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DEQUALINIUM: A NEW APPROACH ON LEUKAEMIA TREATMENT?

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Dequalinium: A New Approach to Leukaemia Treatment?

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Abstract

Haematologic malignancies are neoplasias that arise from a disturbance in the normal hematopoietic process and are increasingly common. Current cancer therapies seek to induce cell death, but the lack of specificity for cancer cells limits its efficacy.

The primary function of mitochondria is oxidative phosphorylation, however this process induces Reactive Oxygen Species (ROS) production which has a relevant role in inducing cell proliferation and death.

Delocalized lipophilic cations (DLCs) penetrate the hydrophobic barriers of the plasma and mitochondrial membranes and accumulate in mitochondria in response to the negative transmembrane potentials. The higher plasma and/or mitochondrial membrane potentials of neoplastic vs. normal cells, in general, account for greater uptake of these compounds in neoplastic cells.

Dequalinium (DQA) is a DLC that interferes with the mitochondria and so our goal is to evaluate the therapeutic potential of DQA in several haematological malignancies, namely in B-cell Chronic Lymphocytic Leukaemia (B-CLL), Chronic Myeloid Leukemia (CML), Myelodysplastic Syndrome (MDS), Acute Promyelocytic Leukaemia (APL) and Diffuse Large B Cell Lymphoma (DLBCL).

For this purpose we treated five haematological cancer cell lines, the EHEB (B-CLL), K562 (CML), F36P (MDS), HL-60 (APL) and Farage (DLBCL) cells, with different concentrations of DQA either by single dose administration or by daily dose administration, in monotherapy and/or in association with conventional anticarcinogenic agents. Flow cytometry was used to evaluate the mitochondrial membrane potential, the levels of ROS (H_2O_2; $O_2^{\bullet -}$), the antioxidant defense, Reduced Glutathione (GSH), and the levels of caspase 3, cytochrome c, Cyclin D1 and p53.

Our results show that DQA induces a decrease in cell viability inducing cell death by late apoptosis/necrosis in a time, dose and cell type dependent manner. The most sensitive cells appear to be the K562, Farage and HL-60 cells (IC_{50} of 2,5 µM), followed by the F36P (IC_{50} of 5 µM) and the least sensitive being the EHEB cells (IC_{50} between 5 and 7,5 µM). Our results suggest that the therapeutic combination of DQA with FDN, Imatinib, Ara-C and ATRA, respectively in EHEB, K562, F36P and HL-60 cells, increases the cytotoxic effect observed for the same compounds alone. The daily dose administration seems to be the better therapeutic strategy for F36P and HL-60 cell lines, since it allows an overall lower dose usage. These effects may be mediated by oxidative stress as we have observed and increase in ROS production and a decrease in GSH levels and in the mitochondrial membrane potential. Moreover, an increase in caspase 3 and cytochrome c indicate an activation of the intrinsic apoptotic pathway, which may be modulated by p53, whose levels were significantly higher in the EHEB, the K562 and the HL-60. Cyclin D1 was also found to be elevated in the K562 cells.

In summary, our results suggest that DQA may be used as new therapeutic approach in haematological neoplasias both in monotherapy and in association with the conventional therapy.

Resumo

As neoplasias hematológicas são doenças que se originam a partir de alterações no normal processo de diferenciação hematopoiética e são cada vez mais comuns. As actuais terapias para o cancro tentam induzir morte celular mas a falta de especificidade para a célula tumoral limita a sua eficácia.

A principal função mitocondrial é a fosforilação oxidativa, no entanto, este processo induz a síntese de espécies reactivas de oxigénio (ROS) que têm um papel importante na indução de proliferação e/ou morte celulares.

Os catiões lipofílicos possuindo uma carga positiva deslocalizada (DLCs) penetram as barreiras hidrofóbicas das membranas citoplasmática e mitocondrial para se acumularem na mitocôndria em resposta ao seu potencial transmembranar negativo. O facto das células neoplásicas terem maior potencial de membrana citoplasmático e/ou mitocondrial em relação às células normais, pode permitir uma maior acumulação destes compostos nas células neoplásicas.

O Dequalinium (DQA) é um DLC que interfere com a mitocôndria e, assim, o nosso objectivo é avaliar o potencial terapêutico do DQA em neoplasias hematológicas, nomeadamente em Leucemia Linfocítica Crónica (LLC), Leucemia Mielóide Crónica (LMC), Síndrome Mielodisplásico (SMD), Leucemia Promielocítica Aguda (LPA) e Linfoma Difuso de Grandes Células B (LDGCB).

Para atingirmos este objectivo utilizámos cinco linhas celulares, incubámos as células EHEB (LLC), K562 (LMC), F36P (SMD), HL-60 (APL) e Farage (LDGCB), com diferentes concentrações de DQA quer em administração de dose única, quer por administração diária, em monoterapia e/ou em associação com a terapêutica anticancerígena convencional. Recorremos à citometria de fluxo para avaliar o potencial de membrana mitocondrial, os níveis de ROS (H_2O_2; $O_2^{\bullet -}$), da defesa antioxidante Glutationa Reduzida (GSH) e dos níveis

de caspase 3, citocromo c, ciclina D1 e p53.

Os nossos resultados mostram que o DQA induz diminuição da viabilidade celular por apoptose tardia/necrose num modo dependente de tempo, de dose e do tipo celular. As linhas celulares mais sensíveis parecem ser as K562, as Farage e as HL-60 (IC_{50} de 2,5 µM), seguidas pelas F36P (IC_{50} de 5 µM) e por fim as menos sensíveis EHEB (IC_{50} entre 5 e 7,5 µM). Os nossos resultados sugerem que a associação de DQA com FDN, Imatinib, Ara-C e ATRA, respectivamente nas EHEB, K562, F36P e HL-60, aumenta o efeito citotóxico observado para os mesmos compostos sozinhos. A adição diária parece ser a melhor estratégia terapêutica para as F36P e para as HL-60, visto que permite o uso de uma dose menor. Estes efeitos parecem ser mediados por indução de stress oxidativo, uma vez que observámos um aumento de ROS e uma diminuição dos níveis de GSH e do potencial de membrana mitocondrial. Esta perda do potencial mitocondrial e o aumento de caspase 3 e citocromo c indicam uma activação da via intrínseca da apoptose, que pode ser modulada pela proteína p53, cujos níveis estavam significativamente aumentados nas células EHEB, K562 e HL-60. Também observamos um aumento dos níveis de ciclina D1 nas células K562.

Em resumo, os nossos resultados sugerem que o DQA pode ser utilizado como nova abordagem terapêutica em neoplasias hematológicas tanto em monoterapia como em associação com a terapêutica convencional.

Highlights

- DQA induces cell death depending on time, dose and cancer haematological cell line.
- Late apoptosis/necrosis appears to be the primary mechanism of action.
- Cell death appears to be mediated by oxidative stress and ψ_{mit} depolarization.
- Caspase activation and Cytochrome c mediate apoptosis induced by DQA.
- DQA may be used as new therapeutic approach in haematological neoplasias.

Keywords

- ✓ Dequalinium
- ✓ Haematological neoplasias
- ✓ Reactive Oxygen Species
- ✓ Mitochondria
- ✓ Apoptosis

Abbreviations

A- Aggregates

ALL- Acute lymphoblastic Leukemia

AML – Acute Myeloid Leukemia

APL – Acute Promyelocytic Leukemia

ARA-C – Cytosine Arabinoside

ATRA – All-trans-retinoic acid

CLL – Chronic Lymphocytic Leukemia

CML – Chronic Myeloid Leukemia

DLBCL –Diffuse Large B Cell Lymphoma

DLC – Delocalized Lipophilic Cations

DQA – Dequalinium

Et – Ethidium

FDN - Fludarabine

GSH – Reduced Glutathione

H2DCF-DA - 2',7'-dichlorodihydrofluorescin diacetate

HE – Hydroethidine

IC_{50} – Half-maximal inhibitory concentration

IMA - Imatinib

JC-1 - 5,5',6,6'-tethrachloro-1,1'3,3'-tethraethylbenzimidazolcarbocyanine iodide

M- Monomer

MDS – Myelodysplastic Syndrome

MFI – Mean Fluorescence Intensity

MO – Mercury orange

ROS – Reactive Oxygen Species

VCR - Vincristine

Ψmit - Mitochondrial membrane potential

I. Introduction

Haematopoiesis is the physiological process in which all blood cell lines are formed from a pluripotent stem cell – the hematopoietic stem cell. Haematological malignancies are neoplasias that arise from a disturbance in this normal hematopoietic process, either in the myeloid or the lymphoid lineage, at any stage of cell differentiation. Depending on the clinical and biological characteristics of the cells involved in each specific malignancy, these diseases may be further classified as acute or chronic leukaemias, with different long-term survival rates and prognosis. These neoplasias are increasingly common and, as in most malignancies, there is still a long way to go until treatment is really effective.[1]

The therapy of human leukaemia has come a long way since 1947, when the first remarkable clinical remission was achieved. Despite most children with Acute Lymphoblastic Leukaemia (ALL) being cured, the outlook for adults with leukaemia remains less promising, as the relapse after conventional therapy is frequent. Molecular remissions have been described in Chronic Lymphocytic Leukaemia (CLL) with purine analogues and alkylating agents combination therapy, in Chronic Myeloid Leukaemia (CML) with Imatinib and in Myelodysplastic Syndromes (MDS) with azacytidin, however the impact of these remissions on long-term survival is not well established and/or the development of drug resistance remains a problem. As far as the acute leukaemias or lymphomas are concerned, namely Acute promyelocytic leukaemia (APL) and Diffuse Large B cell Lymphoma (DLBCL), several chemotherapy regimens may be used and despite disease remission may be achieved, it is usually short-longing and drug resistance and subsequent disease progression may occur. Thus, new effective targeted therapies and minimally toxic, remains a need for all these neoplasias.

Current therapies for cancer seek to induce cell death in neoplastic cells in order to limit tumour growth and spreading. These chemotherapies have only achieved limited success in cancer treatment largely due to their lack of specificity for cells of tumorigenic origin. It is

important, therefore, to investigate treatment strategies aimed at novel cellular targets that are sufficiently different between normal and cancer cells that can provide a basis for selective tumour cell killing.

Several signalling pathways modulate cell growth, spreading and death, and the deregulation of such pathways is implicated in cancer development and influences tumour cell proliferation, differentiation and apoptosis. Upon ligand binding to the cell surface receptors one or several pathways may be activated, which relates to clinical progression of the tumour.[2] The mitochondria plays an important role in some of these pathways, mainly those related to cell death induction, as apoptosis mediated by lipid damage and mitochondrial dysfunction, cytochrome c release to the cytoplasm and caspase activation.[2, 3]

The primary metabolic function of the mitochondria is oxidative phosphorylation, an energy generating process that couples substrate oxidation in mitochondria respiratory chain to the synthesis of ATP under aerobic conditions. On the other hand, as mitochondria are involved in apoptosis or programmed cell death, it may constitute an approach on cancer therapy. Considering the mitochondria as the main site for Reactive Oxygen Species (ROS) production, it has been reported that they may have a relevant role in inducing cell death.[2, 4] A selective inhibition of oxidative phosphorylation, that supplies a majority of the cellular ATP required to sustain normal cellular life and functions in neoplastic cells, may therefore provide a novel and effective strategy for the treatment of cancer.[4, 5] In turn, ROS may also be implicated in carcinogenesis, by activating several intracellular pathways [6] that leads to cell proliferation at low levels of ROS, and to initiate apoptosis at high levels.[2]

Lipophilic cations possessing a delocalized positive charge (i.e., delocalized lipophilic cations or DLCs) penetrate the hydrophobic barriers of the plasma and mitochondrial membranes and accumulate in mitochondria in response to the negative transmembrane

potentials. The higher plasma and/or mitochondrial membrane potentials of neoplastic *vs.* normal cells, in general, accounts for greater uptake of these compounds in neoplastic cells and may be a way to selectively target these cells since DLCs exhibit mitochondrial toxicity at high concentrations. Examples of DLCs are rhodamine 123 (Rh 123), thiopyrylium AA-1 and rhodacyanine MKT-077 that compromise mitochondrial respiration by inhibition of F0F1-ATPase. Moreover, DLCs as a single anti-cancer agent have shown promising results.[7, 8]

Dequalinium (DQA) is a DLC that has been shown to have a potent anticancer effect by interfering with the normal functioning of mitochondria mainly by inducing changes in mitochondrial transmembrane potential and ROS production. This compound exhibits antimicrobial and anticancer effects by selective interactions with various enzymes such as mitochondrial F1-ATPase, protein kinase C, calmodulin-dependent phosphodiesterase and calcium-activated potassium channels.[9, 10]

A number of DLCs have already displayed varying degrees of efficacy in selective carcinoma cell killing *in vitro* and/or *in vivo*. [7, 9] However the therapeutic efficacy of these DLC, mainly DQA, in haematological neoplasias is not fully studied.

As such, the aim of this study is to evaluate the therapeutic potential of DQA in several haematological neoplasias, namely in CLL, CML, MDS, APL and DLBCL, either alone, in single dose administration and in daily administration, or in combination with conventional anticarcinogenic drugs.

II. Material and methods

Cell culture

All cell lines, EHEB, K562, F36P, HL-60 and Farage cells were routinely grown in a RPMI-1640 medium (L-glutamine 2 mM, HEPES-Na 25 mM, penicillin 100U/mL and streptomycin 100 µg/mL) supplemented with 10% or 20% heat-inactivated foetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. The F36P cells were also supplemented with 10 ng/mL of recombinant interleucin-3 (rh-Il-3).

DQA was prepared by sonication and then sterilized by passing through a filter with a 0.20 µm porous membrane filter.

All cell lines were incubated in the absence and in the presence of increasing DQA concentrations, ranging from 1 µM to 20 µM, alone, and in combination with the conventional anticarcinogenic agent, namely, with 5 µM of Fludarabine for the EHEB, 10 nM of Imatinib for the K562, 50 nM of cytarabine (Ara-C) for the F36P, 25 µM of All-trans-retinoic acid (ATRA) for the HL-60, and 1 nM of vincristine for the Farage cell line.

Cell viability assays

Cell viability was assessed by the Resazurin Metabolism Assay. Resazurin was prepared as a stock solution of 100 µg/mL in phosphate buffer (PBS) and stock solution was filtered through a sterile 0.20 µm-pore filter and stored in the dark at -20°C. After each 24h of treatment, a final concentration of 10 µg/ml of resazurin was added to cells two hours prior to the measurement of absorbance at 570 nm and 600 nm in a spectrophotometer (Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments). The IC_{50} was evaluated by a dose response curve. Results are expressed in percentage, normalized to the control.

Assessment of cell death

Cell death was evaluated by optical microscopy through morphological assessment of May-Grünwald-Giemsa stained slides and by flow cytometry using the Annexin V and

Propidium Iodide double staining.

Smears were made to evaluate morphological characteristics of treatment-induced cell death by optical microscopy. Cells incubated in the absence (control) and presence of DQA during 48h were transferred to slides, fixed and stained using the May-Grünwald-Giemsa stain to further be observed by light microscopy, using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Camera DXm 1200F.

One of the earliest signs of apoptosis is the externalization of phosphatidylserine, a negative phospholipid that in apoptotic cells goes from the inner to the outer layer of plasmatic membrane. Annexin V (AV), who has a natural affinity for this phospholipid, when conjugated with a fluorochrome, can detect the redistribution of phosphatidylserine by flow cytometry. In contrast to apoptosis, in necrosis the cell membrane becomes permeable and small charged molecules that normally do not traverse the cell membrane will then enter the cell. Propidium iodide (PI) have a high affinity to DNA, and the binding results in an increase of their fluorescence, which makes them suitable for live cell imaging.[4]

Cells were stained simultaneously with Annexin V (AV), labelled with the fluorescent probe fluorescein isothyocianate (FITC) and PI. This assay discriminates among intact cells (AV^-/PI^-), early apoptotic cells (AV^+/PI^-), late apoptotic or necrotic cells (AV^+/ PI^+) and necrotic cells (AV^-/PI^+). After 48h of DQA treatment, cells were stained with AV-FITC and PI according manufacturer's recommended protocol (Immunotech Kit). Briefly, $0.5x10^6$ cells incubated in the absence (control) and presence of DQA during 48h were washed with ice-cold PBS (centrifuged at 1 000 xg for 5 min), resuspended in 100 µL of binding buffer and incubated with 5 µL of AV-FITC solution and 5 µL of PI solution for 10 min in the dark. After incubation time, cells were diluted in 400 µL of binding buffer and analysed in a FACSCalibur^{TM} flow cytometer (Becton Dickinson, San Jose, CA) and at least 10.000 events were collected by acquisition using CellQuest software (Becton Dickinson) and analysed

using Paint-a-gate software (Becton Dickinson). Results are expressed in percentage, normalized to the control.

Reactive oxygen species production

The ROS production was determined by the oxidation of 2',7'-dichlorodihydrofluorescin diacetate (H_2DCF-DA) and hydroethidine (HE). These probes measure peroxides and superoxide anion ($O_2^{\bullet -}$), respectively, and have been used to determine changes in ROS levels in cells lines. H_2DCF-DA is a stable nonfluorescent lipid permeable compound that is converted to H_2DCF by intracellular esterases and then oxidized by intracellular ROS to form the impermeable fluorescent compound DCF that emits green fluorescence, upon excitation at 488 nm, that is proportionally to intracellular ROS levels.[11] Superoxide anion can oxidize HE to ethidium (Et), which binds to DNA. An increase in the fluorescence of Et/DNA detect superoxide production and can be examined by flow cytometry using excitation at 488 and emission at 620 nm.[4]

One million of cells incubated in the absence and presence of DQA for 48h were collected and incubated with 5 µM H_2DCF-DA for 30 minutes and with 5 µM of HE during 15 min, at 37°C, in the dark. Then, cells were washed twice with PBS (centrifugation at 300 xg during 5 min), resuspended and the fluorescence was detected by flow cytometry.

GSH levels measurements

GSH intracellular levels were measured using the mercury orange (MO) dye (1-(4-Chloromercuriophenylazo)-2-naphthol) by flow cytometry. Mercury orange and GSH reaction occurred faster than with thiols protein allowing the detection of GSH using the excitation in 488 nm line of an argon laser. After a short incubation time, more than 75-80% of the GSH had reacted, while at least an 8-h period was required for the mercury orange to

react with protein SH groups. Briefly, after incubation, $1x10^6$ cells incubated in the absence and presence of DQA during 48h were washed with PBS (centrifugation at 300 xg during 5 min) and incubated for 5 min at room temperature with mercury orange at final concentration of 40 µM. Cells were then washed twice, resuspended in PBS, and analysed by flow cytometry. Results are expressed as medium fluorescence intensity (MFI) normalized to control and represents mean±SD of fluorescence intensity detected in the cells of at least 3 independent experiments.

Mitochondrial membrane potential measurement

The integrity of the inner mitochondrial membrane can be measured by observing the potential gradient across this membrane. This can be achieved by determination of mitochondrial membrane potential (ψ_{mit}) using the fluorescent dye 5,5',6,6'-tethrachloro-1,1'3,3'-tethraethylbenzimidazolcarbocyanine iodide (JC-1) (Molecular Probes). 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 are associated with a large shift in emission (590 nm, greenish-orange fluorescence) as the mitochondrial membrane becomes more polarized. The nature of this probe allows detection of changes in mitochondrial membrane potential by the determination of M/A ratio.

Briefly, after incubation, $1x10^6$ cells were washed with PBS (centrifugation at 300 xg during 5 min) and incubated with 5 µg/mL of JC-1 at final concentration, for 15 min at 37°C, in the dark. At the end of the incubation period, the cells were washed twice in PBS, resuspended in a total volume of 500 µL and analysed by flow cytometry. Results are expressed in mean±SD of monomer/aggregate ratio of JC-1 and this ratio was calculated as the fraction of MFI observed for each molecule.

Evaluation of apoptotic and cell cycle proteins expression by flow cytometry

The caspase-3, cytochrome c, p53 and cyclin D1 expression levels were analysed in cells cultured in the absence and in the presence DQA. Cells were centrifuged and incubated of monoclonal antibodies anti-caspase-3 labelled with the fluorescent probe phycoerythrin (PE) (BD Biosciences), anti-cytochrome c-FITC (Santa Cruz Antibodies, Heidelberg, Germany), anti-p53-FITC (Immunostep, Salamanca, Spain) and anti-cyclin D1-PE antibodies (Santa Cruz Antibodies), according with manufactured protocols. Briefly, $1x10^6$ cells were incubated with 100 μL of fix solution (IntraCell; Immunostep) for 15 min and washed by centrifugation at 300 xg for 5 min. Then, cells were permeabilized and incubated for 15 min with 100 μL of permeabilization solution (IntraCell; Immunostep) and 1 μg of each antibody. After a wash step, cells were analysed by flow cytometry and at least 10000 events were acquired. The levels of cellular fluorescence, proportional to the concentration of these proteins in each cell, were measured by flow cytometry and the results were plotted in Mean Fluorescence Intensity (MFI) arbitrary units. This value represents the medium fluorescence intensity detected in the cells, which is proportional to the number of molecules labelled by the antibody.

Statistical analysis

The student's t test was used to compare quantitative data. A significance level of $p < 0.05$ was considered as statistically significant and values of $p<0.01$ and $p<0.001$ were considered as very significant and highly significant, respectively.

III. Results

Cytotoxic Activity of DQA

Initially, we tested the cytotoxic effect of DQA in single dose and observed that this compound induces a decrease in cell viability in a time, dose and cell type dependent manner. As we can see in Figure 1, in all cell lines we observe a decrease in cell viability, with a half-maximal inhibitory concentration (IC_{50}) at 48 hours of exposure of approximately 2,5 µM (K562, Farage and HL-60 cells), 5 µM (F36P cells) and between 5 and 7,5 µM (EHEB cells), and so the K562, the Farage and the HL-60 cells seem to be the most sensitive to DQA.

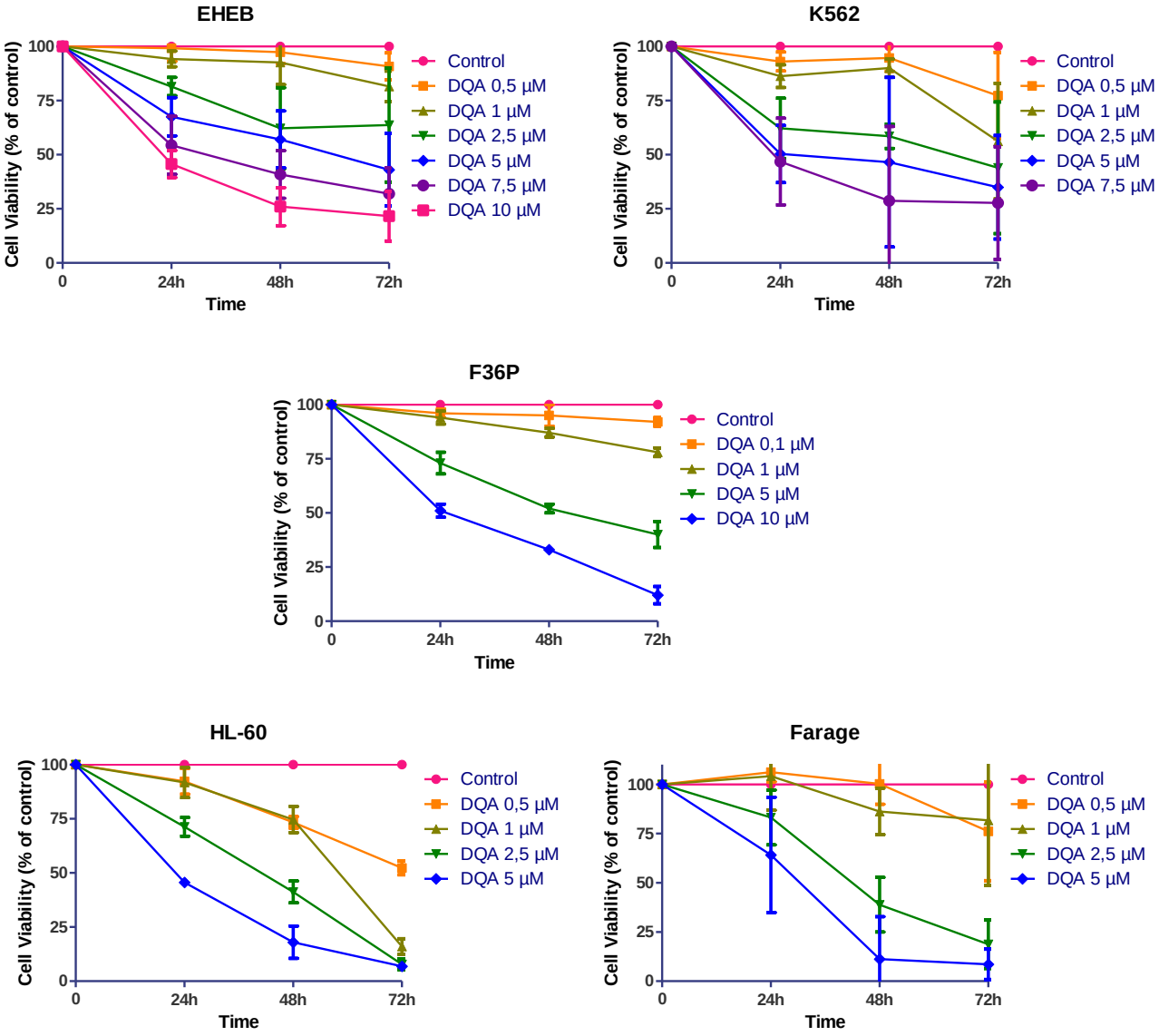

Figure 1. Dose-response curves of dequalinium in EHEB, K562, F36P, HL-60 and Farage cell lines. Cells were incubated in a density of 0.5 x 10^6 cells/ml, for 72 hours, in the absence or in the presence of increasing concentrations of dequalinium (DQA), as indicated in the figure. Viability dose response curves were established by resazurin assay each 24 hours, as described in Methods and Materials. Cell viability is expressed in cell percentage (%) normalized to control. Data are expressed as mean±SD obtained from 3 to 5 independent experiments. $p<0,05$

When we cultured the cells in the presence of DQA in a daily dose administration we achieved a significant decrease in cell viability with a lower concentration of the drug compared to the approximate dose used in single administration in the F36P and HL-60 cell lines (Figure 2). In fact, after 72h of exposure, the effect of the daily dose induced an increase of about 45% and 20% in cell death in F36P and HL-60 cells, respectively, relatively to single dose.

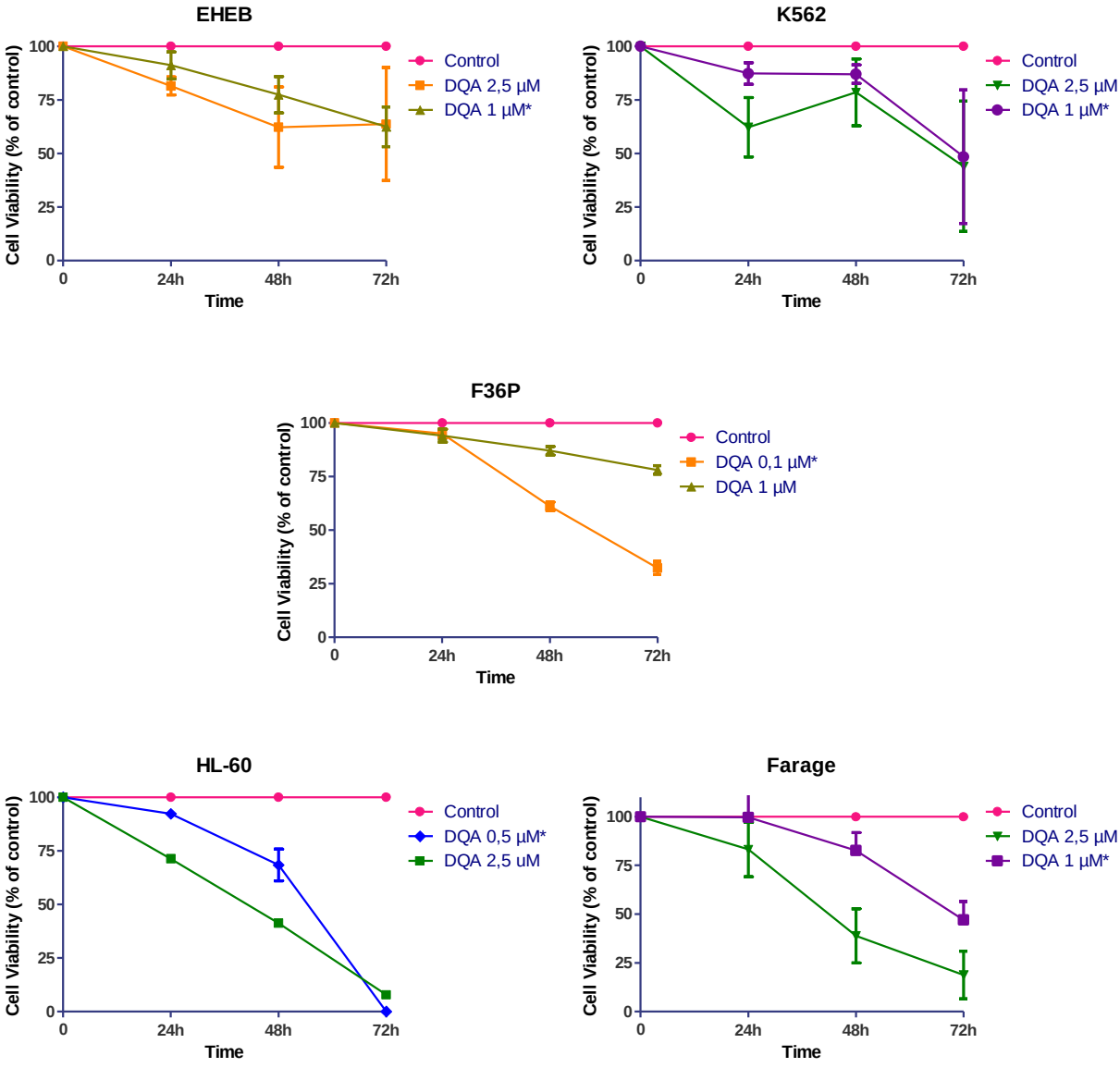

Figure 2. Cytotoxic effect of daily administration of DQA in EHEB, K562, F36P, HL-60 and Farage cell lines. Cells were incubated in a density of 0.5 x 10^6 cells/ml, for 72 hours, in the absence or in the presence of DQA in single or daily administration (*), as indicated in the figure. Viability dose response curves were established by resazurin assay each 24 hours, as described in Methods and Materials. Cell viability is expressed in cell percentage (%) normalized to control. Data are expressed as mean±SD obtained from 3 to 5 independent experiments. p<0,05

However, in the other cell lines, this effect was not observed. In EHEB and K562 cells we observed a similar effect independently of the administration scheme, and in Farage cells daily administration induce a less cytotoxic effect than single administration.

Finally, we evaluated the therapeutic potential of DQA in combination with conventional anticarcinogenic drugs, FDN, Ara-C, ATRA, VCR and IMA, respectively in CLL, MDS, APL, DLBCL and CML cells.

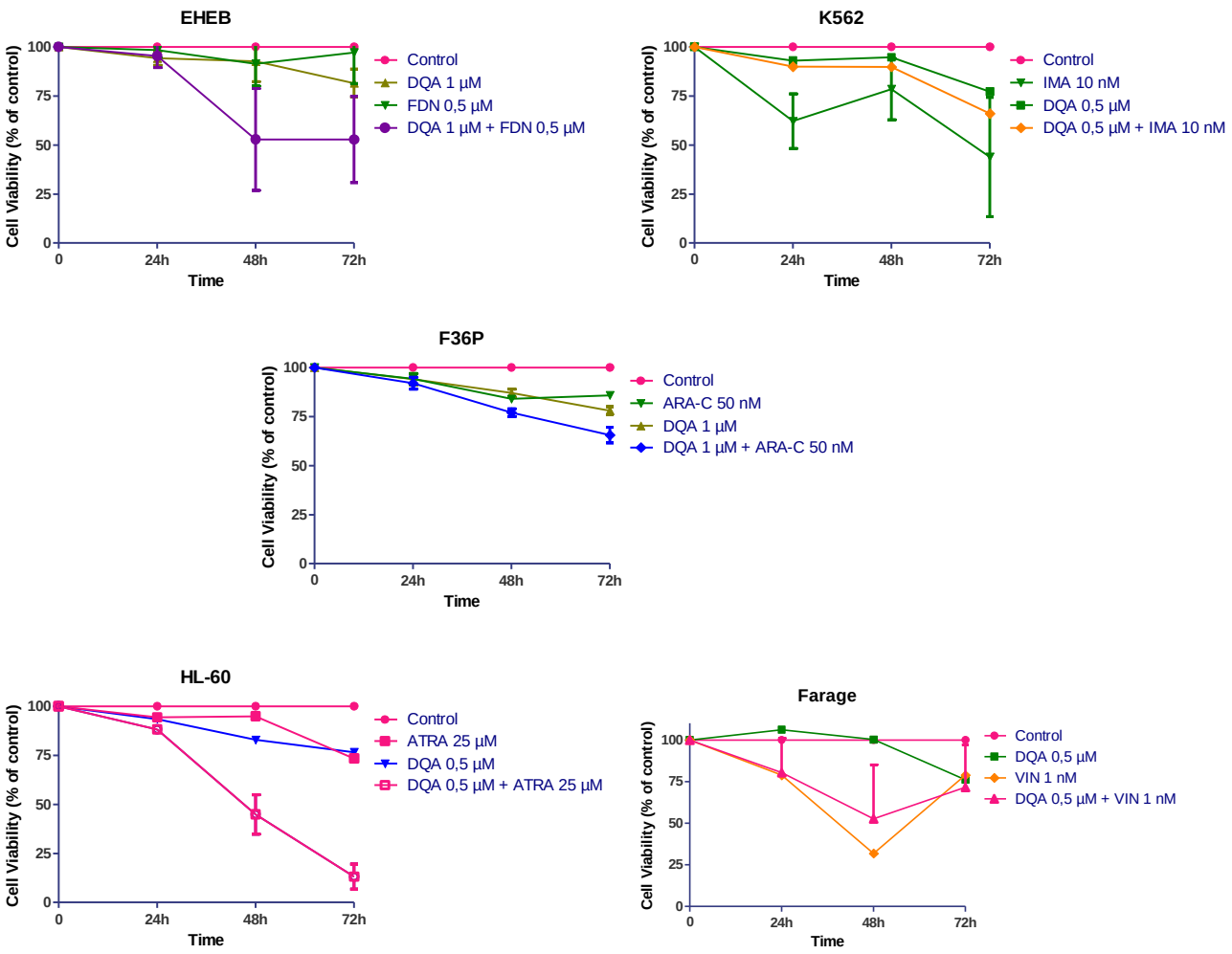

Figure 3. Therapeutic efficacy evaluation of the combination of DQA with conventional anticarcinogenic drugs in EHEB, K562, F36P, HL-60 and Farage cell lines. Cells were incubated in a density of 0.5 x 10^6 cells/ml, for 72 hours, in the absence or in the presence of DQA and/or FDN, IMA, Ara-C, ATRA and VCR, respectively, as indicated in the figure. Viability dose response curves were established by resazurin assay each 24 hours, as described in Methods and Materials. Cell viability is expressed in cell percentage (%) normalized to control. Data are expressed as mean±SD obtained from 3 to 5 independent experiments. $p<0,05$

As shown in Figure 3, we observed a potentiation synergistic effect (the same cytotoxic effect is achieved at lower concentrations than that obtained for the tested drugs in monotherapy) in all cell lines, except for the Farage cell line. In fact, this synergistic cytotoxic effect was more marked in HL-60 and EHEB cells. When HL-60 cells were culture in the presence of 25 µM of ATRA plus 0.5 µM of DQA cell death increased from 25% in monotherapy to 85% in combination (increase of approximately 60%), and in EHEB cell death increased from approximately 20% in monotherapy to 50% in the combination of 0.5 µM of FDN plus 1 µM of DQA.

Cell death, ROS and antioxidant defences analysis

Cell death was analysed by flow cytometry using annexin V/propidium iodide incorporation, as we can observe in Figure 4. As shown, DQA induced a highest cytotoxic effect in myeloid cell lines (K562: 35% ± 7%; HL-60: 38 ± 7%; F36P: 48% ± 4%) then in lymphoid cell lines (EHEB: 55% ± 2%; Farage: 55% ± 3%). Besides that, DQA induced cell death mainly by late apoptosis/necrosis in EHEB and Farage cells and by late apoptosis/necrosis and necrosis in K562, HL-60 and F36P cells.

So as to confirm the results obtained from the previous analyses, cell death was also evaluated by optical microscopy. To attain this, all cell lines were stained with May-Grünwald-Giemsa stain and observed under a microscope. As shown in Figure 5, cells cultured in the presence of DQA during 48h mainly exhibit morphological characteristics of apoptosis, such as blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation. These findings support the previous results obtained by flow cytometry indicating apoptosis as a major player in cell death induced by DQA.

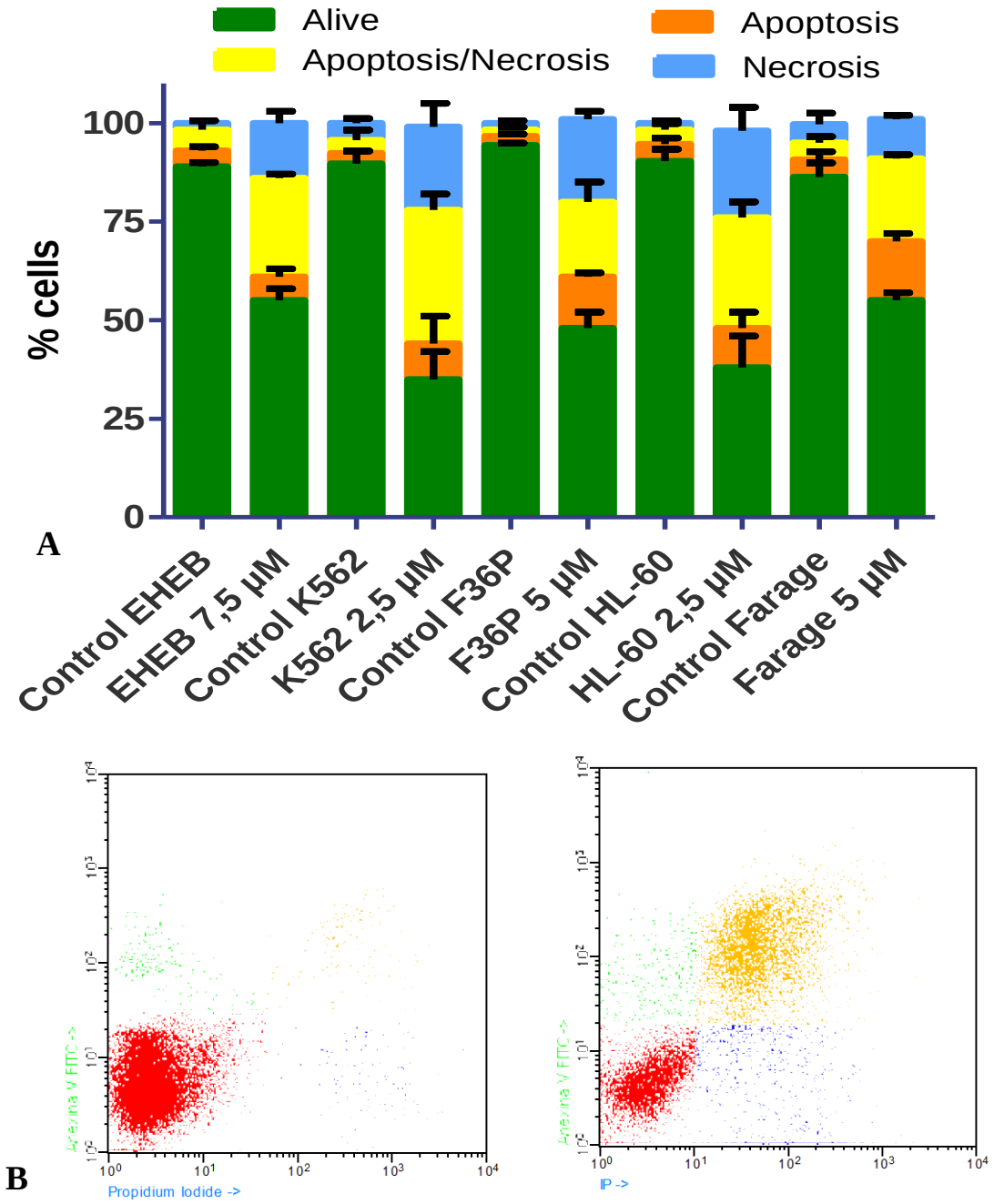

Figure 4. Cell death analysis induced by DQA in leukemic cells, by flow cytometry. A. EHEB, K562, F36P, HL-60 and Farage cells treated with DQA were incubated in a density of 0.5 x 10^6 cells/ml, during 48 hours, in the absence or in the presence of 2,5 µM (K562 and HL-60), 5 µM (F36P and Farage) and 7,5 µM (EHEB) of DQA, as indicated in the figure. Cell death was detected by annexin v and propidium iodide staining and analyzed by flow cytometry. Data are expressed as percentage (%) of viable cells (V), late apoptotic/necrotic cells (A/N) and early apoptotic cells (AE) as mean±SD obtained from at least 3 independent determinations. *** $p<0.001$; ** $p<0.01$; * $p < 0.05$. **B.** An example of the dot plots obtained from the flow cytometry assays. In this case, untreated F36P cells (left) and treated F36P cells (right). Red dots represent viable cells (AV-/PI-), blue dots represent necrotic cells (AV-/PI+), green dots represent the apoptotic cells (AV+/PI-) and yellow cells are late apoptotic/necrotic cells (AV+/PI+).

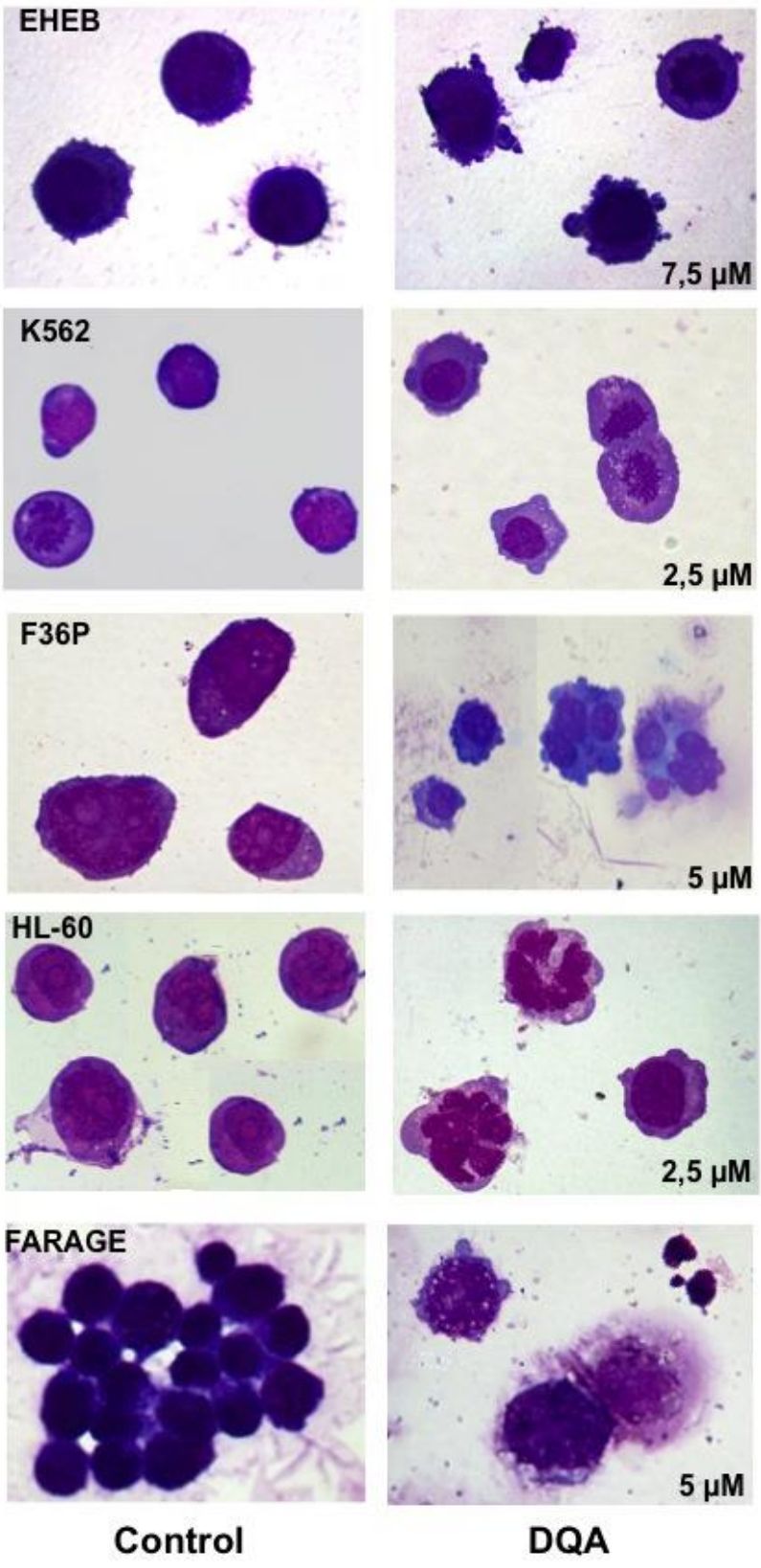

Figure 5. Cell analysis by optical microscopy, in EHEB, K562, F36P, HL-60 and Farage cells treated with DQA. The cells were incubated in a density of 0.5 x 10^6 cells/ml, for 48 hours, in the absence or in the presence of 2,5 µM (K562 and HL-60), 5 µM (F36P and Farage) and 7,5 µM (EHEB). Cells were then stained using a May-Grünwald-Giemsa stain being after transferred to slides and fixed. Finally, the smears were analysed using a light microscope.

To clarify the cytotoxic mechanisms involved in the cell death mediated by DQA, the intracellular expression of H_2O_2, $O_2^{\bullet -}$ and GSH were analysed. Our study suggests that DQA can induce cell death through oxidative stress considering the increase in the peroxide and superoxide anion levels observed in all cell lines. In fact, the increase in peroxide production range from 1,53-fold in K562 cells to 2,05-fold in Farage cell line and superoxide levels range from 1,22-fold in K562 cells to 2,78-fold in F36P cell line. This more oxidant state was accompanied by a reduction in the levels of reduced glutathione in all cell lines (figure 6). The reduction in GSH levels range from 22% in HL-60 cells to 59% in EHEB cells.

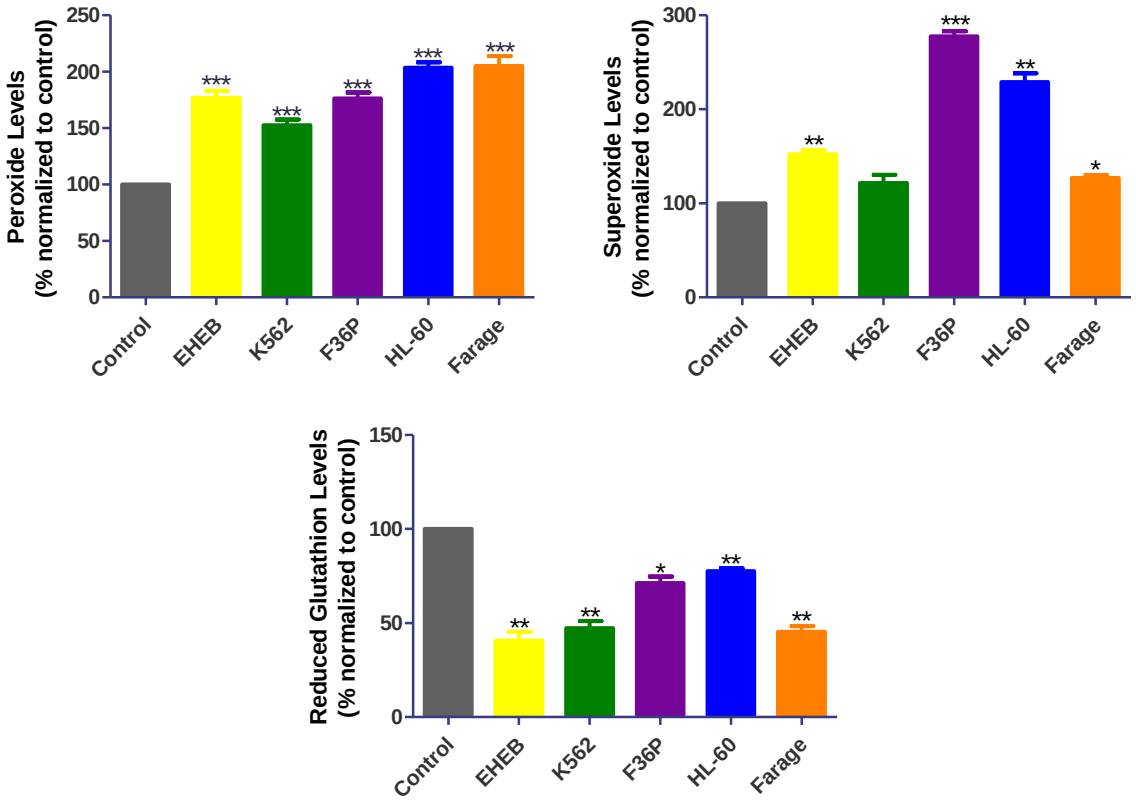

Figure 6. ROS and GSH expression levels in EHEB, K562, F36P, HL-60 and Farage cells treated with DQA, by flow cytometry. ROS levels, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$), were analyzed by flow cytometry using the fluorescent probes, $DCFH_2$-DA and dihydroethidine (DHE) respectively, and reduced glutathione (GSH) were analysed using Mercury Orange (MO). The cells were incubated in a density of 0.5 x 10^6 cells/ml, for 48 hours, in the absence or in the presence of 2,5 µM (K562 and HL-60), 5 µM (F36P and Farage) and 7,5 µM (EHEB). Results are expressed as medium fluorescence intensity (MFI) normalized to control and represents mean±SD of fluorescence intensity detected in the cells of at least 3 independent experiments. *** $p<0.001$; ** $p<0.01$; * $p < 0.05$.

Mitochondrial dysfunction

Initially, we analysed the mitochondrial membrane potential of all cell lines (Figure 7-A) and observed a different basal mitochondrial membrane potential between cell lines (EHEB: 1,16 ± 0,06; Farage: 1,33 ± 0,06; F36P: 1,41 ± 0,05; K562: 1,65 ± 0,10; HL-60: 2,24 ± 0,20) that could be related to DQA sensibility. In fact, HL-60 cells show the highest mitochondrial membrane potential and EHEB cells the lowest (Figure 7-A).

Then, we investigated the mitochondrial involvement in the cell death induced by DQA through the analysis of mitochondrial membrane potential of treated and untreated cells (control), by flow cytometry using the JC1 dye. In apoptotic cells, mitochondrial membrane potential collapses, and JC-1 can't accumulate within the mitochondria, remaining in the monomeric form in cytosol. These cells, exhibit a higher monomer/aggregate ratio of JC1 (M/A) than viable cells.

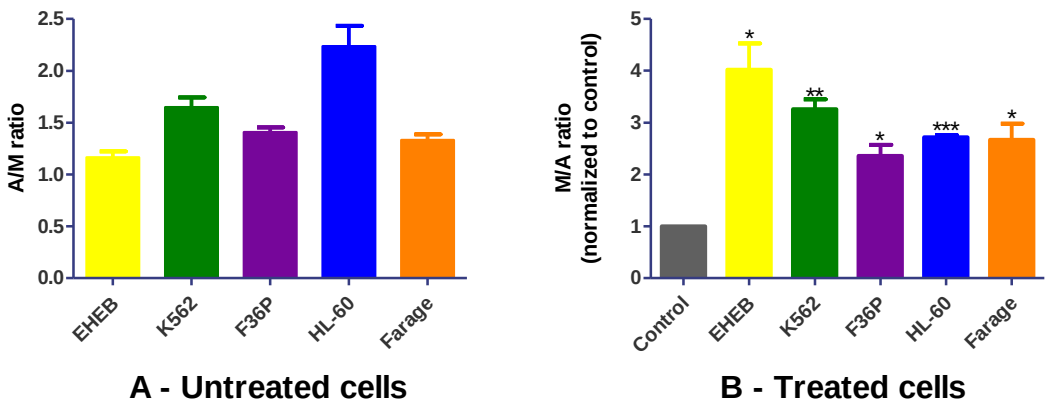

Figure 7. Analysis of mitochondrial membrane potential, by flow cytometry, in EHEB, K562, F36P, HL-60 and Farage cells treated with DQA. The mitochondrial membrane potential was analysed by flow cytometry using the JC1 fluorescent probe, as describe in material and methods. **A. Untreated cells** Basal mitochondrial potential of untreated cells. **B. Treated Cells** The cells were incubated in a density of 0.5 x 10^6 cells/ml, for 48 hours, in the absence (control) or in the presence of 2,5 µM (K562 and HL-60), 5 µM (F36P and Farage) and 7,5 µM (EHEB). JC-1 probe coexist in monomeric (M) or aggregate (A) forms depending on the mitochondrial membrane potential. An increase in the monomer/aggregate ratio (M/A ratio) indicates a decrease in the mitochondrial membrane potential. Results are expressed in mean±SD of monomer/aggregate ratio of JC-1 and this ratio was calculated as the fraction of MFI observed for each molecule. *** $p<0.001$; ** $p<0.01$; * $p < 0.05$.

In agreement with the previous results, we found that DQA induces a decrease in the mitochondrial membrane potential in all cell lines, which is correlated with apoptosis induction. In EHEB and K562 cells, we observed an approximately 4 and 3,2-fold decrease in mitochondrial membrane potential, respectively, and about 2,5 times in F36P, HL-60 and Farage cells.

Evaluation of apoptotic and cell cycle proteins

In order to further investigate the involvement of mitochondria in apoptotic cell death induced by DQA in haematological malignant cells we evaluated the expression levels of the apoptotic proteins, caspase 3 and cytochrome c.

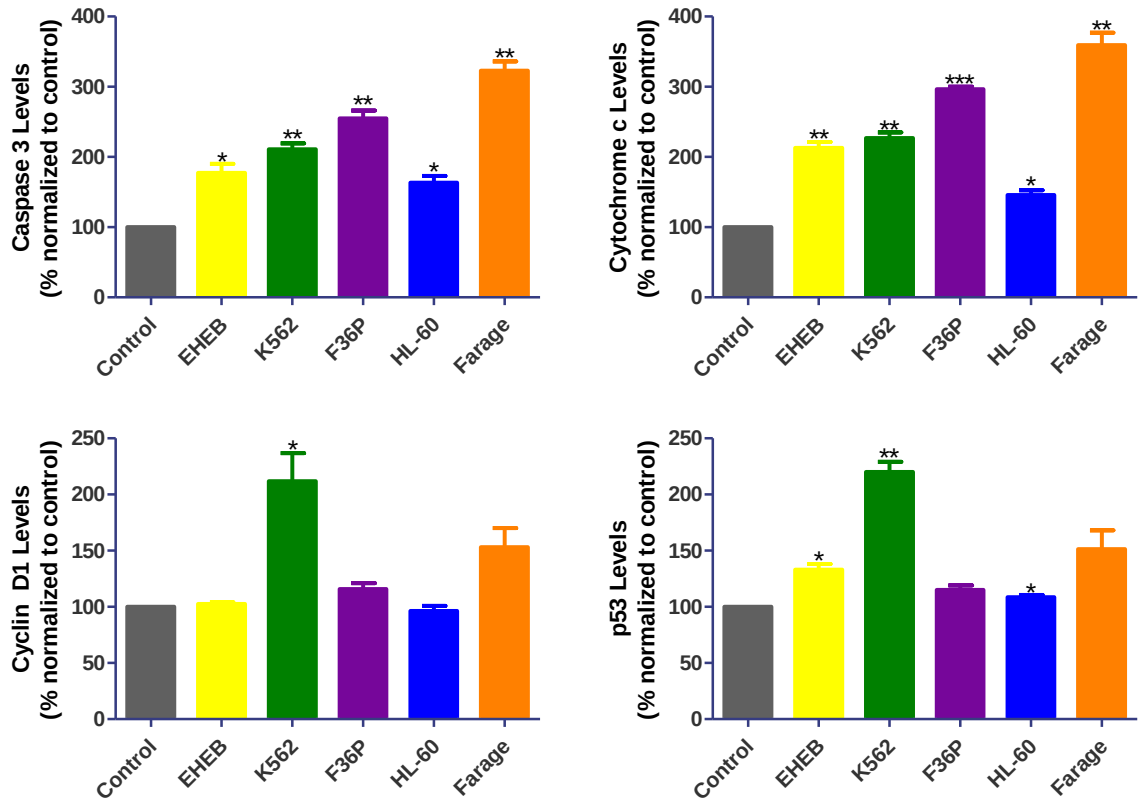

Figure 8. Analysis of apoptotic and cell cycle proteins by flow cytometry, in EHEB, K562, F36P, HL-60 and Farage cells treated with DQA. The expression of Caspase 3, Cytochrome C, Cyclin D1 and p53 were analyzed by flow cytometry, as described in material and methods. The cells were incubated in a density of 0.5 x 10^6 cells/ml, for 48 hours, in the absence or in the presence of 2,5 µM (K562 and HL-60), 5 µM (F36P and Farage) and 7,5 µM (EHEB). Results are expressed as medium fluorescence intensity (MFI) normalized to control and represents mean±SD of fluorescence intensity detected in the cells of at least 3 independent experiments. *** $p<0.001$; ** $p<0.01$; * $p < 0.05$.

As we can see in figure 8, there is an increase of caspase 3 and cytochrome c in all cell lines. The increase in caspase 3 expression level range from 1,6 times in HL-60 cells to 3,2 in Farage cells and cytochrome c expression levels range from 1,5 times in HL-60 to 3,6 times in Farage cells.

As far as the cell cycle proteins are concerned, we analysed the role of cyclin D1 and p53 in cells treated with DQA and observed a significantly increase in Cyclin D1 expression level in K562 cells (2,1-fold) and an increased p53 for the EHEB (1,3-fold), Farage cells (1,5-fold) and K562 cells (2,2-fold).

IV. Discussion

In this study, we evaluate the therapeutic efficacy of Dequalinium (DQA), a lipophilic cation (DLC) that crosses the cell membrane and accumulates in the mitochondria, in leukemic cell lines. Considering that, in general, the neoplastic cells have a higher mitochondrial transmembrane potential than normal cells (which gives them a greater capacity for accumulation and retention of lipophilic cations), DQA may be a new selective therapeutic strategy for leukemic cells.[7] Its anti-cancer effect has been described in cells of various neoplasms.[9, 10, 12] However, most studies with DQA have been focused in epithelial tumour cells. [8, 9, 12]

Our results show that DQA induces a cytotoxic effect in haematological neoplastic cell lines in a time, dose and cell type dependent manner. In fact, we found that it was more effective, requiring a lower dose, in the K562 and HL-60 cells, the CML and APL cell lines model, respectively, which may be related to the higher proliferative potential and so the higher mitochondrial membrane potential found in these cell lines (K562: 1,65 ± 0,06; HL-60: 2,24 ± 0,20) compared to the other studied cells lines (EHEB: 1,16±0,06; Farage: 1,33±0,06; F36P: 1,41±0,05). Despite, in general, CML cells not being considered highly proliferative, this cell line was established from a patient with CML in blast crisis, which correlates to a more aggressive phenotype, with higher proliferation and a clinical behaviour similar to that of an acute leukaemia, which can explain these results. Galeano *et al.* (2005), like in our study, have also found an IC_{50} of 2,5 µM for K562 cells and found an IC_{50} of 2 µM for the NB4 cell line (an APL cell line), which is in accordance to our findings for the HL-60 cells (IC_{50} of 2,5 µM). [12]

The therapeutic use of these compounds in combination with anti-cancer conventional agents was also evaluated. Our results suggest that the therapeutic combination of DQA with FDN, Imatinib, Ara-C and ATRA, respectively in EHEB, K562, F36P and HL-60 cells, increases the cytotoxic effect observed for the same compounds alone. Since the

pharmacological combination is based on reducing the concentration of used compounds, those combinations may minimize the toxicity of new and/or conventional anticancer drugs and improve the quality of life of patients with these hematologic malignancies. On the other hand, the potentiation effect was not observed in Farage cell lines incubated with the combination of DQA plus VCR. However, we didn´t study other anticarcinogenic agents used in the standard treatment of this kind of lymphoma.

In this work we analysed different drugs administration schemes and observed that daily dose administration seems to be the better therapeutic strategy for F36P and HL-60 cell lines, since it allows a lower dose usage, hence a reduction in the potential side effects of these anti-carcinogenic drugs. It is interesting to note that these cells have higher proliferative and mitochondrial potential, and are the ones in which this effect can be seen.

We also show that the cytotoxicity induced by DQA may be mediated by oxidative stress, as we have observed an increase in ROS production in all cell lines (increased peroxide and/or superoxide levels) production and a decrease in antioxidant defences, namely reduced glutathione. These mechanisms may be implicated in cell death induction. These results are in agreement with previously reported results, in other leukemic cells by Galeano, *et al*. (2005) and Sancho, *et al*. (2007).[10, 12]

When we investigated if these changes in the redox balance were related to changes in the mitochondrial membrane potential, we realized there was an increase in M/A ratio, which translates to a disruption of the mitochondrial membrane potential. This proves that DQA induces changes in the mitochondrial function and also corroborates the cytotoxicity induced by this compound , considering that an early key-feature of cell death (apoptosis and necrosis) is the dissipation of the mitochondrial transmembrane potential. As expected, the cells with the greater basal mitochondrial potential (and consequently a higher proliferation rate) required a lower dose of DQA to induce the same cytotoxic effect. Previous studies have

shown that DQA exhibits different levels of cytotoxicity depending on the cell line. The mechanisms underlying the cytotoxic effects of DQA as well as the particular cellular response seems to be associated with changes in mitochondrial transmembrane potential of the tumour cells.[12]

When we evaluated caspase 3 and cytochrome c expression, we found that there is an increase in the levels of these molecules suggesting that the DQA-induced apoptosis is a process mediated by mitochondrial activation, which is supported by the increased levels of cytochrome c in the cytosol and the loss of mitochondrial potential. Besides that, we also evaluated the expression of cell cycle proteins Cyclin D1 and p53. Cyclin D1 is a cell-cycle regulator protein, involved in the transition from G1 to S phase, during mitosis, whose overexpression has been associated with important cell cycle changes and G1 phase acceleration, which in turn can lead to abnormal cell proliferation. On the other hand, it is well known that p53 inhibits cell growth and induces DNA repair and/or apoptosis. In this study, DQA leads to an increase in p53 protein that is responsible for transient G1 arrest and induction of apoptosis in K562, EHEB and HL-60 cells and an increase in cyclin D1 in the K562 cells. Since p53 is one of the negative regulators of cyclin D1, these findings suggest that cyclin D1 may be responsible for the highest increase in p53 expression level in K562 or that p53-mediated apoptosis that occurs in these cells is dependent on cyclin D1 levels. Moreover, and considering that p53 is a mediator of apoptosis and that cyclin D1 can induce apoptosis in some cell types, a mechanism whereby cyclin D1 influences p53 levels is likely to occur in K562 cells. [14-16] In fact, Kranenburg *et al*, 1996, found that cyclin D1 is capable of inducing neuronal and non-neuronal cell death and that moderate overexpression results in growth stimulation, whereas high overexpression results in apoptotic cell death. They also found that p53 is not required for cyclin D1-induced apoptosis.[15] Clearly, further studies are needed to fully understand the activity of cyclin D1.

Pajuelo *et al* (2011) studied the effect of DQA in mononuclear cells isolated from human CLL and found that DQA selectively targets the neoplastic cells of patients with CLL without effect in non-neoplastic cells obtained from healthy donors.[17] Besides that, in our lab we already tested the same concentration of DQA use on these cells lines in mononuclear cells isolated from healthy donors and observed a low or null cytotoxic effect (data not shown). This is yet another argument in favour of DQA as a new therapeutic approach in haematological neoplasias.

V. Conclusion

In summary, we found that DQA induces cell death in neoplastic haematological cell lines depending on time, dose and cell line. DQA-induced apoptosis is mediated by changes in the redox balance towards a state of oxidative stress, since we observed an increase in ROS production and a decrease in GSH. Moreover, the loss of mitochondrial transmembrane potential and the increase in caspase 3 and cytochrome c indicate an activation of the intrinsic apoptotic pathway modulated by p53.

In conclusion, DQA may be used as a new therapeutic approach in haematological neoplasias in monotherapy and/or in association with conventional chemotherapy. However, further studies are required to better assess dosage, administration scheme and tumoral selectivity.

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VII. Attachments

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