Scientific report

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Detailed analysis of apoptosis and delayed luminescence of human leukemia Jurkat T-cells after proton-irradiation and treatments with oxidant agents and flavonoids

Introduction. As we have already shown in the first phase of the project, epigallocatechine-3-gallate (EGCG) and quercetin (QC; 3,5,7,3',4'-pentahydroxyflavone) are two well-investigated flavonoids which inhibit cell proliferation and induce apoptosis in various cancer cell types [1-6]. Continuing our previous studies concerning the effects of these two compounds on the human leukemia Jurkat-T [7], we have investigated into more detail the relationship between apoptosis and delayed luminescence (DL) in this cell type, following various treatments. In order to induce the oxidative stress we have used menadione (MD) and hydrogen peroxide (H_2O_2) as well as the two flavomoids, QC and EGCG, applied alone or in combination with MD or H_2O_2. Because in the therapy of various cancer types there are often used combined treatments, drugs-radiations, 62 MeV proton beams were used to irradiate cells under a uniform dose of 2 or 10 Gy, respectively. We have assessed the apoptosis, the cell cycle distributions and the delayed luminescence. We have shown that menadione, H_2O_2 and quercetin were potent inducers of apoptosis and DL inhibitors. Quercetin decreased clonogenic survival and the NAD(P)H level in a dose-dependent manner. Proton-irradiation with 2 Gy but not 10 Gy increased the apoptotic rate. However, both doses induced a substantial G_2/M arrest. Quercetin reduced apoptosis and prolonged the G_2/M arrest induced by radiation. DL spectroscopy indicated that proton-irradiation disrupted the electron flow within Complex I of the mitochondrial respiratory chain, thus explaining the massive necrosis induced by 10 Gy of protons, and also suggested an equivalent action of menadione and quercetin at the level of the Fe/S center N2, which may be mediated by their binding to a common site within Complex I, probably the rotenone binding site.

Materials and methods. The method has been described into detail in the scientific report *in extenso* of the project, as well as in the publication resulted from the present study [8]. In short, we have performed measurements of cellular viability with trypan blue solution, and the apoptosis and the cell cycle have been assessed with a flow cytometer, for cells incubated with a propidium iodide. The measurements have been performed on cell cultures of human leukemia Jurkat T-cell lymphoblasts in suspension, in MegaCell RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37ºC in a humidified incubator with a 5% CO_2 atmosphere. ***Proton-irradiation.*** Cell suspensions (7 ml) were irradiated in 50 ml- centrifuge tubes fixed in a vertical position. 62 MeV proton beams accelerated by the superconducting cyclotron at LNS-INFN, Catania (Italy) were used for proton-irradiation at a dose rate of 11.76 Gy/min. A plane-parallel advanced PTW 34045 Markus ionization chamber was adopted as a reference dosimeter. ***Delayed luminescence spectroscopy.*** We used an improved version of the ARETUSA set-up [39], a highly sensitive equipment able to detect single photons. The cell samples were excited by a Nitrogen Laser source (Laser Photonics LN 230C; wavelength 337 nm, pulse-width 5 ns, energy 100 ± 5 μJ/pulse). A multi-alkali photomultiplier tube (Hamamatsu R-7602-1/Q) was used as a detector for photoemission signals with wavelengths in the visible range (VIS, 400-800 nm), in single photon counting mode. ***Spectrofluorimetry*****.** For determination of the relative level of intracellular nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate in their reduced form (NADH and NADPH, respectively), denoted generically as NAD(P)H, exponentially growing cells were washed twice in a standard saline solution (SS) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, pH 7.2/NaOH, resuspended in SS at ~10^6 cells/ml and transferred into a 2 ml quartz cuvette maintained at 37ºC under continuous stirring in a Horiba Jobin Yvon spectrofluorimeter. Every 22 s the cell sample was excited at 340 nm and emission was collected at 450 nm. After stabilization of the fluorescence signal, QC at the indicated dose was added directly to the cuvette and the kinetic recording was carried on for an additional 45-60 min. ***Statistics.*** Unless indicated otherwise, the data are presented as median ± s.e.m. of at least three different measurements.

Statistically significant differences were determined using Student's *t*-test. A level of $P < 0.05$ was considered significant in all statistical tests. Other details in [8].

Results and discussion

Effects of proton radiation, MD, H_2O_2, QC and EGCG on apoptosis and cell cycle

Continuing a previous preliminary study, we performed more extensive measurements and we have done a more detailed analysis of the experimental results. We have firstly assessed the apoptosis and the cell cycle distributions in the Jurkat cells under various treatments. In agreement with our previous investigations [7, 10], quercetin induced apoptosis in Jurkat cells in a dose- and time-dependent manner, while 0.5 μM EGCG applied for 24 h did not affect the apoptotic rate or cell cycle distribution, but could enhance the apoptosis induced by MD or H_2O_2. Consistently with the already mentioned studies, in the present study we have found that proton irradiation with high energy protons of 2 Gy but not with 10 Gy produced a significant increase of the apoptotic rate at 48 hr. after irradiation. Trypan blue exclusion tests confirmed high rates of cell death, namely 18.4 ± 3.2% and 46.6 ± 6.8% at 24 h and 48 h after irradiation with 10 Gy of protons, respectively.

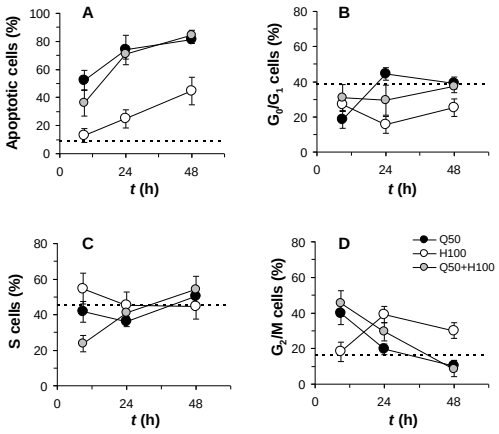


Fig. 1. Time course of the apoptotic rate and cell-cycle distribution after treatment of Jurkat cells with 50 μM QC for 24 h (Q50, solid circles), 100 μM H_2O_2 for 20 min. (H100, open circles) or combination of the two (50 μM QC pre-incubation followed by addition of 100 μM H_2O_2 for 20 min.; treatment denoted as Q50 + H100, gray circles). Apoptotic rates (**A**), G_0/G_1 (**B**), S-phase (**C**) and G_2/M (**D**) cell fractions are indicated. The dashed line represents the average obtained from control cell samples.

From kinetic measurements one can notice that quercetin can arrest the Jurkat cells in the G_2/M phase (Fig. 1). Moreover, the G_2/M cell fraction in the case of the cells treated with 50 μM QC for 24 h decreased from 39.8 ± 6.4% at 9 h, to 10.3 ± 3.0% at 48 h after the treatment (Fig. 1D). The G_2/M block was associated with a significant reduction in the G_0/G_1 cell fraction (Fig. 1B), whereas the S-phase distribution was unaltered (Fig. 1C). The cells also displayed a consistent apoptotic rate (52.2 ± 7.3%) 9 h after drug removal, which then increased gradually up to 81.5% during the probing interval (Fig. 1A).

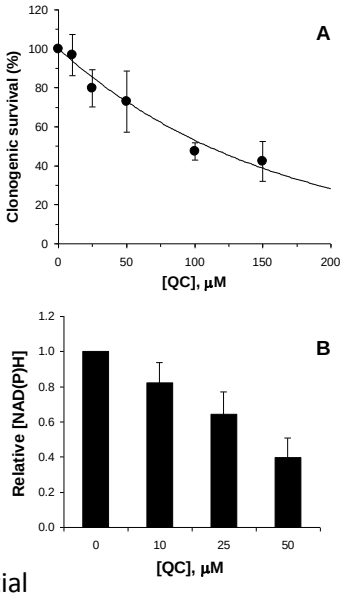
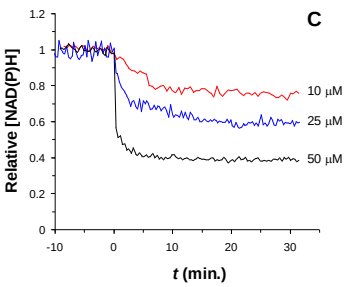


Fig. 2. Quercetin decreases clonogenic survival and the cellular content of NAD(P)H in Jurkat cells. (**A**) Dose-response of clonogenicity (*S*) was fitted to an exponential function (curve) of the form S (%) = 100 × exp(-D/D_0), where D represents the dose of QC applied for 1 h and the characteristic dose derived from the fit was D_0 = 158.5 μM. Data are expressed as mean ± standard deviation of 4 - 6 separate determinations. (**B**) The ratio between NAD(P)H fluorescence of treated vs. control cells (relative NAD(P)H) obtained in steady state after addition of QC to cell suspensions decreases with the level of QC. (**C**) Representative recordings of NAD(P)H fluorescence relative to the resting value in cell suspensions before and after addition of QC at various levels indicated near each trace.

In treatments with duration of 1 h, QC decreased clonogenic survival in an exponential manner, with an estimated dose for reduction of clonogenicity to 50%, $D_{50\%}$ =109.8 μM (Fig. 2A). In separate spectrofluorimetry experiments, QC also decreased the cellular content of NAD(P)H in a dose-dependent manner, with an effective dose for half-maximal effect IC_{50} = 39.5 μM (Fig. 2B). In Fig. 2C we present some examples of NAD(P)H fluorescence recordings in Jurkat cell suspensions exposed to different



concentrations of QC. After addition of QC, the NAD(P)H fluorescence signal decreased slowly (in up to ~15 min.) to a steady value which appeared to be dose-dependent. Fig. 2B summarizes the steady state data obtained from recordings like those in Fig. 2C.

In a different set of experiments, we investigated the effects of pre-incubating Jurkat cells with 50 μM QC for 1 h on apoptosis and cell cycle distribution after irradiation with 2 Gy of protons (Fig. 3). Quercetin exercised an inhibitory effect on apoptosis (Fig. 3A) and appeared to prolong significantly the G_2/M arrest induced by proton-irradiation (Fig. 3D), which may indicate an enhanced capacity for DNA repair and maintenance of the G_2/M checkpoint active. The parallel reduction in the S cell pool (Fig. 3C) and conservation of the G_0/G_1 cell fraction (Fig. 3B) suggest that cells surviving irradiation may experience an additional G_0/G_1 but not S-phase arrest after 48 h from irradiation.

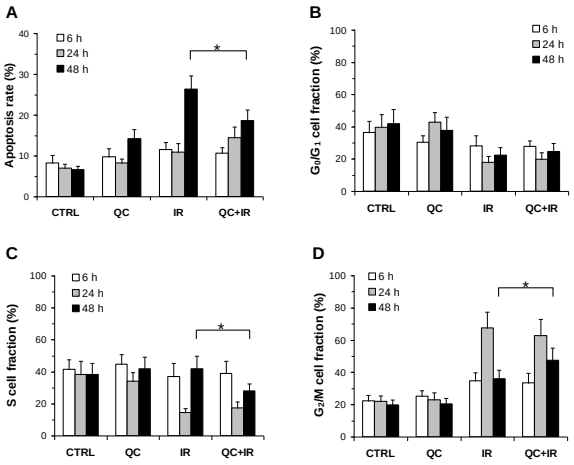


Fig. 3. Apoptosis and cell-cycle distributions assessed at 6, 24 and 48 h after treatment of Jurkat cells with the vehicle (Ctrl), with 50 μM QC for 1 h (QC), with 2 Gy of proton radiation (IR) or with 2 Gy of proton radiation after preincubation with 50 μM QC for 1 h (QC + IR). Apoptotic rates (**A**), G_0/G_1 (**B**), S-phase (**C**) and G_2/M (**D**) cell fractions are illustrated. The star denotes significant difference between the treatments IR and QC + IR.

Effects of proton radiation, MD, H_2O_2, QC and EGCG on delayed luminescence

DL of Jurkat cells irradiated with 10 Gy of high energy protons exhibited different characteristics when probed at 1 h or 24 h after irradiation. Hence, a reduction of 34.1 ± 9.6% in the DL-III relative quantum yield in VIS was observed after 1 h from irradiation, whereas the cell samples probed at 24 h after irradiation exhibited an increase of 27.3 ± 8.5% in the DL-II relative quantum yield and an increase of 41.8 ± 14.3% in the time domain 10 - 100 ms, while all the other components of the DL emission in VIS were not significantly different from the resting DL emission (Fig. 4B).

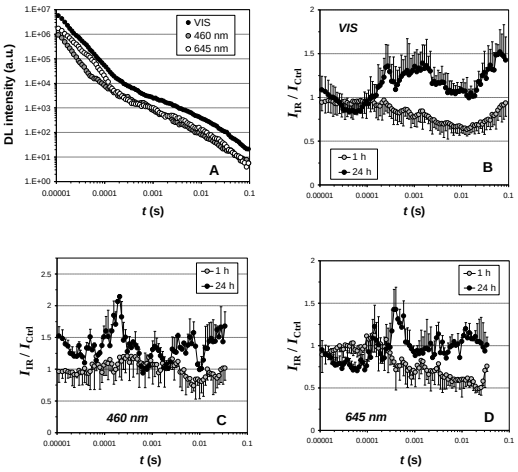


Fig. 4. Kinetics of DL emission of Jurkat cells under control conditions (**A**) or after irradiation with 10 Gy of protons (**B**-**D**). In (**A**) some representative photoemission curves are shown for the entire visible domain (VIS), as well as for detection of 460 nm and 645 nm light emitted by the same cell sample. In (**B**-**D**) the intensity of light emission of irradiated cells (I_{IR}) is normalized to the DL intensity of sham-irradiated cultures (I_{Ctrl}). Measurements were done after 1 h and 24 h from irradiation, as indicated. Results are presented for VIS (**B**), 460 nm (**C**) and 645 nm (**D**) emitted light.

Shortly after irradiation, DL emitted at 460 nm was similar to that of control cells; however, the surviving cells exhibited 24 h later a significant overall DL enhancement with about 35% of the control intensity (Fig. 4C). A remarkable augmentation, up to ~1.7 fold, of blue light emission was detected for a DL component with an established time constant of 178 μs (value derived from fitting analysis, not shown; however, the distinctive peak centered on ~180 μs is clearly visible in Fig. 4C). 1 h after irradiation, delayed emission of red light presented a significant reduction of the DL-III component, with 38.3 ± 11.5%, whereas 24 h later DL-I decreased to 76.1 ± 13.8% of control emission and there was a significant increase of a DL component with an estimated time constant of 379 μs (Fig. 4D, and data analysis not shown). At increasing doses, quercetin inhibited DL progressively (Fig. 5A). The most sensitive DL region was DL-III, which decreased by one order of magnitude after the treatment

with 50 μM QC for 24 h, whereas DL-I was only slightly affected by QC. EGCG exerted a qualitatively different effect on DL by producing a fairly uniform reduction of the photoemission intensity along the entire timescale.

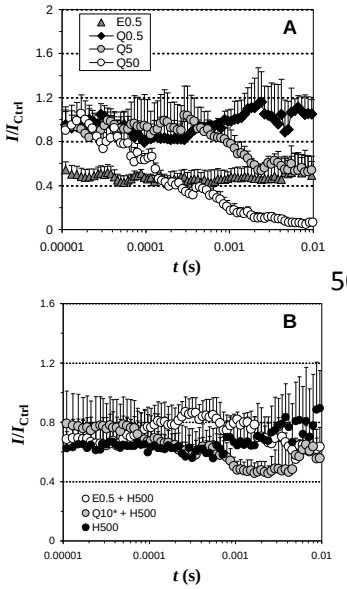


Fig. 5. Kinetics of DL emission of Jurkat cells after various treatments with flavonoids (**A**) or with H_2O_2 alone or in combination with EGCG or QC (**B**). Treatments are labeled as in Fig. 1. The intensity of light emission of treated cells (*I*) is normalized to the DL intensity of control cells (I_{Ctrl}) [7].

500 μM H_2O_2 applied for 20 min. reduced DL significantly over the regions DL-I and DL-II (Fig. 5B, Fig. 6A, B). Pre-treatment with 0.5 μM EGCG for 24 h was able to induce a significant recovery of DL-II emission, whereas preincubation with 10 μM QC for 1 h further reduced the DL-III intensity. The lower dose of 100 μM H_2O_2 had a modest effect on DL and inhibited photoemission by ≈22% over the entire timescale (Fig. 6A-C). Preincubation with 50 μM QC for 24 h restored DL-I emission, but inhibited substantially DL-II and DL-III.

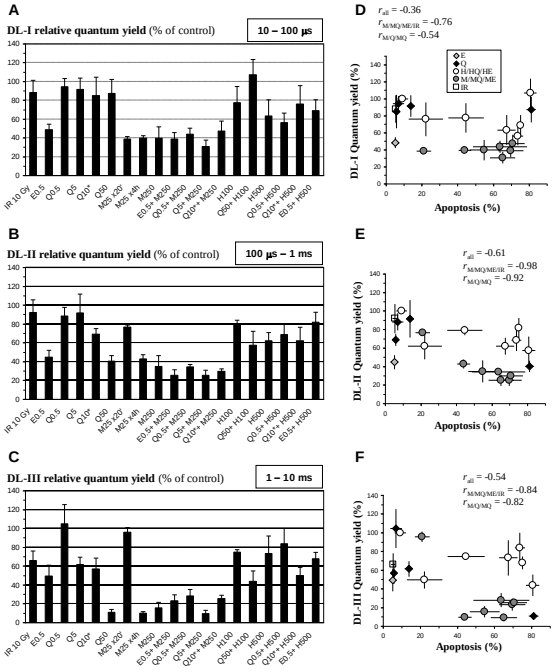


Fig. 6. DL-quantum yield relative to control (**A**-**C**) and its correlation to the apoptotic cell fraction (**D**-**F**) under various treatments indicated in Fig. 1. Q, E, M/MQ/ME, H/HQ/HE and IR denote single QC- or EGCG-treatments, MD- treatments with or without QC- or EGCG-preincubation, H_2O_2- treatments with or without QC- or EGCG-preincubation, and irradiation with 10 Gy of protons, respectively. Pearson correlation coefficients are shown for all treatments ($r_{M/MQ/ME/IR}$) and for the M/MQ/ME/IR Results obtained for separate DL treatments (r_{all}), for the M/Q/MQ are displayed individually for DL-I treatments ($r_{M/Q/MQ}$). DL in a dose-dependent time-domains indicated inside boxes (**A**, **D**), DL-II (**B**, **E**) and DL-III (**C**, **F**).

Menadione also inhibited modest effect of QC on DL-I, manner, and, at variance with the photoemission in the DL-I MD reduced substantially strong even at the lowest region (Fig. 6A).This inhibition was dose of 25 μM menadione. DL-II was inhibited to a similar extent by high doses of MD (Fig. 6B), whereas DL-III exhibited a drastic reduction and thus, in the M250 treatment, the DL-III quantum yield reached 15.5 ± 6.1% of its resting value (Fig. 6C). Preincubation with the two flavonoids generally induced partial recovery of DL-III up to ~25% of the resting value, except in the case of pre-treatment with 5 μM QC for 24 h, when a further reduction to 9.2 ± 3.8% was recorded.

At the moment, the effects of QC or EGCG on apoptosis induced in Jurkat T-cells by the flavonoids themselves or in conjunction with menadione and hydrogen peroxide are poorly known. Here we found that a 24-hour treatment with physiological levels (0.5 - 5 μM) of QC and EGCG can potentiate the antiproliferative activity of menadione by enhancing drug-induced apoptosis in human leukemia Jurkat T cells. In agreement with previous reports that QC is a more potent inhibitor of hydroxyl radical formation than a scavenger of superoxide anions [11], none of the quercetin-based treatments used in the present work exercised protective effects against MD, whereas a short incubation with 10 μM QC for 1 h offered consistent protection against H_2O_2 and induced G_2/M cell cycle arrest, hence allowing time for repair of H_2O_2-induced damage. In addition, preincubation for 24 h with a very low level (0.5 μM) of EGCG increased significantly the G_2/M cell fraction after exposure to 250 μM MD. Nevertheless, albeit long-term administration of QC or EGCG may improve significantly the menadione-based treatment of leukemia, it is important to establish the critical level of flavonoid that is no longer beneficial to normal cells. Our investigations

suggest a connection between the ability of quercetin to decrease the level of NAD(P)H and the induction of apoptosis, which is probably mediated by the failure to maintain the ATP-dependent electrochemical gradient across the inner mitochondrial membrane, and the consequent dissipation of the mitochondrial membrane potential. Similarly to the protective effect of QC against H_2O_2 discussed above, our results suggest that short treatments with quercetin could be able to improve cell survival after proton-irradiation, most likely by inhibiting hydroxyl radical formation after irradiation and protecting against cellular oxidative DNA damage. The data presented here indicate that DL of proton-irradiated cells probed shortly after irradiation was dominated by light emission in the red region of the spectrum and was characterized by a significant reduction in the millisecond DL-III region. On the contrary, DL emission of irradiated cells that survived the subsequent 24 h was dominated by light emission in the blue region of the spectrum and exhibited a significant increase in the sub-millisecond DL-II region. Moreover, cells that survived 1 day post-irradiation revealed two distinctive DL states, namely a blue-light emitting state with a characteristic lifetime of 178 μs, and a red-light emitting state with a characteristic lifetime of 379 μs. In agreement with our previous results [7] and a series of data we have obtained with rotenone-treated cells (not shown), as well as with established electron transfer rates within Complex I [12], we propose that the red-light emitting state is characteristic to the Fe/S center N2 in reduced form. Having in view the growing interest of using DL spectroscopy in clinical applications [13-16], our results lend further support for the development of this methodology as a valuable tool of investigation and diagnosis The results of this study have been published in the ISI journal *Oxidative Medicine and Cellular Longevity*, having an impact factor of 2.8 [8].

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