EFFECTS OF MENADIONE AND QUERCETIN ON APOPTOSIS AND DELAYED LUMINESCENCE OF HUMAN LEUKAEMIA JURKAT T CELLS

Irina Baran[a,*], Constanta Ganea[a], Agata Scordino[b,c], Francesco Musumeci[b,c], Salvatore Tudisco[b,c], Simona Privitera[b,c], Rosaria Grasso[b,c], Vincenza Barresi[d], Daniele F. Condorelli[d], Virgil Baran[e], Eva Katona[a], Maria-Magdalena Mocanu[a], Marisa Gulino[b,c], Mihaela Surcel[f], Cornel Ursaciuc[f]

a) "Carol Davila" University of Medicine and Pharmacy, Dept. of Biophysics, 8 Eroii Sanitari, Bucharest, Romania
b) Istituto Nazionale di Fisica Nucleare – Laboratori Nazionali del Sud, 62 S. Sofia, Catania, Italy
c) Dip. di Metodologie Fisiche e Chimiche per l'Ingegneria, Università di Catania, I-95125 Catania, Italy
d) Dip. di Scienze Chimiche, Università di Catania, 6 A. Doria, I-95125 Catania, Italy
e) Faculty of Physics, University of Bucharest and NIPNE-HH Bucharest, Romania
f) "Victor Babes" National Institute, Dept. of Immunology, 99-101 Spl. Independentei, Bucharest, Romania
**Corresponding author, e-mail: baran@ifin.nipne.ro*

Abstract

Menadione (vitamin K_3), a redox cycling quinone, is an effective cytotoxic drug used for the treatment of leukaemia. Quercetin is a natural flavonoid with both antioxidant and pro-oxidant properties. We investigated the effects of menadione and quercetin on apoptosis induction and delayed luminescence (DL) of human leukaemia Jurkat T cells following treatment with various doses of the oxidant agent menadione, in the presence or absence of the bioflavonoid at different concentrations. A consistent decrease in DL intensity is observed in both menadione- and/or quercetin-treated cells, suggesting that superoxide anions and inhibition of the mitochondrial electron transport chain alter significantly the DL emission in this cell type. Quantification of these effects can help answer the question on the origin of DL, which at the moment is still a matter of debate.

INTRODUCTION

Menadione (vitamin K_3), a redox cycling quinone, is a clinically important chemotherapeutic agent, used as an effective cytotoxic drug for the treatment of leukaemia [1]. Menadione (Men) can produce intracellularly large amounts of superoxide ($O_2^{-\bullet}$) by redox cycling reactions [2], and can also inhibit the Complex I of the mitochondrial respiratory chain [3]. Quercetin (Que) is a natural flavonoid with both antioxidant and pro-oxidant properties [4]. It is known that quercetin can transfer one electron to $O_2^{-\bullet}$ by forming stable aroxyl radicals [5]. Scavenging of oxygen reactive species such as $O_2^{-\bullet}$ or $OH^{\bullet}$ by low levels of quercetin can protect cells from oxidative injury [6-8], however high concentrations of the flavonoid appear to enhance production of free radicals (especially $O_2^{-\bullet}$ and $OH^{\bullet}$) and display cytotoxic effects [4, 7, 8]. Quercetin can also inhibit the Complexes I and III of the mitochondrial respiratory chain [9], and participate in quinone redox cycling through enzymatic activation of quercetin in reversible oxido-reduction processes [10]. Consequently, both menadione and quercetin can lead to consistent intracellular production of oxygen reactive species while inhibiting the electron transport chain within the mitochondria.

In this study we investigate the effects of menadione and quercetin on apoptosis induction and delayed luminescence (DL) of cells. Quantification of these effects can help answer the question on the origin of DL, which at the moment is still a matter of debate [11-14]. It has been suggested that one cause for DL in living cells is the presence of free radicals which form in oxidation reactions [12, 15, 16].

MATERIALS AND METHODS

Cell cultures

Human Jurkat T cell lymphoblasts were cultured in suspension in MegaCell RPMI 1640 medium (Sigma M3817) supplemented, according to the manufacturer's instructions, with 5% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Cells in logarithmic phase were brought to 0.15×10^6 cells/ml and pre-incubated with quercetin for 24 hours or 1 hour, as indicated. We used menadione sodium bisulphite (Sigma M2518) dissolved in phosphate buffer saline (PBS) at 100 mM and stock solutions of dihydrated quercetin (Sigma Q0125) prepared in DMSO (up to 24 mg/ml). For each treatment, a certain volume of these solutions was added to the cell culture to reach the specified final concentration. Non-treated (control) cells were prepared in the same manner, except the addition of quercetin or menadione. The final concentration of the vehicle DMSO in all cell cultures was 0.1%. Menadione treatments were conducted for either 20 min. or 4 hours, with the culture flasks kept inside the incubator. After the treatment, cells were collected by centrifugation, washed with PBS and resuspended either in PBS (for DL samples, $\sim 50 \times 10^6$ cells/ml) or in complete medium (for further culturing and

apoptosis evaluation, ~0.2 × 10^6 cells/ml). DL cell samples were analyzed immediately by DL spectroscopy. In parallel, 25 μl- aliquots of the DL samples were diluted with PBS, stained with 0.4% trypan blue solution and counted on a Burker haemocytometer. DL emission intensity was always normalized with respect to the number of living cells in the sample, and the PBS background emission was subtracted from all DL data.

Flow cytometry

24 h and 48 h after the treatment, samples containing 10^6 cells were fixed in 70% ethanol and kept at -20°C until flow cytometer (FC) determinations. Propidium iodide (PI) staining was performed using a PI/RNAse staining buffer (PHARMINGEN 550825). The ethanol-fixed samples were washed with PBS and incubated with the staining buffer for 30 minutes at 37ºC in the dark. Cells were then analyzed for DNA content by using a Becton Dickinson FACS Calibur flow cytometer, and the CellQuestas data acquisition software. The freeware WinMDI 2.8 was used for raw data analysis, and the histogram deconvolution software Cylchred for cell cycle distribution quantization. Percentages of cell populations in various cell cycle phases were calculated as averages of values given by the WinMDI software with marker defined histogram regions, and of values given by the deconvolution software Cylchred. Apoptosis was evaluated as the fraction of hypoploid cells [6], corresponding to the cells with fragmented DNA content (the sub-G_0 cell fraction).

Delayed Luminescence Spectroscopy

In order to measure the DL of cell cultures an improved version of the ARETUSA set-up was used [14]. This highly sensitive equipment is able to detect single photons and has a very low background signal; moreover it presents a high efficiency in collecting the luminescence originating from the cell sample, and a low delay time (11 μs) between the end of the illumination pulse and the beginning of signal acquisition.

The 25 μl- DL cell samples were excited by a Nitrogen Laser source (Laser Photonics LN 230C), characterized by a wavelength λ = 337 nm, a 5 ns pulse width, an energy of 100 ± 5 μJ/pulse. The laser power was reduced, in some cases, to prevent the dimpling of the photomultiplier. A multi-alkali photomultiplier tube (PMT) (Hamamatsu R-7602-1/Q), selected for single photons counting, was used as a detector. The detected signals were acquired by a Multi-channel Scaler (MCS) (Ortec MCS PCI) able to collect analogical or logical signals as a function of the time with a minimum dwell time of 200 ns.

RESULTS AND DISCUSSION

We determined the effects of menadione and quercetin on cell cycle progression and apoptosis induction. We observed that a 24 h-treatment with 5 μM quercetin only slightly increases the apoptotic cell fraction, whereas 50 μM quercetin induces apoptosis in about 80% of the cells and a slight blockage of the cell cycle in the G_0/G_1 phase (Fig. 1). We obtained a very good agreement (not shown) with previous data regarding the apoptotic cell fraction (~45%) assessed shortly after the 24 h- treatment with 50 μM Que [17]. In addition, our results are also consistent with previous reports on the cytotoxicity of quercetin in Jurkat T cells, which is characterized by an IC_{50} ~8 μM for treatment durations ≥ 24 hours [4, 17, 18].

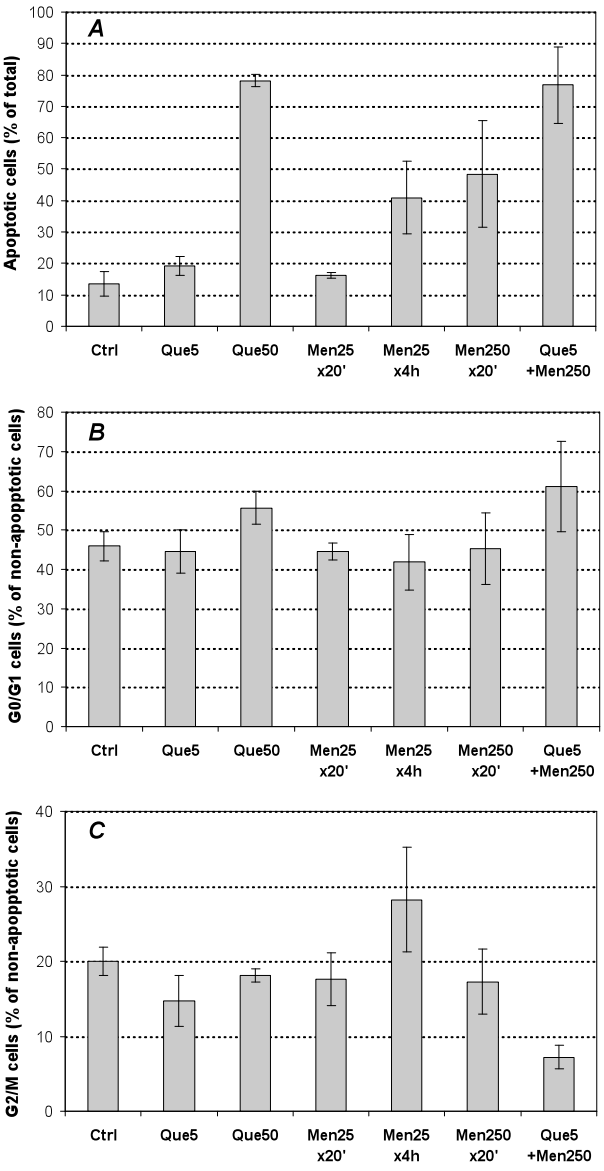

Figure 1: Apoptosis induction (*A*) and fractions of G_0/G_1 cells (*B*) or G_2/M cells (*C*) after treatments with 5 or 50 μM quercetin (Que5, Que50) for 24 h, menadione for 20 min. or 4 h (Men25: 25 μM, Men250: 250 μM), or a combined treatment (5 μM Que for 24 h, followed by 250 μM menadione treatment for 20 min.; last column). Data are represented as average and standard deviation of at least three measurements performed at 24 h and 48 h after the treatment.

Low concentrations of menadione (25 μM) applied for short times (20 min.) had no significant effect on

apoptosis or the cell cycle (Fig. 1), in agreement with other reports [19, 20], whereas in the case of longer treatment duration (4 h) the apoptotic cell fraction increased to 52% at 48 h after the treatment (not shown), and there was a partial block in the G_2/M phase of the cell cycle (Fig. 1), indicating the presence of DNA damage and activation of the G_2/M checkpoint. Other studies indicated that exposure of Jurkat T lymphoblasts to 25 μM menadione for 4 h leads to depression of the mitochondrial transmembrane potential (an early step in apoptosis) in about 70% of the cells [20], which is consistent with our apoptosis data.

Menadione at very high concentration (250 μM for 20 min.) induced apoptosis in 38% and 68% of the cells when assessed at 24 h and 48 h after the treatment, but did not affect the cell cycle (Fig. 1). It has been shown previously that high levels of menadione (100 μM) can induce immediately (20 min. after the exposure) the collapse of the mitochondrial membrane potential in about 70% of the cells [20], in agreement with our results.

Pre-treatment with 5 μM quercetin for 24 h enhanced apoptosis induced by 250 μM menadione and partially blocked the cell cycle in G_0/G_1 (Fig. 1), whereas the G_2/M cell fraction decreased considerably, suggesting that cells with aberrant division pass to G_1 and initiate apoptosis.

We next investigated the DL emission of Jurkat cells exposed to menadione and/or quercetin. We observed that light emission of quercetin-treated cultures is lower than in not treated cultures, and the effect is more pronounced at increasing concentrations of the flavonoid (Fig. 2).

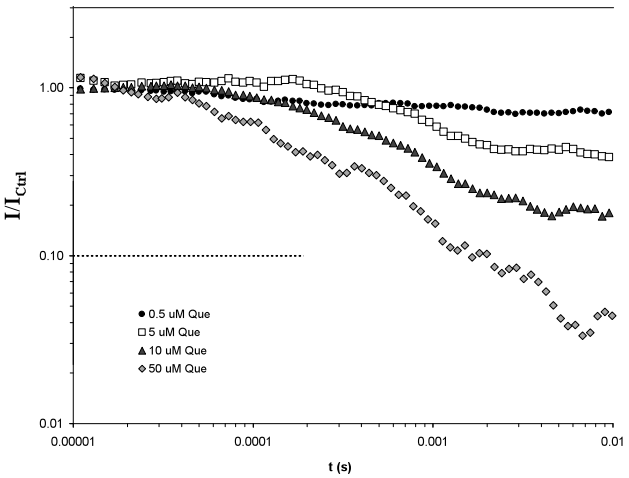

Figure 2: Time variation of DL intensity of cell cultures treated with different doses of quercetin. Intensity of light emission (I, rate of photon emission) is normalized with respect to the DL intensity of non-treated cultures (I_{Ctrl}). Data are obtained by average of values from at least 4 different experiments.

DL emission of menadione-treated cells is also reduced as compared with emission of control cells (Fig. 3). A mild stress induced shortly by a low level of menadione (25 μM for 20 min.) affects DL emission up to about 200 μs only, at variance with the case of a more severe oxidative stress induced by 25 μM menadione for 4 h, where DL emission between 200 μs and 10 ms is dramatically reduced up to about 7% of control emission. A similar behaviour was observed after a severe oxidative stress induced in short time with a high dose of menadione (250 μM for 20 min.); in addition, light emission up to 200 μs was even more decreased than under a milder menadione-stress. There were no significant differences between DL emission in the range 200 μs - 10 ms of cells treated with 25 μM menadione for 4 h or with 250 μM menadione for 20 min., with or without pre-incubation with 0.5 μM quercetin (Fig. 3). A further reduction in DL emission was observed in 5 μM quercetin pre-treated cells, whereas pre-incubation with 0.5 μM quercetin led to partial recovery of DL emission up to about 100 μs.

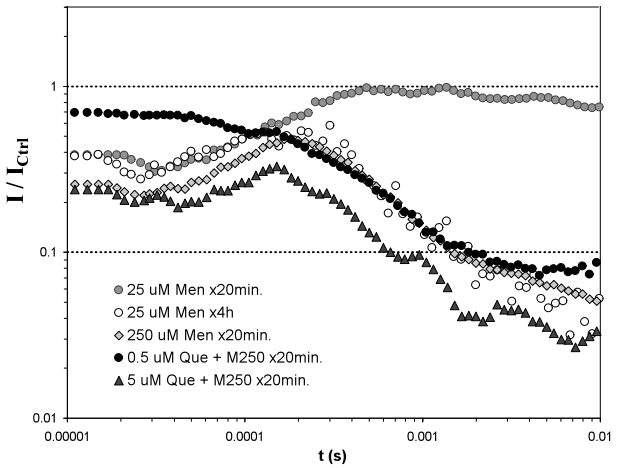

Figure 3: Time variation of DL intensity of cell cultures exposed to menadione-induced oxidative stress, with or without pre-treatment with quercetin for 24 hours (other details as in Fig. 2).

In Fig. 4 we summarize the main DL characteristics for all the quercetin/menadione treatments discussed above. Two different regions in the DL decay curve appear to display distinct features (Figs 2, 3). The DL-I region, with photon emission in the time range 11 μs – 200 μs, was partially affected by quercetin only at very high levels, and was substantially reduced by menadione even at low concentrations (Fig. 4) . Interestingly, 25 μM menadione produced a fairly even reduction of the DL-I intensity (~55%), irrespective of the treatment time. In addition, a partial recovery was observed in cells pre-incubated either with 0.5 μM Que for 24 h or with 10 μM Que for 1 h (both antioxidant treatments [8, 21]), suggesting that DL-I emission could be anti-correlated with the intracellular $O_2^{-\bullet}$ level. In favour of this interpretation, our investigations using a strong H_2O_2-oxidative stress indicated a very weak reduction (< 20%) of DL-I emission (not shown), ruling out the involvement of the intermediates products H_2O_2 and $OH^\bullet$, which are easily produced through dismutation of $O_2^{-\bullet}$ to H_2O_2 by cytosolic and mitochondrial superoxide dismutases, and through Fenton reactions, in which $OH^\bullet$ is produced from H_2O_2. Moreover, previous studies reported that quercetin produces noticeable quantities of $O_2^{-\bullet}$ and $OH^\bullet$ in human

normal lymphocytes at levels of 50 μM or higher [21]. It is then conceivable that in our experiments incubation of Jurkat T cells with 50 μM quercetin for 24 h produced only moderate quantities of $O_2^{-\bullet}$ and thus decreased the DL-I emission by 30% (Fig. 4).

The DL-II region, with emission between 200 μs and 10 ms, was considerably altered by all the treatments (either anti-oxidative or pro-oxidative), except the mild menadione-stress (25 μM menadione for 20 min.), indicating that DL-II emission is not correlated with the intracellular level of superoxide anions. A dose-dependent response was observed in quercetin-treatments (Fig. 4). Moreover, both quercetin- and menadione-treated cells displayed a remarkably similar trend of the time-dependent decay within the region II (Figs. 2, 3), suggesting a common working mechanism. The most likely interpretation of our results is that DL-II emission on the millisecond scale is reduced, directly or indirectly, by inhibition of the mitochondrial electron transport chain, which can be induced by both quercetin and menadione. Earlier works described a similar effect of electron transport inhibitors on light emission from mitochondria [22] and chloroplasts [23, 24].

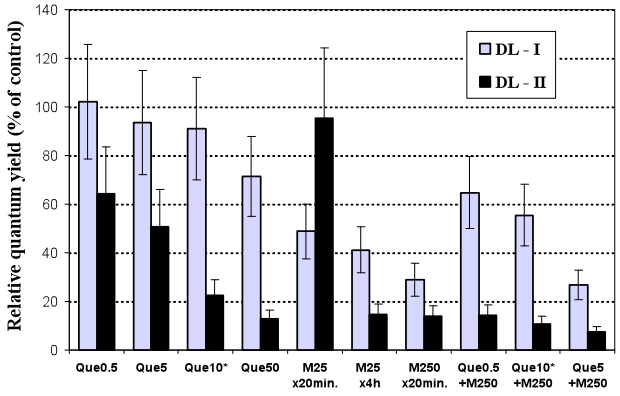

Figure 4: Quantum yield (total no. of emitted photons) of treated cells relative to control cell emission. DL-I: DL emission in the range 11 μs – 200 μs; DL-II: 200 μs – 10 ms. Que10*: treatment with 10 μM quercetin for 1 h; other quercetin treatments: 24 h. Data are represented as average and standard deviation of values from at least 4 different experiments.

CONCLUSIONS

We have investigated the relation between delayed luminescence and the effects of menadione, a potent intracellular generator of superoxide anions, and the bioflavonoid quercetin on apoptosis induction and cell cycle progression in human leukaemia Jurkat T lymphoblasts. The effects of both drugs on DL emission are significant. Delayed light emission on the 10 μs - 200 μs scale appears to be anti-correlated with the intracellular level of superoxide anions. A strong reduction of delayed light emission was observed within the interval 200 μs – 10 ms after sample excitation in quercetin and/or menadione treatments, irrespective of the antioxidant or pro-oxidant character of the treatment, suggesting the involvement of the mitochondrial respiratory chain as an important source of DL emission on a millisecond scale.

Our apoptosis/proliferation data come in favour of other recent studies that point to the need to accurately define the safe concentration domain of the daily quercetin uptake, since elevated levels of quercetin in the blood plasma may be highly toxic. In this work, a 24 hours- treatment with a relatively low level (5 μM) of quercetin enhanced apoptosis induced by menadione in Jurkat T cells. While this could represent a significant advantage in treating leukaemia by a quercetin/menadione combination, it is important that normal cells remain unexposed to toxic levels of flavonoids. However, it is known that malignant cells are more susceptible than normal cells to quercetin cytotoxicity [17, 21], which could therefore represent an additional benefit in a flavonoid-combined treatment used in leukaemia therapy or in cancer prevention.

ACKNOWLEDGEMENTS

This work was partially supported by the Romanian Ministry of Education and Research under the CNCSIS Grant PNII-Ideas No. 1138/2009.

REFERENCES

[1] Laux I., Nel A. 2001. *Clinical Immunology* 101: 335-344
[2] Jamison J.M. et al. 2002. *Biochem. Pharmacol.* 63: 1773-1783
[3] Xu X., Arriaga E.A. 2009. *Free Radical Biol. Med.* 46: 905-913
[4] Rao Y.K. et al. 2007. *Food Chem. Toxicol.* 45: 1770-1776
[5] Furuno K. et al. 2002. *Biol. Pharm. Bull.* 25: 19-23
[6] Kim B.M. et al. 2009. *Toxicol. Appl. Pharmacol.* 239: 87-97
[7] Wilms L.C. et al. 2008. *Toxicology in Vitro* 22: 301-307
[8] Johnson M.K., Loo G. 2000. *Mutation Res.* 459: 211-218
[9] Dorta D.J. et al. 2005. *Chem.-Biol. Interact.* 152: 67-78
[10] Metodiewa D. et al. 1999. *Free Radical Biol. Med.* 26: 107-116
[11] Popp F.A. et al. 1984. *Cell Biophys.* 6: 33-52
[12] Slawinski J. 1988. *Experientia* 44:559-71
[13] Scordino A., Triglia A., Musumeci F. 2000. *J. Photochem. Photobiol.* B 56: 181-186
[14] Tudisco S., Scordino A., Privitera G., Baran I., Musumeci F. 2004. *Nucl. Instr. Meth. Phys. Res. A* 518: 463-464
[15] Rajfur Z. 1994. *J. Biolumin. Chemilumin.* 9: 59-63
[16] Radotic K. et al. 1998. *Gen. Physiol. Biophys.* 17: 289-308
[17] Chen D. et al. 2005. *Biochem. Pharmacol.* 69: 1421-1432
[18] De Vincenzo R. et al. 2000. *Cancer Chemoter. Pharmacol.* 46: 305-312
[19] Dumont A. et al. 1999. *Oncogene* 18: 747-757
[20] Godar D.E. 1999. *J. Invest. Dermatol.* 112: 3-12
[21] Yen G.-C. et al. 2003. *Biosci. Biotechol. Biochem.* 67: 1215-1222
[22] Hideg E. et al. 1991. *Biochim. Biophys. Acta – Bioenergetics* 1098: 27-31
[23] Felker P. et al. 1973. *Biochim. Biophys. Acta – Bioenergetics* 325: 193-196
[24] Katsumata M. et al. 2008. *J. Photochem. Photobiol. B.* 90: 152-162