukemia: keeping resistance in check. *Expert Opin Investig Drugs.* 2008;17(6):865-878.

21.   Neckers L. Heat shock protein 90: the cancer chaperone. *J Biosci.* 2007;32(3):517-530.

22.   Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer.* 2005;5(10):761-772.

23.   Kamal A, Thao L, Sensintaffar J, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature.* 2003;425(6956):407-410.

24. Blagosklonny MV, Fojo T, Bhalla KN, et al. The Hsp90 inhibitor geldanamycin selectively sensitizes Bcr-Abl-expressing leukemia cells to cytotoxic chemotherapy. *Leukemia.* 2001;15(10):1537-1543.

25. Nimmanapalli R, O'Bryan E, Bhalla K. Geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin lowers Bcr-Abl levels and induces apoptosis and differentiation of Bcr-Abl-positive human leukemic blasts. *Cancer Res.* 2001;61(5):1799-1804.

26. Rao R, Nalluri S, Fiskus W, et al. Heat shock protein 90 inhibition depletes TrkA levels and signaling in human acute leukemia cells. *Mol Cancer Ther.* 2010;9(8):2232-2242.

27. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev.* 2007;87(1):99-163.

28. Li JJ, Zhang JJ, Wang X, Sun ZM. Effects of 17-DMAG on diffuse large B-cell lymphoma cell apoptosis. In: *Exp Ther Med.* Vol 14.2017:3727-3731.

29. Georgakis GV, Li Y, Younes A. The heat shock protein 90 inhibitor 17-AAG induces cell cycle arrest and apoptosis in mantle cell lymphoma cell lines by depleting cyclin D1, Akt, Bid and activating caspase 9. *Br J Haematol.* 2006;135(1):68-71.

30. Burrows F, Zhang H, Kamal A. Hsp90 activation and cell cycle regulation. *Cell Cycle.* 2004;3(12):1530-1536.

31.   Maloney A, Workman P. HSP90 as a new therapeutic target for cancer therapy: the story unfolds. *Expert Opin Biol Ther.* 2002;2(1):3-24.

32.   Sedlackova L, Spacek M, Holler E, Imryskova Z, Hromadnikova I. Heat-shock protein expression in leukemia. *Tumour Biol.* 2011;32(1):33-44.

33.   Lee SM, Bae JH, Kim MJ, et al. Bcr-Abl-independent imatinib-resistant K562 cells show aberrant protein acetylation and increased sensitivity to histone deacetylase inhibitors. *J Pharmacol Exp Ther.* 2007;322(3):1084-1092.

34.   Pocaly M, Lagarde V, Etienne G, et al. Overexpression of the heat-shock protein 70 is associated to imatinib resistance in chronic myeloid leukemia. *Leukemia.* 2007;21(1):93-101.

35.   Lancet JE, Gojo I, Burton M, et al. Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia. *Leukemia.* 2010;24(4):699-705.

36.   Smith V, Sausville EA, Camalier RF, Fiebig HH, Burger AM. Comparison of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) in vitro: effects on Hsp90 and client proteins in melanoma models. *Cancer Chemother Pharmacol.* 2005;56(2):126-137.

37.   Kaur G, Belotti D, Burger AM, et al. Antiangiogenic properties of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin: an orally bioavailable heat shock protein 90 modulator. *Clin Cancer Res.* 2004;10(14):4813-4821.

38.   Zhang H, Li H, Xi HS, Li S. HIF1alpha is required for survival maintenance of chronic myeloid leukemia stem cells. *Blood.* 2012;119(11):2595-2607.

39.   Mo JH, Choi IJ, Jeong WJ, Jeon EH, Ahn SH. HIF-1alpha and HSP90: target molecules selected from a tumorigenic papillary thyroid carcinoma cell line. *Cancer Sci.* 2012;103(3):464-471.