Original Research

Raf/MEK/ERK signaling inhibition enhances the ability of dequalinium to induce apoptosis in the human leukemic cell line K562

Pilar Sancho[1], Eva Galeano[1,2], María Cristina Estañ[1,3], Irene Gañán-Gómez[1], María del Carmen Boyano-Adánez[1] and Ana Isabel García-Pérez[1]

[1]Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Campus Universitario, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid; [2]Centro de Investigación Médica Aplicada, Universidad de Navarra, Avda. Pío XII 55, 31008 Pamplona; [3]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain
Corresponding author: Dr Pilar Sancho. Email: pilar.sancho@uah.es

Abstract

Delocalized lipophilic cations, such as dequalinium (DQA), selectively accumulate in mitochondria and display anticancer activity in cells from different malignancies. Previous studies in K562 human leukemic cells indicate that DQA causes cell damage as a consequence of an early disturbance in the mitochondrial function, inducing oxidative stress. These cells turned out to be resistant to apoptosis and died by necrosis when treated with high DQA concentrations (20 μmol/L) for long time periods (48 h). Resistance of K562 cells to DQA-induced apoptosis could be eliminated by inhibition of the kinase activity of the Bcr-Abl protein with imatinib. In this paper, we have studied the effect of DQA on the Raf/MEK/ERK1/2 and PI3K/Akt signal transduction pathways in K562 cells. Our data suggest a DQA downregulatory activity on both ERK1/2 and PI3K protein kinase activity supporting an interaction between both proteins. Moreover, inhibition of ERK1/2 with U0126 enhanced the ability of DQA to potentiate imatinib-induced apoptosis, suggesting a role of the Raf/MEK/ERK pathway and the Bcr-Abl tyrosine kinase in the K562 cell survival. This study contributes to a better understanding of the action mechanism of DQA on K562 cells and encourages the study of DQA in combination with other agents for improving the efficacy of targeted therapies and overcoming resistance to chemotherapeutic agents.

Keywords: Apoptosis, cell signaling, dequalinium, ERK1/2, leukemia, imatinib

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Introduction

Chronic myelogenous leukemia (CML) is a type of worldwide extended hematopoietic malignancy that expresses the oncoprotein Bcr/Abl which exhibits constitutive tyrosine kinase activity. This kinase activates a number of downstream signaling cascades, including the mitogen-activated protein kinases (MAPKs) that regulate cell proliferation, differentiation and death/survival processes.[1–3] As a consequence, these cells are resistant to apoptosis induced by different drugs.[2–4] Despite the clinical effectiveness of some chemical agents against CML, such as the Bcr-Abl tyrosine kinase inhibitors imatinib, nilotinib and dasatinib, the development of new chemotherapeutic approaches such as the combination of these drugs with other antileukemic agents is needed to overcome resistance in some patients.[5–9]

New drug development in cancer therapy focuses on the discovery of effective pharmacological agents that can modulate diverse signaling pathways which control normal cell proliferation and death, inducing malignant cells to undergo apoptosis.[10,11] Inhibition or modulation of such pathways is an important approach to the treatment of cancer, e.g. leukemia, and of several other diseases. In general, it is established that the Raf/MEK/ERK1/2 and PI3K/Akt signaling pathways regulate cell proliferation and survival, protecting cells from apoptosis. Both pathways are currently being considered as targets for therapeutic purposes.[4,12,13]

Strategies to improve therapeutic selectivity of new drugs largely rely on our understanding of the biological difference between tumoral and normal cells, and on the availability of therapeutic agents to target critical biological events for cancer but not for normal cells. A higher negative mitochondrial transmembrane potential has been found in tumor cells as compared with normal cells.[14,15] As a

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consequence, accumulation and retention of some positively charged agents, such as delocalized lipophilic cations (DLCs), are increased in tumor cells, providing an attractive basis for selective tumor cell eradication.[16] Among the wide variety of DLCs, dequalinium (DQA) has been reported to display a potent anticancer activity in cells from different malignancies.[17–20] Our previous studies have shown that DQA behaves as a potential antileukemic agent in NB4 and K562 human leukemic cell lines, inducing apoptosis and/or necrosis by interfering with mitochondrial function and affecting the redox balance.[21–23] However, the specific mechanism of DQA cytotoxicity is not well known.

In this work, we have investigated the effects of DQA on the Raf/MEK/ERK and PI3K/Akt signal transduction pathways involved in cell proliferation and survival in the human leukemic cell line K562. For this purpose, phosphorylation of ERK1/2 and Akt, in the absence or presence of their specific inhibitors U0126 and LY294002, respectively, was detected and related to cell death by apoptosis or necrosis. DQA co-treatment with imatinib was also studied. Our data show a downregulatory role of DQA in the Raf/MEK/ERK and PI3K/Akt pathways, as well as an increased ability of DQA to potentiate imatinib-induced apoptosis in the presence of the ERK1/2 inhibitor U0126.

Materials and methods

Cell cultures

The K562 leukemic cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI 1640 medium enriched with L-glutamine (Gibco – Life Technologies, Paisley, Scotland, UK) supplemented with 8% heat-inactivated fetal calf serum (FCS; Gibco – Life Technologies), gentamicin (80 μg/mL) and 1% penicillin–streptomycin (Gibco). The cells were cultured at a density of $2–3\times10^5$ cells/mL. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

DQA preparation

Micellar solutions of DQA were prepared as previously described.[24,25] Briefly, a 10 mmol/L DQA (MW 527.6; Sigma Chemical Co., St Louis, MO, USA) stock solution was prepared by dissolving an adequate amount of DQA in methanol in a round bottom flask. The organic solvent was removed with a rotary evaporator. The DQA-film obtained was re-suspended in 5 mmol/L N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4 and sonicated for one hour. Finally, the sample was centrifuged (1000 *g*, 5 min) to remove metal particles from the probe as well as larger DQA aggregates. This procedure yielded an opaque solution of liposome-like DQA vesicles, which was then filtered using a 0.2 μmol/L filter. The DQA concentration was determined by fluorimetry (PerkinElmer LS-50 B spectrofluorimeter, Santa Clara, CA, USA; excitation $\lambda = 335$ nm, emission $\lambda = 360$ nm). The DQA standard curve was found to be linear between 0.001 and 0.01 mmol/L DQA ($r^2 = 0.998$).

Cell treatment

The cells were treated for 16 or 48 h with different DQA concentrations in the presence or absence of either the ERK1/2 and PI3K protein kinase inhibitors U0126 and LY294002 (Calbiochem, La Jolla, CA, USA), respectively. Stock solutions of 10 mmol/L U0126 and 50 mmol/L LY294002 were prepared in dimethyl sulfoxide and stored at −20°C. The doses used were 10 μmol/L for U0126 and 25 μmol/L for LY294002.

Imatinib (STI571, Gleevec®) was kindly provided by Novartis Pharma AG (Basel, Switzerland) and the cells were treated with a dose of 2 μmol/L.

Analysis of ERK and Akt phosphorylation by Western blotting

After treatment, the cells were harvested and lysed in 10 μL of lysis buffer (NP40 1%, 150 mmol/L Tris pH 8, 20 mmol/L NaF, 1 mmol/L ethylenediaminetetraacetic acid pH 8, 1 mmol/L phenylmethylsulfonyl fluoride and 10 μg/mL each of aprotinin and leupeptin) by incubation on ice for 30 min. The extracts were then centrifuged at 14,000 *g* for 20 min and the protein concentration determined using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (40 μg) were blotted on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes which were blocked with 5% non-fat milk TTBS (Tween-Tris (hydroxymethyl) aminomethane buffered saline buffer) for one hour to reduce non-specific binding. Next, the membranes were incubated with anti-human primary antibodies directed against p-ERK1/2 (1:500) and p-Akt (1:250) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After overnight incubation at 4°C, the blots were washed and exposed to either horseradish peroxidase-conjugated (1:1500) or infrared-specific fluorochrome-labeled (IRDyes, Li-Cor Biosciences, Lincoln, NE, USA) secondary antibodies for one hour at room temperature. An anti-human β-actin monoclonal antibody (Oncogene, EMD Biosciences Inc., Darmstadt, Germany) was used to assess equal protein load in all lanes. Protein bands were detected by using either the Amersham ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, England, UK) or the Odyssey® Infrared Imaging System (Li-Cor Biosciences).

Analysis of ERK phosphorylation by flow cytometry

The intracellular detection of phospho-ERK1/ERK2 was assessed by flow cytometry with a rabbit monoclonal anti-phospho-ERK1/2 antibody (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Cells (0.5×10^6) were fixed and permeabilized using 4% paraformaldehyde and ice-cold methanol. A total of 10 μL of the antibody (10 μg/mL) was added to the cells in a total reaction volume not exceeding 200 μL. Fluorescence was measured in a FACScan cytometer (Becton Dickinson, San Jose, CA, USA) with an FL-1 detector.

Metabolic activity assays

The metabolic activity of K562 cells was determined using the 3-[4,-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay (Roche, Mannheim, Germany). This assay enables the detection of mitochondrial dehydrogenase activity. Viable cells, with functional mitochondria, are able to reduce the tetrazolium ring to a blue formazan product, whereas the dead cells remain uncoloured. Cell metabolic activity is expressed as a percentage of control cells.

Necrotic and apoptotic cell death assessment

Cell death by necrosis was determined by measurement of the loss of cell membrane integrity by flow cytometry using propidium iodide (PI). For this study, $3-5 \times 10^5$ cells were stained with 20 μg/mL of PI and the emitted fluorescence was analyzed in a FACScan flow cytometer (Becton Dickinson) with an FL2 detector (620 nm band pass filter). Under these conditions, necrotic cells are brightly stained with PI and appear as a peak at very high fluorescence values. Apoptotic cells appear as a dimly fluorescent population. Since apoptosis ultimately leads to a loss of plasma membrane integrity, the necrosis determined here includes the late stages of apoptosis.

The characteristic loss of DNA in the apoptotic process was analyzed by flow cytometry of permeabilized PI-stained cells. Samples containing $3-5 \times 10^5$ cells were incubated with 0.5 mg/mL of RNase A for 30 min. The cells were then permeabilized with 0.1% Nonidet P-40 and incubated with 20 μg/mL of PI. Cell cycle analysis was carried

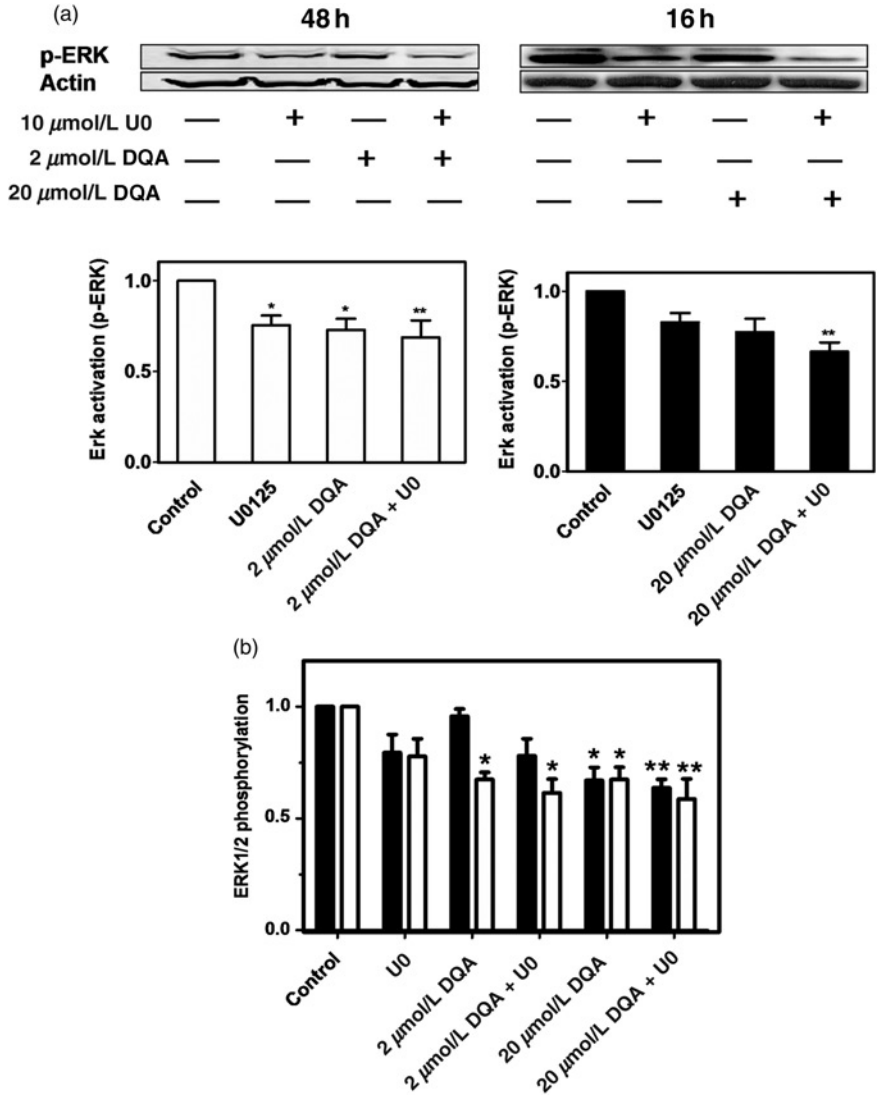

Figure 1 Effect of DQA on ERK1/2 phosphorylation in K562 cells. (a) Representative immunoblotting of ERK1/2 phosphorylation. Cells were treated for 48 h with 2 μmol/L DQA (left-hand side) or for 16 h with 20 μmol/L DQA (right-hand side) in the absence or presence of the MEK inhibitor U0126 (U0, 10 μmol/L) and samples (40 μg proteins) analyzed by immunoblotting. The two bands observed correspond with the 42 and 44 kDa isoforms of ERK1/2. Anti-human actin monoclonal antibody was used to demonstrate equal protein load. The bar graph shows the mean intensities of the bands obtained by Western blots at 48 h (white bars) and 16 h (black bars) of treatment in relation to the control cells. (b) Phosphorylation of ERK1/2 analysed by flow cytometry after cell treatment with 2 and 20 μmol/L DQA and U0, alone or in co-treatment for 16 h (black bars) or 48 h (white bars). Data in bar graphs are presented as the mean $\pm$ SEM of at least three separate experiments. Significant differences compared with control cells are indicated as $^*P$ value <0.05 and $^{**}P$ value <0.01. DQA, dequalinium

out by flow cytometry (FL-2 detector in a linear mode) using WinMDI (Version 2.9, The Scripps Research Institute, San Diego, CA, USA) and Cylchred software (Version 1.0.2, Cardiff University, Cardiff, UK) programs. Permeabilization of cells causes the leakage of the cleaved low-molecular-weight DNA fragments that are produced during apoptosis. As a consequence, apoptotic cells are identified as a hypodiploid peak (sub-G0/G1 content).

Apoptosis was also analyzed by studying the phosphatidylserine exposure on the cell surface. For this purpose, the cells were double-stained with Annexin V-fluorescein isothiocyanate (FITC) and PI (Annexin V-FITC Apoptosis Detection Kit; Calbiochem) and analyzed by flow cytometry (FACScan, Becton Dickinson) according to the manufacturer's instructions.

Statistical analysis

One-way analysis of variance with a *post hoc* Bonferroni test was used to compare quantitative data. P value <0.05 was considered to be statistically significant, $P < 0.01$ very significant and $P < 0.001$ extremely significant.

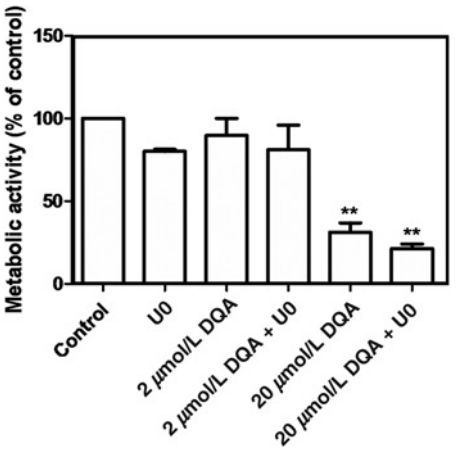

Figure 2 Effect of DQA and U0126 on metabolic activity in K562 cells. Cells were treated for 48 h with 2 and 20 μmol/L DQA, in the absence or presence of the ERK1/2 inhibitor U0126 (U0, 10 μmol/L). Cell metabolic activity was measured by the MTT test and is provided as a percentage relative to the controls. Data are presented as the mean $\pm$ SEM of at least three separate experiments. Significant differences compared with control cells are indicated as $^{**}P$ value <0.01. DQA, dequalinium; MTT, 3-[4,-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide

Results

DQA effect on the Raf/MEK/ERK pathway

Previous studies in our laboratory have demonstrated a cytotoxic effect of DQA on the leukemic K562 cells which died by necrosis in a DQA concentration (from 0.5 to 20 μmol/L)- and time (from 24 to 48 h)-dependent manner.[21] An absence of apoptosis at every DQA concentration analyzed was observed, which supports the reported resistance of these cells to apoptosis.[21,22] In this work, we have first studied the relationship between DQA-induced K562 cell death and the Raf/MEK/ERK pathway. To this aim, the cells were treated with a low (2 μmol/L) or a high (20 μmol/L) DQA concentration for 48 and 16 h, respectively, in the absence or presence of the ERK1/2 specific inhibitor U0126. ERK1/2 phosphorylation was analyzed by both Western blot and flow cytometry. Figure 1a shows that ERK1/2 is constitutively active in K562 control cells, as it has been described by other authors.[26] ERK phosphorylation decreases after 16 h of treatment with U0126 or DQA. However, this effect was statistically significant only at the longest incubation time studied, 48 h. The inhibition of ERK phosphorylation was significantly potentiated in co-treatments with U0126 and DQA. Flow cytometry analysis led us to detect that ERK phosphorylation was significantly inhibited both at a low 2 μmol/L concentration of DQA for a long 48-h incubation time and at a high 20 μmol/L concentration of DQA either for a short 16-h or a long 48-h incubation time (Figure 1b). The ERK1/2 inhibitor U0126 decreases ERK phosphorylation, this effect being potentiated by co-treatment with either high DQA concentrations for short and long times or low DQA concentrations for long times. These results agree with the ones shown in Figure 1a and allow us to point out an inhibition of

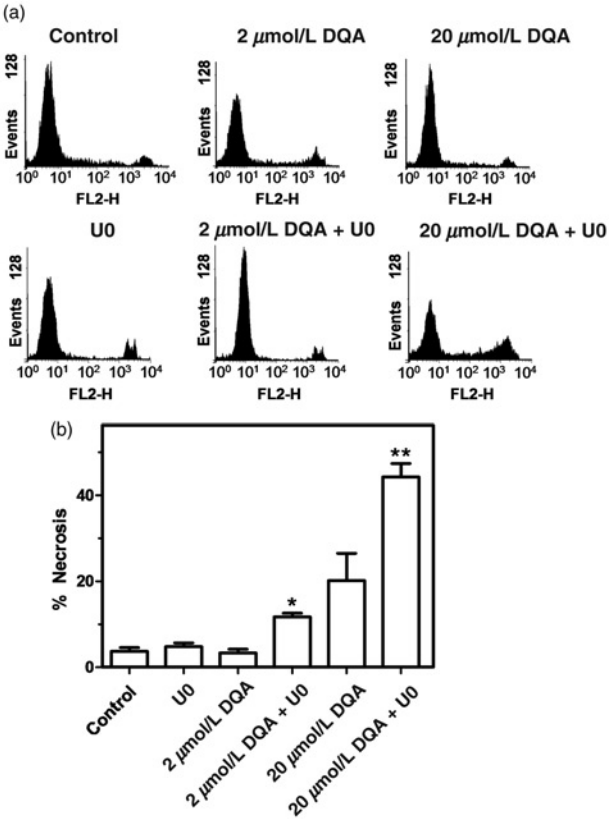

Figure 3 Effect of DQA and U0126 on necrotic cell death in K562 cells. (a) Representative flow cytometry histograms of untreated (control) cells and cells treated with 2 and 20 μmol/L DQA for 48 h in the absence or presence of the ERK1/2 inhibitor U0126 (U0, 10 μmol/L), as detected by propidium iodide-free influx. (b) Percentage of necrotic cells, obtained from the necrotic region of the flow cytometry histograms. Percentages are presented as the mean $\pm$ SEM of at least three separate experiments. Significant differences compared with control cells are indicated as $^{*}P$ value <0.05 and $^{**}P$ value <0.01. DQA, dequalinium

ERK1/2 phosphorylation after U0126 or DQA treatment that is potentiated by the combined effect of both agents.

Implication of the Raf/MEK/ERK pathway on the metabolic activity and cell death of DQA-treated K562 cells

Once the influence of DQA on ERK1/2 activity was demonstrated, the implication of the Raf/MEK/ERK pathway in the metabolic activity of K562 cells in the presence of DQA and U0126 was investigated through a time- and concentration-response study. The cells were treated with 2 and 20 μmol/L DQA for 16 and 48 h in the absence or presence of U0126, and metabolic activity was analyzed by the MTT test. According to our previous results,[21,22] K562 cell metabolic activity after 16 h of treatment with DQA was always higher than 60% (results not shown). However, longer 48-h DQA treatments induced a decrease in metabolic activity even at low DQA concentrations (Figure 2). In this way, DQA decreases cell metabolic activity in a time- and concentration-dependent manner. The ERK inhibition by U0126 produces a decrease in the metabolic activity, this effect being significantly potentiated by co-treatment with high DQA concentrations for long time periods.

The implication of the Raf/MEK/ERK pathway in K562 cell death, either by necrosis or apoptosis, was subsequently analyzed. According to our previous results,[21,22] treatment with DQA for 16 h did not induce K562 cell death. For this reason, results are only shown for treatments at the 48-h incubation time. A characteristic feature of the necrotic process such as the loss of cell membrane integrity was analyzed by PI permeability (Figure 3). Representative flow cytometry profiles of the PI uptake in control cells and cells incubated for 48 h with 2 and 20 μmol/L DQA in the absence or presence of U0126 are shown in Figure 3a. The percentage of necrotic cells with high fluorescence intensity is shown in Figure 3b. As can be observed, at a higher DQA

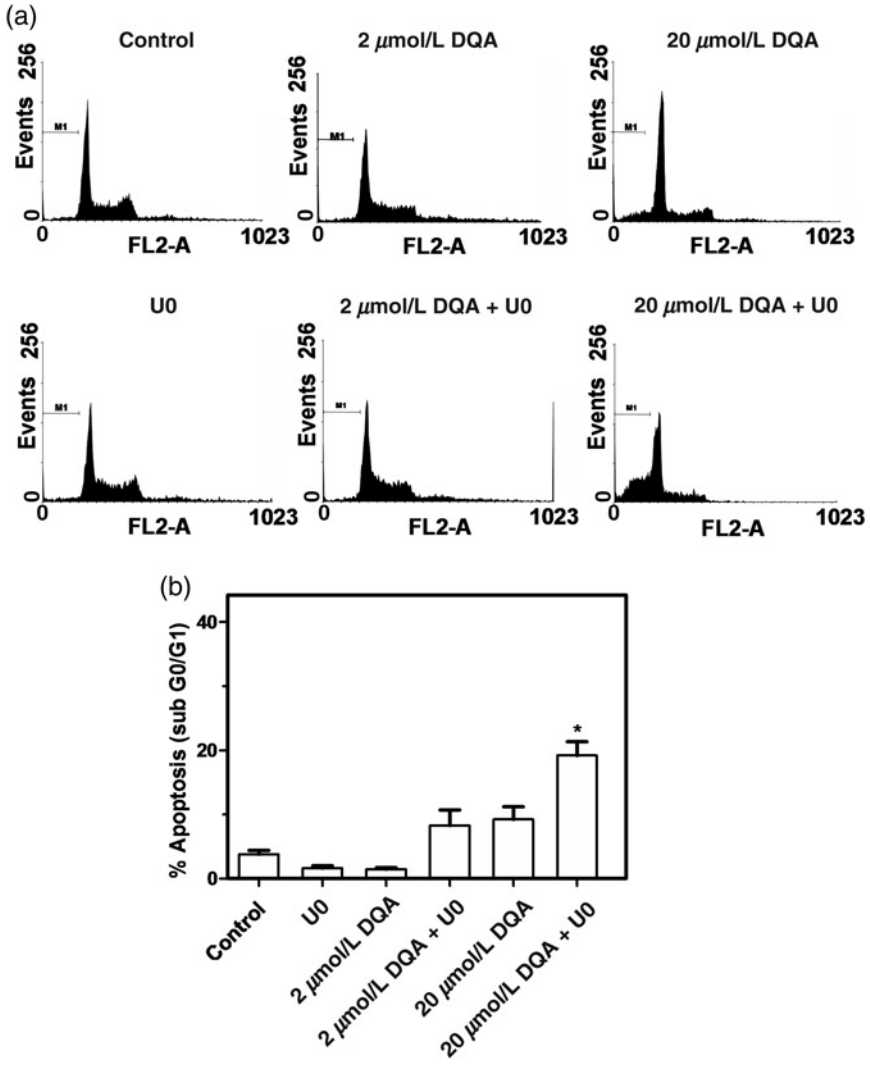

Figure 4 Effect of DQA and U0126 on apoptotic cell death in K562 cells detected by cell permeabilization and propidium iodide staining. (a) Representative flow cytometry histograms of untreated (control) cells and cells treated with 2 and 20 μmol/L DQA for 48 h in the absence or presence of the ERK1/2 inhibitor U0126 (U0, 10 μmol/L). (b) Percentage of cells obtained from the corresponding sub-G0/G1 regions of the DNA content flow cytometry histograms. Percentages are presented as the mean $\pm$ SEM of at least three separate experiments. Significant differences compared with control cells are indicated as $^{*}P$ value <0.05. DQA, dequalinium

concentration, higher necrosis was found. Necrosis was significantly potentiated by the ERK inhibitor U0126 which did not induce necrosis by itself. These and the above results suggest a relationship between the inhibition of the phosphorylation of ERK, loss of metabolic activity and necrosis in K562 cells treated with DQA.

Apoptosis was studied by cell cycle analysis obtained by flow cytometry after PI staining of DNA of previously permeabilized cells. Figure 4a shows a representative example of the PI fluorescence profiles obtained from control cells and cells incubated for 48 h with 2 or 20 μmol/L DQA in the absence or presence of U0126. A DQA concentration-dependent accumulation of cells in the sub-G0/G1 and G0/G1 phases and a decrease in the S and G2/M phases is observed. The percentage of apoptotic cells in the sub-G0/G1 phase is shown in Figure 4b where it can be seen that U0126 potentiates the DQA-induced apoptosis. These results were confirmed by the measurement of the cell surface exposure of phosphatidylserine of apoptotic cells by flow cytometry using double Annexin V-FITC/PI staining. Figure 5a shows representative dot plots diagrams obtained for control and DQA-treated cells for 48 h either

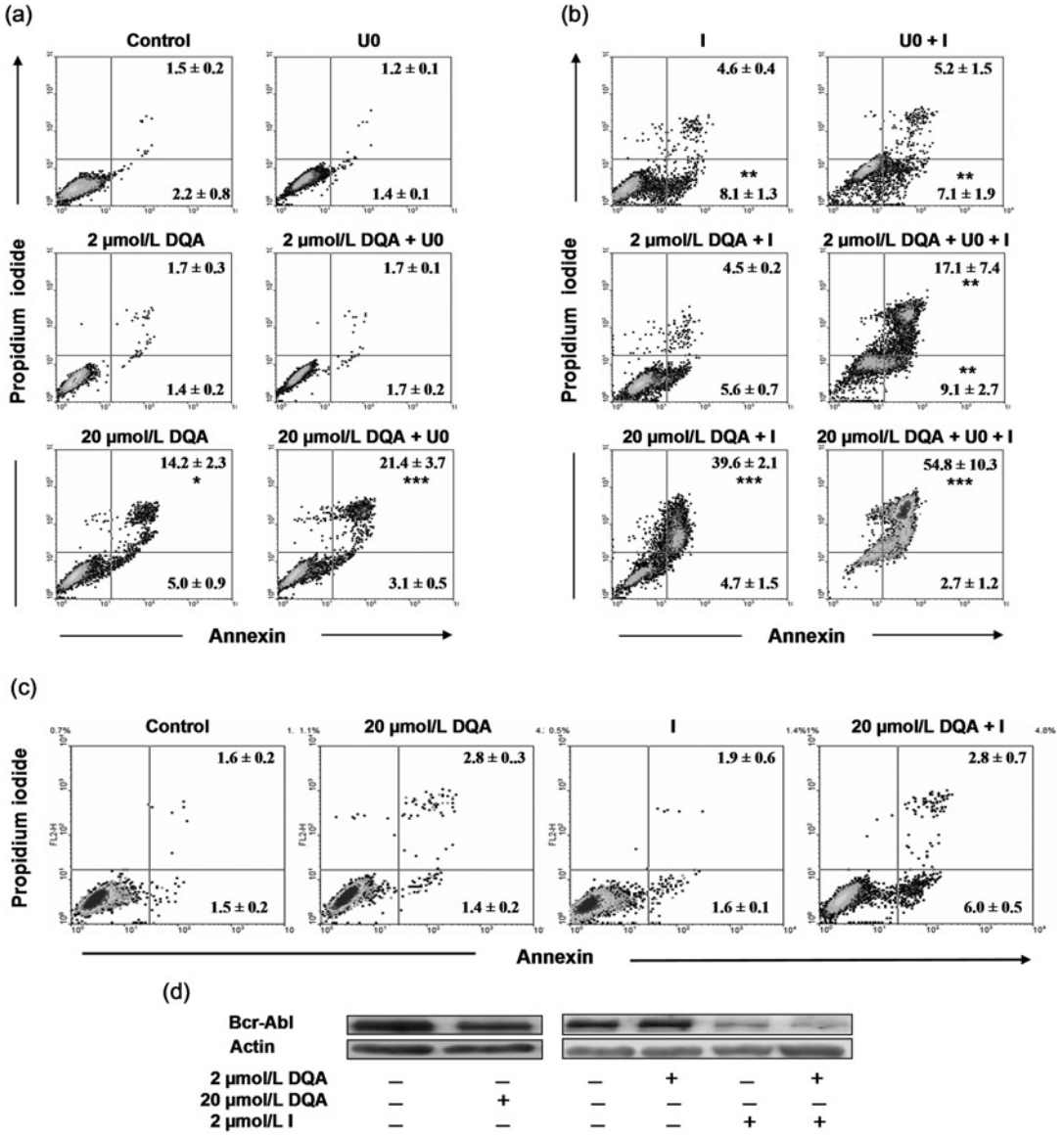

Figure 5 Effect of DQA, U0126 and imatinib on apoptotic cell death in K562 cells. Representative dot plot diagrams obtained by flow cytometry with Annexin V-FITC/PI double-staining of cells treated for 48 h with 2 or 20 μmol/L DQA in the absence (left panels) or presence (right panels) of the MEK inhibitor U0126 (U0, 10 μmol/L) (a) and in the presence of the Bcr-Abl tyrosine kinase inhibitor imatinib (I, 2 μmol/L) in the absence (left panels) or presence (right panels) of the MEK inhibitor U0126 (b). (c) Representative dot plot diagrams of cells treated for 16 h with 20 μmol/L DQA in the absence or presence of imatinib. Lower left (LL) quadrant $FITC^-/PI^-$ corresponds to living cells, lower right (LR) quadrant $FITC^+/PI^-$ corresponds to early apoptotic cells, upper right (UR) quadrant $FITC^+/PI^+$ corresponds to late apoptotic or necrotic cells and upper left (UL) quadrant $FITC^-/PI^+$ corresponds to necrotic cells. Quadrant-inserted data represent the mean $\pm$ SEM of at least three separate experiments. (d) Bcr-Abl phosphorylation detected by Western blotting (40 μg proteins). Cells were treated for 48 h with 2 μmol/L DQA or 20 μmol/L DQA in the absence or presence of imatinib. Anti-human actin monoclonal antibody was used to demonstrate equal protein load. Significant differences compared with control cells are indicated as $^*P$ value <0.05, $^{**}P$ value <0.01 and $^{***}P$ value <0.001. DQA, dequalinium; PI, propidium iodide; FITC, fluorescein isothiocyanate

in the absence (left panels) or presence (right panels) of U0126. The results show that high 20 μmol/L DQA concentrations induce some early (lower-right quadrants) and late (upper-right quadrants) apoptosis which was significantly increased by co-treatment with U0126. These results support that resistance of K562 cells to apoptosis can be overcome by the synergic effect of DQA with the ERK1/2 inhibition by U0126.

Previous studies have shown that resistance of K562 cells to DQA-induced apoptosis can be overcome by the inhibition of the Bcr-Abl protein kinase with the tyrosin kinase inhibitor imatinib.[21] For that reason, we studied the effect of co-treatments with DQA and imatinib either in the absence or presence of U0126 on apoptosis cell death (Figure 5b). As was expected, after 48 h, imatinib induced apoptosis in K562 cells. In co-treatment with DQA, apoptosis was potentiated in a concentration- and time-dependent manner, as can be deduced by comparing Figures 5b and c, reaching levels of 40% at the concentration of 20 μmol/L DQA after 48 h (left panels in Figure 5b). ERK inhibition by U0126 did not show any effect on imatinib action, indicating that apoptosis detected after co-treatment with imatinib and U0126 may only be attributed to imatinib. In co-treatment with U0126 and imatinib, DQA significantly increased apoptosis in a DQA concentration-dependent way, reaching a very high 55% value at the concentration of 20 μmol/L DQA after 48 h.

Western blot analysis allowed us to demonstrate that both high DQA concentrations and imatinib induce the dephosphorylation of the Bcr-Abl kinase after 48 h of treatment, an effect that seems to be potentiated by co-treatment with both agents even at the low 2 μmol/L DQA concentration (Figure 5d). All these results suggest that both ERK1/2 and Bcr-Abl kinase activities are implicated in the resistance of K562 cells to DQA.

Effect of DQA on the PI3K/Akt pathway

Just like the MEK/ERK1/2 pathway, the PI3K/Akt pathway is considered a proliferative and survival pathway. Thus, we next studied the effect of DQA on the phosphorylation of Akt in the presence or absence of the PI3K-specific inhibitor LY294002 after 48 or 16 h of incubation with 2 or 20 μmol/L DQA, respectively. As can be observed in Figure 6, Akt is constitutively active in K562 control cells, as has been described in leukemic cells by other authors.[27–29] Treatment with DQA slightly decreases Akt phosphorylation while LY294002 decreased the phosphorylation of Akt in a time-dependent manner, this effect being potentiated by co-treatment with DQA. The decrease in the phosphorylation of Akt could not be related with a decrease in cell viability, since the latter was always elevated (results not shown).

Relationship between the Raf/MEK/ERK1/2 and PI3K/Akt pathways

The role of the PI3K/Akt pathway in the effects elicited via MAPK signaling has been suggested.[30,31] Therefore, we finally studied the relationship between the Raf/MEK/ERK and PI3K/Akt pathways and its implication in DQA-induced cell death. To this aim, the effect of the ERK1/2 inhibitor U0126 on the phosphorylation of Akt, as well as the effect of the Akt inhibitor LY294002 on the phosphorylation of ERK1/2, was analyzed. As can be seen in Figure 7a, the presence of DQA or U0126 did not significantly affect the phosphorylation of Akt. However, treatment with LY294002 significantly decreased the phosphorylation of ERK1/2 both in the absence or the presence of DQA (Figure 7b). These results suggest a direct relation between the PI3K/Akt and Raf/MEK/ERK pathways in

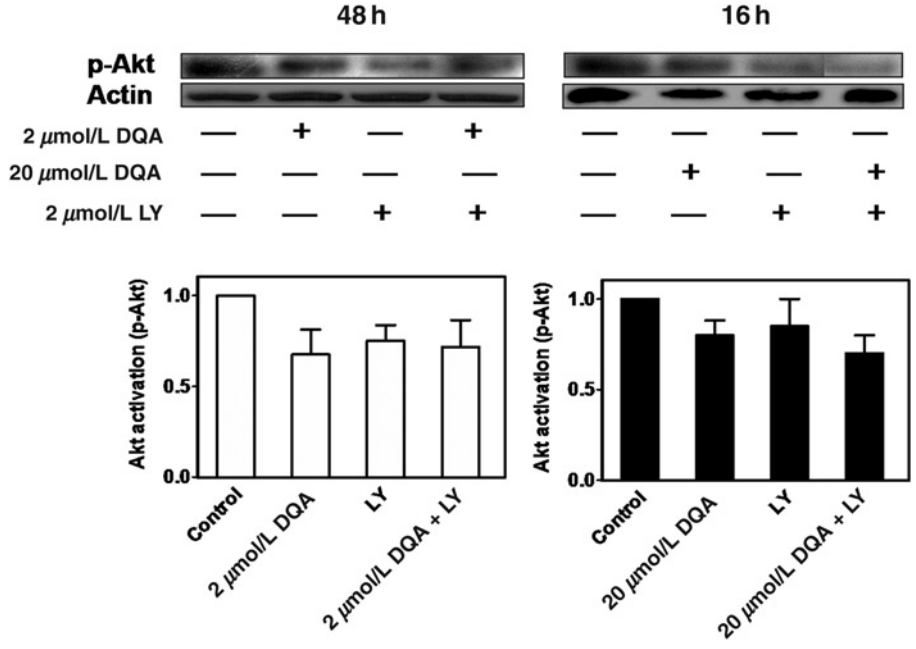

Figure 6 Effect of DQA on Akt phosphorylation in K562 cells. Phosphorylation of Akt as detected by Western blotting (40 μg proteins). Cells were treated for 48 h with 2 μmol/L DQA or for 16 h with 20 μmol/L DQA in the absence or presence of the Akt inhibitor LY294002 (LY, 25 μmol/L). Anti-human actin monoclonal antibody was used to demonstrate equal protein load. DQA, dequalinium

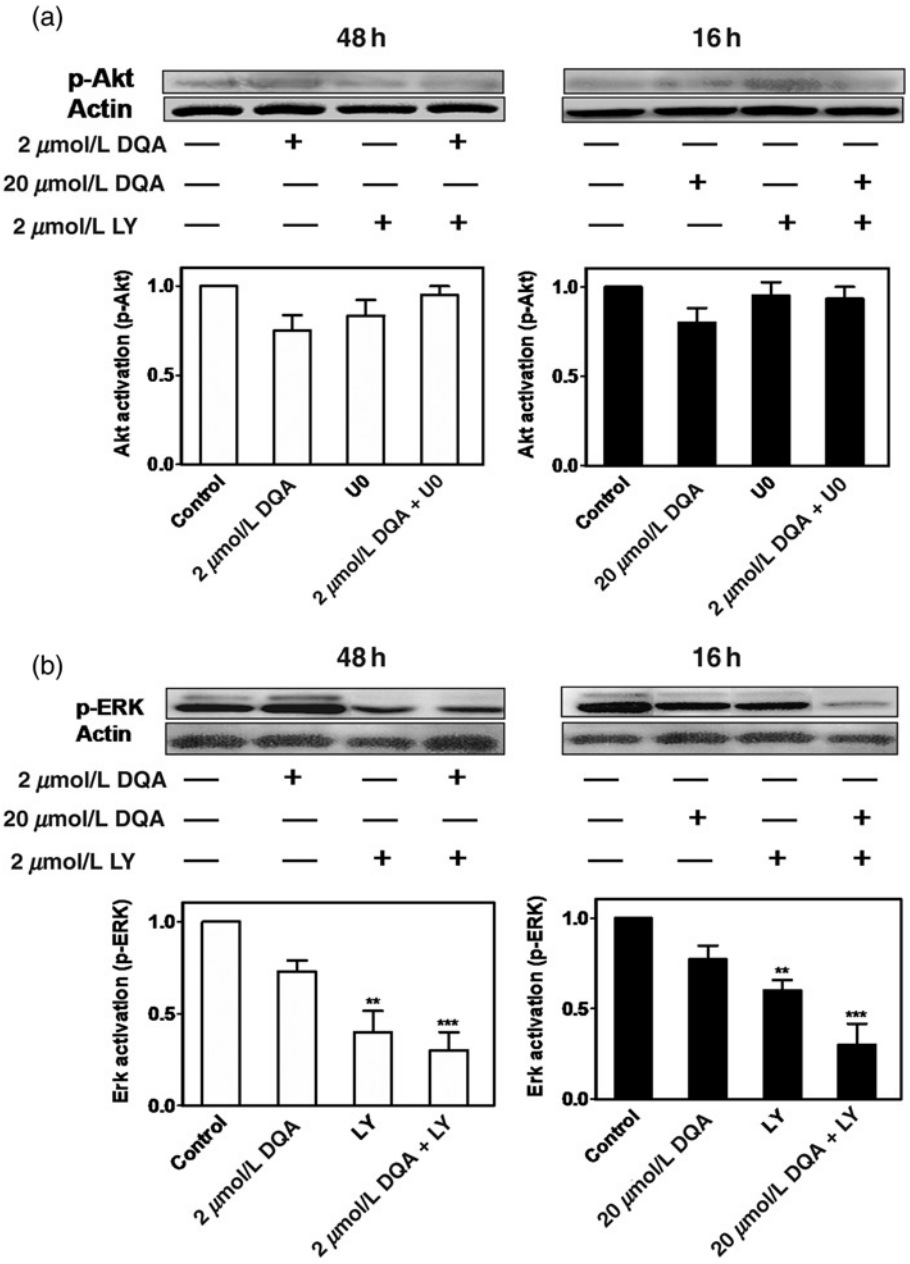

Figure 7 Relationship between Raf/MEK/ERK and PI3K/Akt pathways in K562 cells. Phosphorylation of Akt in the absence or presence of the ERK1/2 inhibitor U0126 (U0, 10 μmol/L) (a) and phosphorylation of ERK1/2 in the absence or presence of the Akt inhibitor LY294002 (LY, 25 μmol/L) (b), as detected by Western blotting (40 μg proteins). Cells were treated for 48 h with 2 μmol/L DQA or for 16 h with 20 μmol/L DQA. The two bands observed correspond with the 42 and 44 kDa isoforms of ERK1/2. Anti-human actin monoclonal antibody was used to demonstrate equal protein load. Significant differences compared with control cells are indicated as $^{**}P$ value <0.01 and $^{***}P$ value <0.001

K562 cells that is potentiated in the presence of high DQA concentration.

Discussion

The cytotoxic activity of DQA on K562 cells has been previously studied by our group.[21–23] These cells showed a progressive decrease of cell viability and metabolic activity, with the increase of both the DQA concentration and the incubation time showing some necrosis (>10%) when treated with a high 20 μmol/L DQA concentration for 16 h or 4 μmol/L DQA onwards for 48 h, being resistant to apoptosis. It has been described that targeting the Raf/MEK/ERK and PI3K/Akt/mTOR pathways may be an effective approach for therapeutic intervention in drug-resistant cancers that have mutations which activate these pathways.[13,26] In the present study, we investigated the role of the Raf/MEK/ERK and PI3K/Akt signal transduction pathways in the resistance of K562 cells to DQA-induced apoptosis.

The obtained results show that Raf/MEK/ERK1/2 and PI3K/Akt signaling pathways are constitutively activated in K562 control cells, indicating an important role of these pro-survival signaling pathways in leukemogenesis and proliferation of CML cells. Such activation has also been observed by other authors in K562 and other cancer cell

lines and has been related to cell survival and proliferation processes.[27–33] Therefore, the inhibition of Raf/MEK/ERK1/2 and PI3K/Akt cell signaling pathways is being considered as a possible therapeutic target in order to fight against leukemia.[4,12,13,34–37]

DQA induced a slight downregulation of the Raf/MEK/ERK1/2 pathway in K562 cells, while metabolic activity decreased and necrosis increased. These effects were enhanced in the presence of the ERK1/2 inhibitor U0126 which in part reverted the resistance of K562 cells to DQA-induced apoptosis, suggesting a role of the Raf/MEK/ERK pathway in the survival of K562 cells. The studies carried out in this paper show that the ERK1/2 inhibition by U0126 does not increase the imatinib-induced cell death significantly. However, U0126 and imatinib in co-treatment with DQA increase cell death in a DQA concentration-dependent way. These results suggest an implication of both proteins Bcr-Abl and ERK1/2 in cell survival and in the resistance of K562 cells to DQA. Moreover, the combination of imatinib and DQA seems to be necessary for the synergistic effect with U0126, as shown by the co-treatment with DQA, indicating that other mechanisms different from the inhibition of the Raf/MEK/ERK pathway might be involved. Recent studies have shown that the fusion protein Bcr-Abl activates ERK1/2, favoring the proliferation of CML cells.[33] The studies carried out in this paper show that the co-treatment with the Bcr-Abl tyrosine kinase inhibitor imatinib, the ERK1/2 inhibitor U0126 and DQA increases cell death in a DQA concentration-dependent way, suggesting an implication of the cell survival proteins Bcr-Abl and ERK1/2 in the resistance of K562 cells to DQA. The results also suggest that DQA is not only able to induce an inhibition of the Raf/MEK/ERK1/2 pathway, but also offset the effects of Bcr-Abl on ERK1/2, which results in an inhibition of cell survival. In other words, the survival rate of K562 cells could be decreased by the inhibition of ERK1/2 either directly by U0126 or indirectly by decreasing Bcr-Abl activity, which supports a relationship between ERK and Bcr-Abl. Thus, DQA was able to increase the apoptosis induced by the Bcr-Abl inhibitor imatinib, this effect being enhanced by the presence of U0126. The best results were obtained at a low 2 μmol/L DQA concentration. Other authors have shown a higher cytotoxic action of imatinib when combined with other drugs. Thus, it has been described that inhibition of Bcr/Abl activity by imatinib increases the toxicity of melphalan and allows an efficient killing of leukemic K562 cells.[38]

On the other hand, DQA induced a slight downregulation of the PI3K/Akt pathway that was enhanced by the addition of the Akt inhibitor LY294002 without affecting cell death. The present results also show an interaction between the Raf/MEK/ERK and PI3K/Akt pathways. It has been described that although it depends on the cell type, among some other factors, Akt seems to activate Raf in order to prevent apoptosis in hematopoietic cells and other tumor cells.[39–42] In our hands, the dual inhibition of both ERK1/2 and Akt protein kinase activities of LY294002 could sensitize K562 cells to DQA, thus reducing cell proliferation and favoring apoptosis. In other words, a positive modulation of PI3K/Akt on Raf/MEK/ERK1/2 in K562 cells could be favoring the resistance of K562 cells to DQA-induced apoptosis. Thus, PI3K/Akt pathway inhibition may provide a solution to one aspect of the K562 apoptotic resistance phenomenon.

To conclude, our findings in K562 cells demonstrate that inhibiting the Raf/MEK/ERK pathway enhances the ability of DQA to induce apoptosis, supporting the role of ERK in cell survival. Besides, ERK inhibition in combination with DQA significantly potentiates imatinib-induced apoptosis, which is a promising finding for the development of combination therapies against imatinib-resistant leukemias. This study broadens our knowledge on the action mechanism of DQA and establishes its implication in the signaling transduction pathways related to the survival or death of human leukemic cells. This work also supports the use of DQA in co-treatment with other agents as a new therapeutic option for patients with CML.

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