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DOI: 10.1515/dch-2015-0005

Quercetin, Menadione, Doxorubicin combination as a potential alternative to Doxorubicin monotherapy of acute lymphoblastic leukemia

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Abstract:

Doxorubicin is a widely used chemotherapeutic drug, effective on patients with acute lymphoblastic leukemia, but associated with significant long term cardiotoxicity. Menadione (vitamin K3) and the flavonoid quercetin are known as strong apoptogens in human leukemia Jurkat T cells. We explored the potential synergic cytotoxic effects of doxorubicin in association with quercetin and menadione in this cellular model for acute lymphoblastic leukemia.

Apoptosis, necrosis and cell cycle distributions were determined by flow cytometry on Jurkat lymphoblasts labeled with Annexin V-FITC/7-Aminoactinomycin D and propidium iodide, respectively. Oxidative stress was assessed by flow cytometry using CM-H2DCFDA/7-Aminoactinomycin D labeling.

Results indicate a dose-dependent oxidative-stress generation, cell cycle arrest and apoptosis induction by doxorubicin alone, correlated with a decrease of the required doses when the anticancer drug was associated with quercetin and menadione. Data also support the theory of an additive cytotoxic effect of the three agents on leukemia cells.

Introducing quercetin-menadione combinations in leukemia doxorubicin-based treatment could significantly increase the treatment's efficacy. The main mechanism responsible for this effect appears to be the increase in the affinity of doxorubicin for DNA, which enables lowering of the therapeutic dose.

Keywords: *doxorubicin, acute lymphoblastic leukemia, oxidative stress, apoptosis, necrosis, cell cycle*

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Introduction

In the past years, a great interest has been focused on the health benefits of natural flavonoids. They are chemical compounds widely found in fruits, leaves and vegetables, responsible for their bright colors, and also well-known potent antioxidants. The possible antioxidant effects of flavonoids were suspected after it was shown that a red wine rich Mediterranean diet was associated with a lower cardiovascular risk [1]. Numerous studies have shown that they possess antiatherogenic - cardioprotective effects and exert antitumoral, anti-mutagenic and anti-inflammatory activities [2-4].

Quercetin (QC) is one of the best characterized flavonoids, found prevalently in fresh parsley, onions, olives and green salad [1]. This flavonoid is known to have pro-oxidative properties under various conditions, and a number of both in vitro and in vivo studies indicate a greater susceptibility of malignant cells to the cytotoxic effect of the flavonoid with respect to their normal counterparts [5,6].

Flavonoids appear to bind to a sugar molecule and originally it was considered that only free flavonoids could be absorbed in the human intestine. Quercetin in its native state is usually bound to a glucose or rutinose molecule. Studies have indicated a superior absorption of the quercetin-glucose complex, compared to the quercetin-rutinose one, with a 20 times higher peak plasma level reached in 10 times less time from the ingestion, suggesting a small intestine absorption for the first compound and a large intestine post-deglycosylation for the latter [7].

The main limitation of the clinical use of quercetin is linked to its poor bioavailability and its rapid metabolic

conversion in the liver. Pharmacokinetic studies had shown that tolerable QC plasma levels efficient for cancer therapy (>10 μM) are maintained for about 30 minutes after intravenous administration, the average half-life being little above 40 minutes [8,9]. Liposomal carriers used to deliver quercetin can increase the half-life and the bioavailability of the flavonoid [10] and thus could lower the threshold level required for efficient cancer treatment protocols.

Flavonoids possess at least 3 antioxidant mechanisms, as well as the capacity of stimulating the natural protective capacity. One mechanism (probably responsible for the anti-atherogenic effect) consists of direct reactive oxygen species scavenging through the high hydroxyl functional group reactivity, resulting in the inactivation of radical species [7]. A second cellular pathway stimulates the nitric oxide (NO) production. Studies conducted on acute lymphoblastic leukemia cells after quercetin exposure show increased nitric oxide levels and NO synthase inhibition by 1-(2-Mercaptoethyl)guanidine increases the apoptotic fraction of leukemic post-antioxidant treatment cells. The increase in NO production appears to be a protective mechanism at physiological low concentrations, but at high levels it reacts with reactive oxygen species, forming peroxynitrite, with deleterious effects on cell membranes [11]. The third antioxidant mechanism can be explained by quercetin's iron chelation properties, demonstrated in in vitro studies on mice erythrocytes after glutathione depletion. In the absence of flavonoids, significant membrane lipid peroxidation and hemolysis may occur due to iron release, while association with quercetin can exert protective effects via iron chelation [12].

In the human leukemia Jurkat T cell line, quercetin activates the ryanodine receptor (a calcium channel), hence inducing calcium release from the endoplasmic reticulum, acting as a potent apoptogen and enhancing apoptosis induced by menadione (MD) [11,13,14].
Menadione, also known as vitamin K3, is a well-known pro-oxidant agent, with antitumoral activity in various cellular lines, including Jurkat T cells. MD is a liposoluble synthetic form of vitamin K, which is metabolized in the human body to vitamin K2. Recent studies have shown a chemosensitizing effect of menadione in different cancer models [5,15], as well as a powerful corrector of doxorubicin resistance in leukemia cells. More than half of the intracellular metabolism of menadione is developed through redox cycling, which can generate high quantities of free radicals [16].

Both menadione and quercetin are known as pro-apoptotic agents acting via the calcium-dependent mitochondrial pathway, promoting calcium release from the endoplasmic reticulum and opening of the mitochondrial permeability transition pore. The opened pore induces the collapse of the mitochondrial transmembrane potential and the release of the cytochrome c from the mitochondria. Cytochrome c is associated with the internal mitochondrial membrane and is an essential component of the electron transport chain. When released into the cytoplasm, it activates the apoptotic protease activation factor-1, thus triggering apoptosis [17,18].

Doxorubicin (DOX), also known as adriamycin, is a broad spectrum chemotherapeutic drug compared to other antineoplastic drugs, first extracted from Streptomyces peucetius in the 1970s. Its cytotoxic properties are either a consequence of DNA intercalation and topoisomerase II-mediated DNA repair disruption, or a result of free oxygen radicals generation, that determine lipid peroxidation, DNA and protein injury, thus triggering apoptosis [19]. Doxorubicin enters the cells through passive diffusion [20] and once it has entered the cell, it can bind to the proteasome. The doxorubicin-proteasome complex is translocated into the nucleus, where it dissociates and DOX inserts itself between the strands of DNA, thus inhibiting the cellular metabolism [21]. Doxorubicin molecules that did not intercalate into the DNA stabilize the complex between topoisomerase II and DNA strings, leading to an increase in the damaged genomic material, associated with G2/M arrest and necrosis/ apoptosis [22,23].

During the intracellular metabolic conversion of doxorubicin, one electron is added to the quinone, leading to the formation of a semiquinone, which is converted back to its quinone form when entering a redox reaction. Reactive oxygen species are then produced leading to cellular oxidative stress [24].

A major limitation in the clinical use of doxorubicin is its cardiotoxicity. The myocardial involvement is dependent on the cumulative dose and may occur even decades after finishing the treatment [24]. It appears that this severe side effect is determined by iron release and free radicals generation. Doxorubicin is reduced to doxorubicinol, an active metabolite which interferes with intracellular iron deposits. This theory is supported by the fact that dexrazoxane, an iron chelator, is protective against doxorubicin induced toxicity. In this case, association of quercetin could not only improve the therapeutic index of doxorubicin, but also decrease its cardiac toxicity, through its iron chelating properties [25,26].

Methods

Jurkat cells (clone E6-1, ATCC TIB-152) were cultured in GLUTAMAX-I and HEPES-containing RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100

mg/ml streptomycin, at 37°C in a humidified incubator with a 5% CO2 atmosphere. Menadione sodium bisulphite (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS), whereas doxorubicin (Tocris), dihydrated quercetin (Sigma-Aldrich) and CM-H2DCFDA (Invitrogen) were dissolved in dimethyl sulfoxide (DMSO).

For evaluation of the cell cycle distribution, 106 cells were washed twice with PBS, incubated for 15 min. in PI (propidium iodide)/RNAse staining buffer (Pharmingen) containing 0.1% Triton X-100 and 25 M digitonin, in the dark, at room temperature and then analyzed on a Beckman Coulter Gallios flow-cytometer. PI staining, which has a red fluorescence, was used to measure the DNA content. For data acquisition and analysis we used CellQuest and WinMDI 2.9 software, together with a Gaussian deconvolution algorithm as described [11]. Apoptosis was evaluated as the fraction of hypodiploid cells (the sub-G0/G1 cell fraction). The G0/G1, S, and G2/M cell fractions were calculated for the non-apoptotic cell population, after excluding the hypodiploid events from the cell cycle analysis.

For apoptosis/necrosis assessment, 106 cells were washed twice with PBS and double stained with Annexin V-FITC (fluorescein isothiocyanate) (Pharmingen) and PI (Pharmingen), according to the manufacturer's instructions. The samples were analyzed immediately on a Beckman Coulter Gallios flow-cytometer. The excitation wavelength was 488 nm. Emission was recorded in FL1 (525 nm, bandpass 40 nm) for Annexin V-FITC, and FL3 (620 nm, bandpass 30 nm) for PI. Data analysis was performed using the software WinMDI 2.9. Cells negative for both Annexin V-FITC and PI were considered as living cells, those positive for Annexin V-FITC and negative for PI were considered as early apoptotic cells and those positive for both dyes, due to their permeable plasmatic membrane, were considered as late apoptotic/necrotic cells.

For oxidative stress evaluation, cells were washed with standard saline (SS, containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 20 mM HEPES, 10 mM glucose, pH 7.4/NaOH), resuspended in SS containing 0.5 M CM-H2DCFDA (a general oxidative stress indicator that is largely sensitive to H2O2 and OH, but can also sense peroxynitrite) and incubated for 10 min. at 37ºC in the dark. Cells were then centrifuged and resuspended in 0.1 ml SS containing 7-AAD (7-Aminoactinomycin D; Pharmingen), according to the manufacturer's instructions. After staining for 15 min. at room temperature in the dark, samples were diluted with 0.4 ml SS and measured immediately on a Beckman Coulter Gallios flow-cytometer. CM-H2DCFDA and 7-AAD emission was recorded on FL1 and FL4 (675 nm, bandpass 20 nm), respectively.

Unless otherwise specified, data are expressed as median ± s.e.m. of at least three different measurements.

Results

Doxorubicin is known to undergo several redox reactions to form a doxorubicin semiquinone radical, an unstable metabolite which is converted back to doxorubicin in a process that is generally accompanied by a dose-dependent production of free radicals. To determine whether the cytotoxic effects of DOX and DOX combinations are mediated by intracellular ROS generation, we evaluated simultaneously the cellular oxidative status, by labeling Jurkat cells with the fluorescent indicator CM-H2DCFDA, and the cellular viability using 7-AAD as a fluorescent dye. In agreement with our expectations, we observed that after exposure for 18 h, DOX induced the production of reactive oxygen species and decreased the viable cell fraction in a dose dependent manner, with a half-maximal inhibitory concentration IC50 = 0.57 μM ***(Fig. 1 A)***. The derived Hill coefficient, H = 1.44, suggested that cooperativity of two DOX molecules was required to induce the observed cytotoxic effect.

The combinations 15 μM QC/7.5 μM MD and 15 μM QC/15 μM MD applied for 18 h produced by themselves a cell death rate of 31% and 52%, respectively, and generated significant oxidative stress *(Fig. 1 A)*. In both cases, DOX-combined treatments exhibited additive cytotoxicity, with relatively similar IC50 and Hill coefficient values (IC50 = 2.26 μM, H = 1.73, and IC50 = 1.25 μM, H = 1.70, respectively) ***(Fig. 1A)***. In particular, the equimolar combination QC/MD (15 μM) enhanced considerably the cytotoxic effect of doxorubicin and induced a dramatic decrease in the viable cell fraction over a wide DOX concentration domain ***(Fig. 1A)***. The bivariate density plots obtained by flow cytometry on CM-H2DCFDA/7-AAD double stained cells (e.g., Fig. 1B) indicated that cell death induced by DOX, QC/MD and their combinations is preceded by oxidative stress generation and that all three agents enhance dose-dependently the cellular red autofluorescence, probably originating from oxidized cytochromes and porphyrines.

The cytotoxic effect of DOX was associated with cell cycle arrest in different cell cycle phases, depending on the dosage. Thus, untreated cells displayed G0/G1, S and G2/M, cell fractions of 49%, 33% and 18%, respectively, whereas exposure to 100 nM DOX for 18 h induced a consistent G2/M arrest, with 63%, 29% and 8% of the cells detected in the G2/M, S and G0/G1 phase, respectively ***(Figs. 2, 3)***. Addition of equimolar QC/MD combinations reduced progressively the G2/M arrest and augmented the pre-replicative cell percentage. Furthermore, addition of QC and MD up to 7.5 μM each concomitantly increased the S-cell fraction ***(Fig. 2, 3)***.

A higher level of doxorubicin (1000 nM) induced instead a considerable S-phase blockage (16%, 73% and 11% G0/G1, S and G2/M cell fractions, respectively; Figs. 2, 3), as well as a significant sub-G0/G1 population, indicating an increased number of apoptotic cells. Adding QC and MD to the treatment up to 2.5 µM each led to a very high percentage (89%) of S-phase arrested cells (Fig. 2). Exceeding these doses, the cell cycle arrest was gradually attenuated and, in the presence of 15 µM QC/15 µM MD, the cell cycle distribution approached the normal profile observed with untreated cells ***(Fig. 2)***.

In conclusion, addition of equimolar QC/MD up to 7.5 µM to 100 nM DOX and up to 2.5 µM to 1000 nM DOX increased the S-cell fraction, whereas higher levels apparently inhibited the cell cycle arrest. However, additional measurements based on the Annexin V-FITC/PI apoptosis assay indicated that these severe DOX/QC/MD treatments induced high apoptotic rates associated with unaltered cell cycle distributions (not shown), suggesting that cell cycle progression was stopped in all of its phases, and that apoptosis was triggered following persistent growth arrest in cell cycle phases carrying irreparable lesions.

In particular, high levels of DOX (1000 nM) triggered a significant apoptotic rate, measured by the Annexin V-FITC/PI assay, within 18 h, but not 6 h of exposure ***(Fig. 4)***. In comparison, the 15 µM equimolar QC/MD combination produced high apoptotic rates both at 6 h and 18 h after exposure, suggesting a fast progression along the apoptotic pathway (Fig 4). Association with DOX significantly increased the apoptotic cell fraction at both sampling times ***(Fig. 4)***.

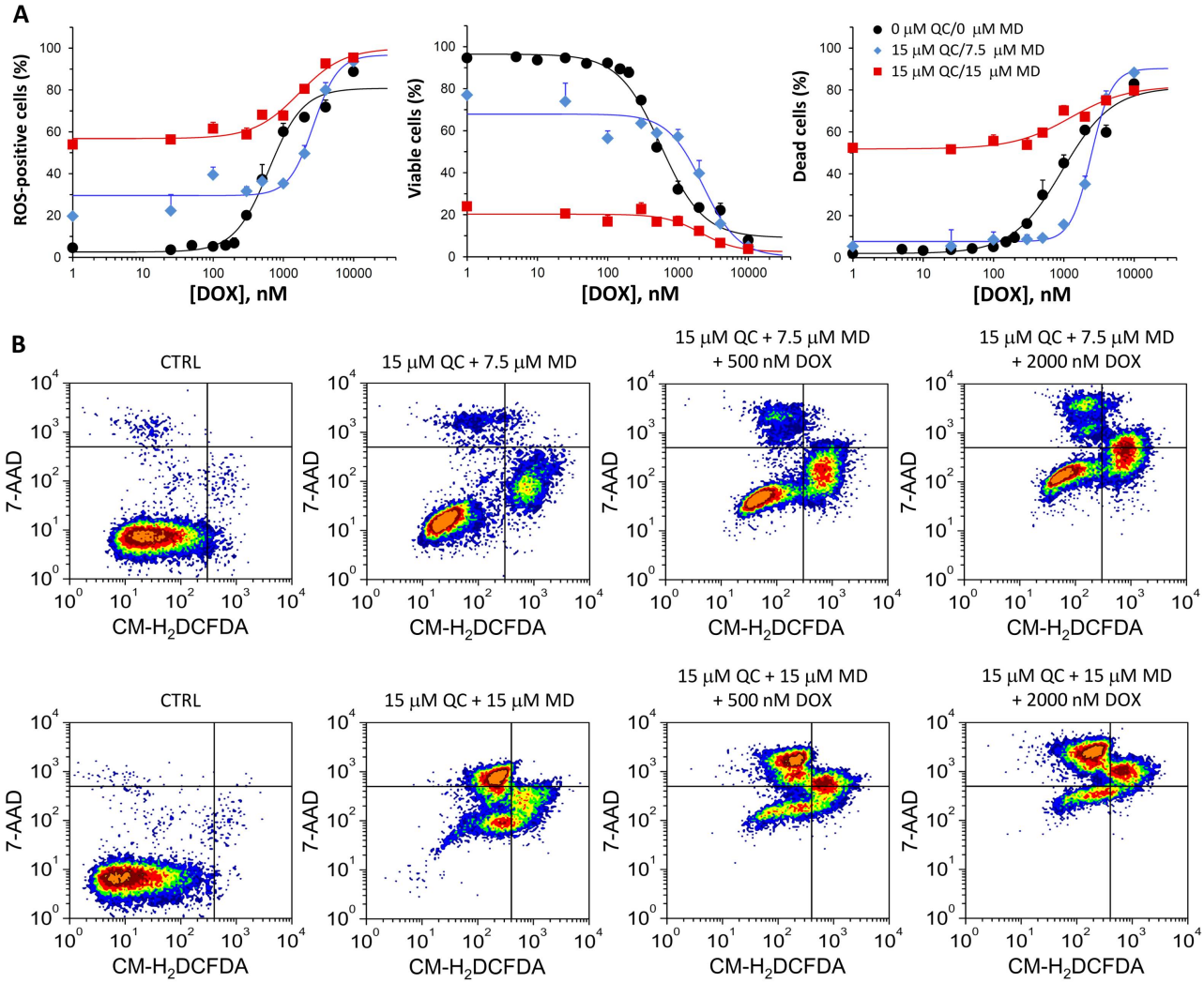

Fig.1. Oxidative stress generation and cytotoxicity induced in Jurkat cells by doxorubicin alone or in association with QC and MD at indicated doses for 18 h. (A) Dose-dependent effects of DOX in the absence or presence of QC/MD. ROS-positive and dead cells denote cells positive for CM-H2DCFDA and 7-AAD, respectively. Viable cells represent cells negative for both CM-H2DCFDA and 7-AAD. (B) Typical bivariate plots obtained by flow cytometry on CM-H2DCFDA/7-AAD double stained cells.

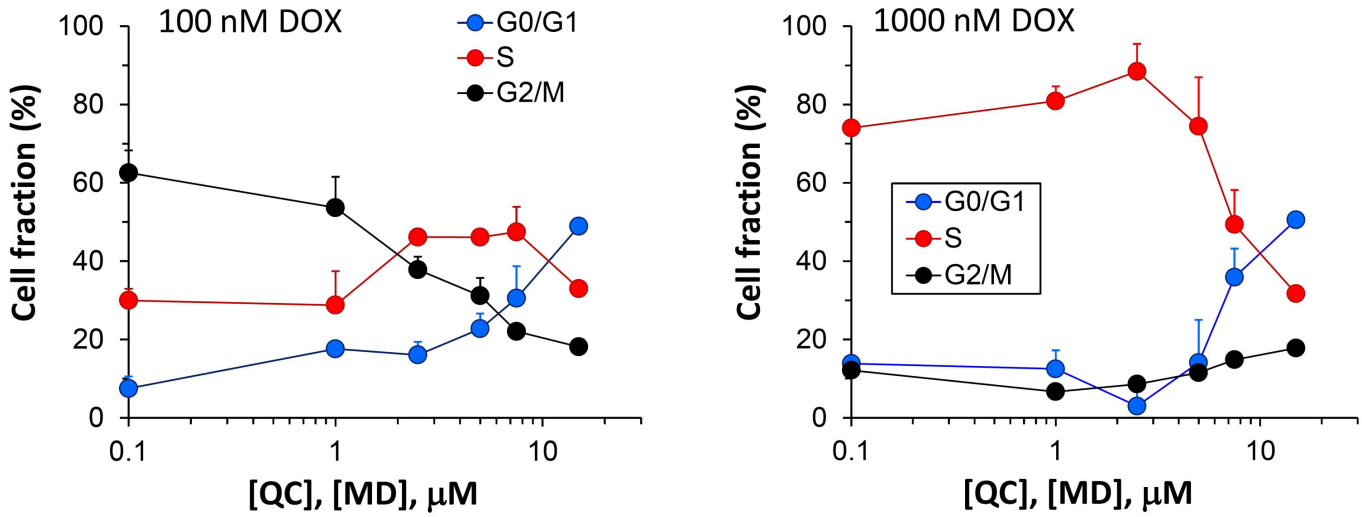

Fig.2. Cell cycle distribution of Jurkat cells exposed for 18 h to 100 nM (left) or 1000 nM (right) DOX, in the presence of equimolar QC/MD combinations at indicated doses.

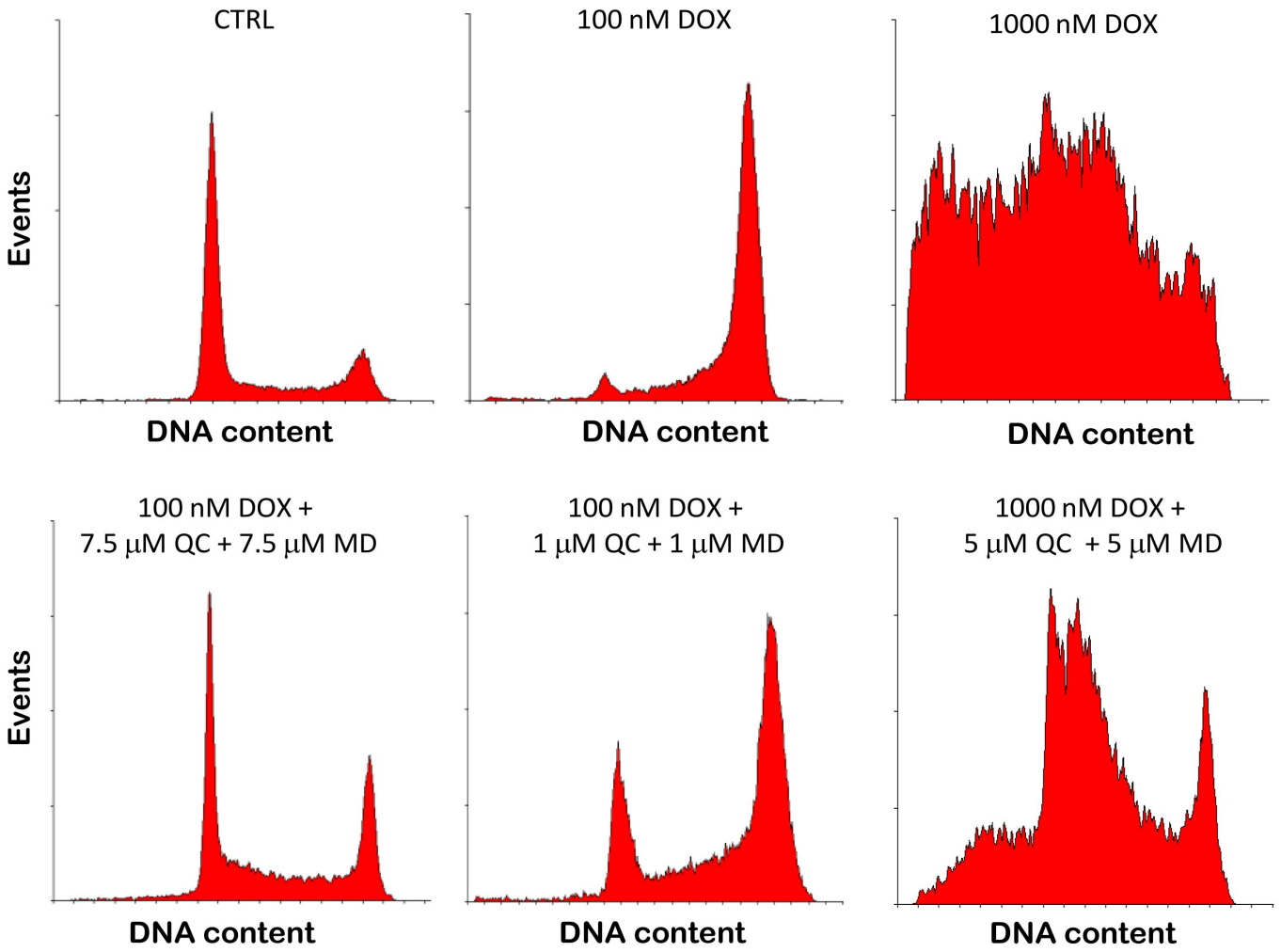

Fig.3. Representative histograms of the cellular DNA content after 18 h treatments with indicated drugs.

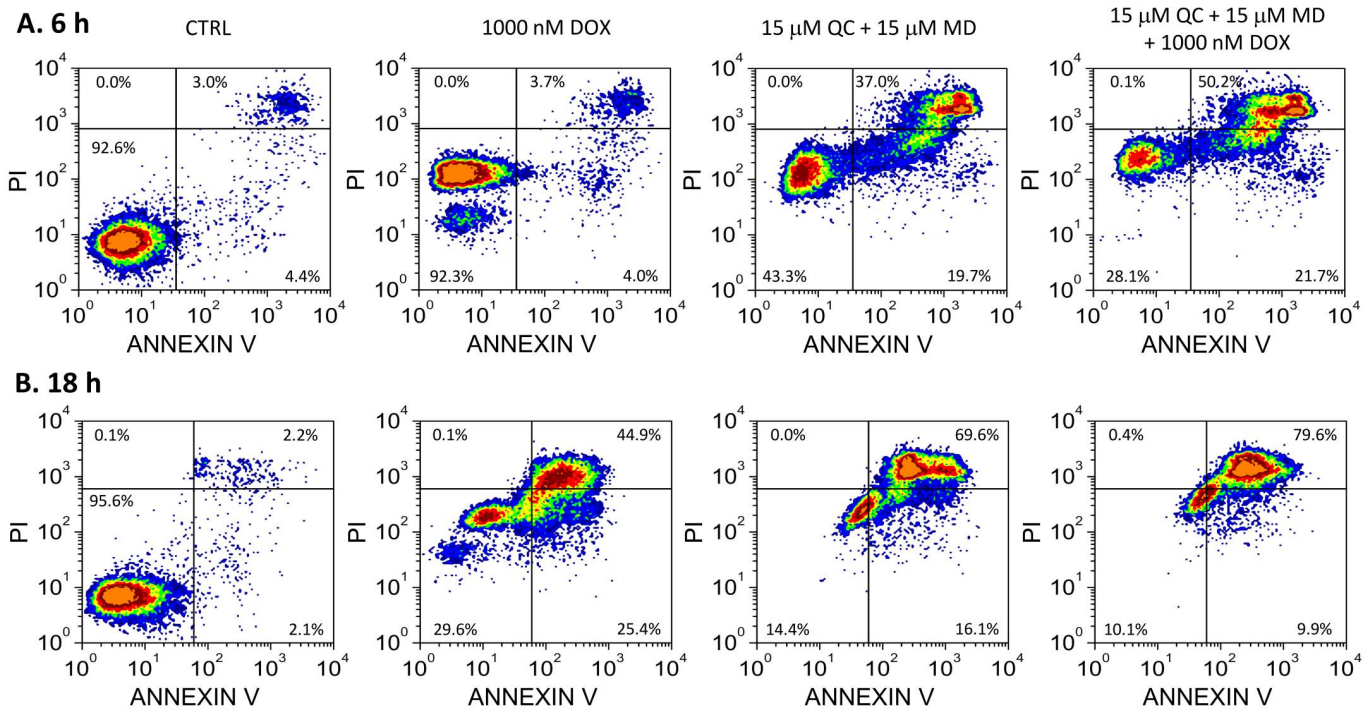

Fig.4. Apoptosis induction determined by the Annexin V/PI assay after exposure for 6 h (A) or 18 h (B) to indicated drugs.

Discussion

Current data suggest that the inclusion of the QC/MD combination in doxorubicin-based treatment schemes for leukemia could improve the growth-suppressive effect of the therapeutic drug by promoting cell cycle arrest, oxidative stress generation and apoptosis induction, in a dose dependent manner.

The primary mechanisms responsible for the cytotoxic effect of doxorubicin are generally recognized to be the formation of DNA adducts leading to topoisomerase II inhibition, and oxidative stress generation with subsequent release of intracellular reactive oxygen species. Doxorubicin's cardiotoxicity is most likely due to the latter mechanism, which may be enhanced considerably by the high mitochondrial density in cardiomyocytes and the high affinity for cardiolipin exhibited by doxorubicin.

Quercetin generally exhibits both a pro-oxidant and an antioxidant character, with a primarily antioxidant effect exerted on healthy cells and a major pro-oxidant effect in neoplastic cells. This dual behavior could not only prevent healthy cell injury promoted by doxorubicin treatment, but also play a significant role as a protective agent against doxorubicin-induced cardiac toxicity.

A relevant effect of doxorubicin observed in our current investigations at low doses of DOX (100 nM) consisted in G2/M phase cell cycle arrest, likely determined by topoisomerase II inhibition and oxidative DNA injury. When using higher doxorubicin doses (1000 nM), S-phase arrest was detected, probably due to DNA breakage and ATM (ataxia telangiectasia mutated) activation. High levels of doxorubicin also induced consistent apoptotic rates in Jurkat cells. QC/MD equimolar combinations at physiological levels maintained the DOX-induced cell cycle arrest and enhanced DOX-induced apoptosis.

In conclusion, this study shows that doxorubicin induces apoptosis, cell cycle arrest and oxidative stress in a dose-dependent manner in human leukemia Jurkat T cells. The cytotoxic effects of DOX and QC/MD at clinically relevant levels appear to be additive, which could allow for a reduction of the therapeutic doses of doxorubicin.

Acknowledgments

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS - UEFISCDI, project number PN-II-ID-PCE-2011-3-0800. Work for I.T.T. was supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/187/1.5/S/156069. Work for R.G.S., O.E.B., V.C. and M.T.I. were supported by four Young Researchers Fellowships of the Romanian

Ministry of Education, UEFISCDI, project numbers 6/2014, 8/2015, 6/2015 and 7/2015, respectively. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abbreviations

CM-H2DCFDA: 5-(and-6)-chloromethyl-2',7'dichlorodihydrofluorescein diacetate, acetyl ester
DMSO: dimethyl sulfoxide
DOX: doxorubicin
FITC: fluorescein isothiocyanate
MD: menadione (2-methyl-1,4-naphthoquinone)
NO: nitric oxide
PBS: phosphate buffer saline
PI: propidium iodide
QC: quercetin
ROS: reactive oxygen species
SS: standard saline
TriX: Triton X-100
7-AAD: 7-Aminoactinomycin D

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