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Effect of new synthetically prepared quinolone ethyl-1,4-dihydro-8-nitro-4-oxoquinoline-3-carboxylate on human leukemia cell line HL-60 without/with presence of UVA irradiation

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Abstract

The present study demonstrated cytotoxic/phototoxic effects of new synthetically prepared quinolone ethyl-1,4-dihydro-8-nitro-4-oxoquinoline-3-carboxylate (DNQC) on human leukemia cells HL-60. The effects on cell proliferation, cell cycle, induction of apoptotic/necrotic cell death and DNA damage in dark and in the presence of UVA irradiation were evaluated. DNQC induced a different cytotoxic/phototoxic effect which was concentration- and time-dependent. UVA irradiation statistically increased DNQC effect on HL-60 cells. The IC_{50} and IC_{100} values showed that the irradiated HL-60 cells are 5.4 – 27.0 (IC_{50}) and 2.5 – 15.0 (IC_{100}) times more sensitive in comparison to the non-irradiated cells. The cytotoxic/phototoxic concentrations of DNQC induced necrotic death of leukemia cells HL-60. Dead cells had the integrity damage of cytoplasmic membrane and DNA damage. The DNA damage generated by DNQC alone/with combination of UVA irradiation induced cell cycle changes and led to necrosis. The concomitant effect of necrosis was the release of lactate dehydrogenase (LDH) from HL-60 cells with integrity damage of cytoplasmic membrane.

Keywords: ethyl-1,4-dihydro-8-nitro-4-oxoquinoline-3-carboxylate; leukemia cells HL-60, UVA irradiation, Cytotoxicity/Phototoxicity; cell death, DNA damage

Introduction

The condensed nitrogen heterocyclic compounds (including quinoline derivatives) occur in the nature as components of plant or animal organs and often belong to compounds with biological widespectral or specific activity. 4-Quinolones (4-hydroxyquinolines) represent a group of heterocyclic compounds known for more than 40 years, many of which are currently used worldwide in the medical practice (e.g., ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin).

Quinolone derivatives are multitarget agents with a variety of biological activities. They are used mainly as antibacterial (Sharma et al. 2009) anti-HIV (Darque et al. 2009, Santo set al. 2009), antitubercular (Sokolova et al. 2009), antifungal (Meyer et al. 2007), antimycobacterial (Senthilkumar et al. 2009, Dinakaranet al. 2008), antiplatelet (Huang et al. 1998, Liao et al. 1998) and antichlamydial (Oriel 1989) agents. Quinolones have been demonstrated to possess antitumor activity, too (Xia et al. 2001, Drygin et al. 2009, You et al.2009, Chou et al. 2009, Azéma et al. 2009).

Quinolone derivatives are also known as gyrase and topoisomerase IV (Khodursky et al. 1995, Drlica and Zhao 1997**)**, topoisomerase I (You et al. 2009) and topoisomerase II inhibitors (Yamashita et al. 1992), tubulin assembly inhibitor (Chang et al. 2009), farnesyltransferase inhibitors (Medeiros et al. 2007, Asoh et al. 2009), P-glycoprotein inhibitors (Adams et al. 2007, Medeiros et al. 2007), protein kinase CK2 inhibitors (Golub et al. 2006) and photosystem II and cytochrome b_6/f-complex inhibitors (Reil et al. 2001). Quinolones derivatives have an cytotoxic/antiproliferative (Xiao et al. 2008, Foroumadi et al. 2009) and antimitotic activity (Xia et al. 2001, Hadjeri et al. 2004, Chang et al. 2009), they are inducers of apoptotic cell death (Sheng et al. 2008, Chang et al. 2009, Claassen et al. 2009) and some of them have been developed for clinical use in human medicine (Blasi et al. 2006, Leibovitz 2006).

The biological activitities of quinolones can be increased upon activation with suitable radiation (e.g., UVA light) (Diwu et al. 1996, Reszka et al. 1992). Many authors demonstrated the quinolones as photosensitive agents which are able to produce radical species in the presence of UVA irradiation and have phototoxic effect (Spratt 1999, Viola et al. 2004). The phototoxic effects of quinolones on many cancer cell lines (human leukemia cells HL-60, human hematopoietic cell line K562, human epithelial carcinoma cell line A431, human carcinoma of nasopharynx KB, breast cancer cell line MCF-7, breast cancer call line MDA-

MB-468) and non-cancer mouse embryonic fibroblast cells NIH-3T3 were reported (Agrawal et al. 2007, Xie et al. 2008).

The interaction of the compound with light can lead to production of radical species. Such compounds are called photosensitizers. Photosensitizer is described as a compound that generates superoxide radicals (type I reaction) in the presence of oxygen and upon light stimulation, which in turn may form peroxide and hydroxyl radicals or (non-radical) singlet oxygen molecules ($^1O_2$) (Type II reaction) (Jantová et al. 2006, Vrecková et al. 2008a,b).

The interaction of photosensitizer with light in the biological system (cell, tissue, organ, or in the whole organism) may initiate the negative processes – the formation of radicals and reactive oxygen species (ROS) initiates protein-, lipid- and DNA- damage, coupled with genotoxicity, mutagenicity and cancerogenicity. These radical reactions lead to oxidative damage of biomolecules resulting in the apoptotic or necrotic cell death. It was also reported that the photoinstability of some pharmaceutics can cause, during treatment, undesirable side-effects and technical problems upon application (e.g. infusions). Some UVA-absorbing photosensitizers are photocarcinogenic. Recently, it has been demonstrated that some antibiotic fluoroquinolones (ciprofloxacin, fleroxacin, lomefloxacin) are photocarcinogens; they sensitize the UVA-induction of skin tumors in different strains of mice on sunlight exposure during oral application (Bulera et al. 1995, Gocke et al. 1998, Chignell et al. 2003). On the other hand, these processes of photoinduced ROS generation can be used in the therapeutic applications (phototherapy, photochemotherapy). Nowadays, phototherapy represents a special branch of medicine with major applications in dermatological diseases and in a new therapeutic approach for the treatment of chronic lymphocytic leukemia.

Based on the reported effects of quinolone derivatives, a new series of substituted quinolones, fluoroquinolones and selenadiazoloquinolones, were prepared by Bella et al. (2007). In our preliminary studies, these derivatives were screened for antimicrobial and cytotoxic/phototoxic activities *in vitro* (Füzik et al. 2007, Jantová et al. 2008a, b, Koňariková et al. 2009). The highest biological activity on four bacterial strains, four yeast strains, four filamentous fungi strains and human leukemia cells HL60 was found for ethyl-1, 4-dihydro-8-nitro-4-oxoquinoline-3-carboxylate (DNQC). After irradiation, quinolone produced radicals and ROS (Bella et al. 2007, Vrecková et al 2008a, b, Jantová et al. 2008).

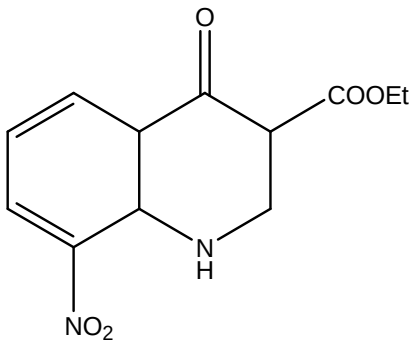
Based upon these preliminary results, in the present study we investigated the *in vitro* antiproliferative/cytotoxic effect of DNQC in the dark and in the presence of UVA light, using human leukemia cell line HL-60. The effect of DNQC alone or in combination with UVA irradiation on the cell cycle, on the induction of apoptosis/necrosis and on DNA damage was monitored.

Material and Methods

Chemicals

Ethyl-1, 4-dihydro-8-nitro-4-oxoquinolin-3-carboxylate (DNQC, Scheme 1) was prepared by Bella et al. (2007). Chromatographically pure quinolone was dissolved in 100% dimethylsulfoxide (DMSO). The stock solution of DNQC for incubation with cells was prepared in DMSO and further diluted in the cell culture medium. The final DMSO concentration in the medium was 0.1% (in either control or treated samples), which did not affect the cell viability. DMSO was purchased from Merck (Slovakia).

Superoxide dismutase (SOD), propidium iodide (PI), normal melting point agarose (NMP agarose), Triton-X 100, trypan blue, ethidium bromide (EtBr), RNase, and Hoechst 33342 were from Sigma-Aldrich (Slovakia). Low melting point agarose (LMP) was purchased from Invitrogen (Great Britain) and phosphate-buffered saline (Dulbecco A) (PBSa) was from OXOID (Great Britain). Ethylenediaminetetraacetic acid (EDTA), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), and sodium hydroxide (NaOH) were from Lachema (Brno, Czech Republic). Proteinase K was obtained from BIOCOM Company (Slovakia).



Scheme 1. Chemical structure of ethyl 1,4-dihydro-8-nitro-4-oxoquinoline-3-carboxylate (DNQC).

Cell lines

Human leukemia HL-60 cells (obtained from ATCC, Rockville, MD, USA) grown at 37°C in humidified 5%-CO_2 and 95%-air atmosphere were in complete RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, penicillin G (100µmol.L^{-1}) and streptomycin (100µmol.L^{-1}). All culture medium compounds were obtained from Sigma-Aldrich (Slovakia), bovine serum and fetal calf serum were from BIOCOM Company (Slovakia).

Light irradiation of cells

Control and DNQC-treated cells were exposed to UVA light (λ > 300 nm) immediately after addition of DNQC. The cells were irradiated directly in Petri dishes using the focused light beam of the HPA 400/30S lamp equipped with a perpendicular prism permitting irradiation in the horizontal position (UVA irradiation of 2 mW.cm^{-2}). HL-60 cells grew as suspension cultures in all culture media. All dishes were completely transparent for the excitation light. The temperature recorded in the culture dishes did not exceed room temperature during the irradiation period.

Antiproliferative assay

HL-60 cells were seeded onto Petri dishes (diameter 60 mm) at 1.5 x 10^6 cells/dish in the exponential phase of growth. After 24 h of incubation at 37°C, the cells were exposed (or not exposed) to DNQC for 24, 48 and 72 h. Final concentration of DNQC added to the cells were 0.0038, 0.38, 3.8, 19.07, 38.14, 95.34 and 190.07 µmol.L^{-1}. Control cells were treated with DMSO, its final concentration never exceeded 0.1%. Immediately after the addition of DNQC, the control and the treated cells were irradiated (or not irradiated) using UVA light and an irradiation time of 90 s (UVA dose 180 mJ cm^{-2}). Then, the cells were cultured for 24, 48 and 72 h in an incubator in the dark. Finally, the cell proliferation was determined by direct counting of cell numbers in a counting chamber. Relative inhibition of cell proliferation or degeneration of the cell population was calculated by the formulae:

$$\%\text{Inhibition} = [(K-E)/(K-K_0) \times 100], \quad (1)$$

$$\%\text{Degeneration} = 100-[(E/K_0) \times 100], \quad (2)$$

where K_0 is the cell number at the time of DNQC addition, *K* is the cell number after 24, 48 or 72 h of cultivation with the solvent and *E* is the cell number after 24, 48 or 72 h of cultivation with DNQC.

After curve fitting using nonlinear regression (Origin 7.0) the IC_{50} and IC_{100} values (the concentration resulting in 50% and 100% of the cell proliferation that was recorded in control experiments) were determined separately for each experiment. The values were calculated from at least three independent experiments.

Monitoring of lactate dehydrogenase (LDH, E.C.1.1.1.27) activity

After 24, 48 and 72 h of incubation, the same volume of the medium with and without cells affected by non-photoactivated/photoactivated DNQC was aspirated for each sample and stored on ice until measurement. Then, the standard solutions for samples with released and total LDH (100 mmol.L^{-1} Tris-HCl buffer, pH 7.1, 0.3 mmol.L^{-1} NADH, 10.0 mmol.L^{-1} pyruvate sodium salt, 0.25 % solution of Triton-X-100) were prepared. The standard solutions were incubated at 31°C for 5 – 10 min before measurement. The enzymatic reaction started by adding sample that was very gently shaken before, into the standard solution. The oxidation of NADH was measured on Philips PU 8750 UV/VIS scanning spectrophotometer at λ = 340 nm. The absorbance decreased linearly with time over 60 s of measurement. To determine the background we used sterile RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin G (100µg.m L^{-1}) and streptomycin (100µg.m L^{-1}). The ratio r of released LDH activity and total LDH activity was calculated by the formulae:

$$r = (\Delta A_R - \Delta A_B)/(\Delta A_T - \Delta A_B) \quad (3)$$

where ΔA_B is the change in background absorbance at 340 nm/60 s, ΔA_R is the change in released LDH absorbance at 340 nm/60 s, ΔA_T is the change in total LDH absorbance at 340 nm/60 s.

Determination of apoptosis/ necrosis by fluorescence microscopy

The untreated (control) HL-60 cells and the cells treated with non-photoactivated and photoactivated DNQC (0.038, 0.38, 3.8, 19.07, 38.14, 95.34 and 190.07 µmol.L^{-1}) for 24, 48 and 72 h, were washed in PBS, trypsinized, and re-resuspended in the cultivation medium. 40 µL of Hoechst 33342 (1 µg.mL^{-1}) and 15 µL of PI (5 µg.mL^{-1}) were added to 1 mL of cell suspension (1 x 10^6) and incubated for 30 min at laboratory temperature. Cells were then

centrifuged at 1500g for 3 min, the pellet was resuspended in 40 µL of the culture medium, and 5 µL of the cell suspension was pipetted on the cover slip and monitored by fluorescence microscopy (Zeiss Jenalumar, Germany).

Cell cycle analysis

Irradiated and non-irradiated control and DNQC-treated HL-60 cells at concentration of 19.07, 38.14, 95.34 and 190.07 µmol.L^{-1} were harvested, washed twice in phosphate-buffered saline (PBS) and exposed to 0.1% Triton X-100 in PBS supplemented with RNase (50µg.mL^{-1}) for 25 min at 37°C. Afterwards, DNA was stained by propidium iodide (50µg.mL^{-1}) for 15 min at 4°C. Samples were analyzed by Beckman-Coulter FC 500 flow cytometer (Beckman Coulter Inc., Fullerton, California, USA) with the use of DNA Cell Cycle Analysis Software distributed by Phoenix Flow Systems – Multicycle AV for Windows. A minimum of 10 000 cells per sample were analyzed at a flow rate of 200 cells/s.

Electrophoretic analysis of apoptosis

HL-60 cells (1 x 10^6) treated with non-photoactivated and photoactivated DNQC (0.038, 0.38, 3.8, 19.07, 38.14, 95.34 and 190.07 µmol.L^{-1}) were harvested, washed with PBS, and then lysed in a 100 µL of solution (10 mmol.L^{-1} Tris, 10 mmol.L^{-1} EDTA, 0.5% Triton X-100) supplemented with proteinase K (1 mg.mL^{-1}). Samples were then incubated at 37°C for 1 h and heated at 70°C for 10 min. Following lysis, RNase (200 µg/mL) was added and repeated incubation at 37°C for 1 h followed. The samples were subjected to electrophoresis at 40 V for 3 h in 2% (w/v) agarose gels supplemented with EtBr (1 µg.mL^{-1}). Separated DNA fragments (DNA ladders) were visualized using a UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, USA).

Detection of DNA damage by alkaline comet assay

Comet assay is based on the ability of DNA strand breaks to migrate in a weak electric field in the direction of the anode, giving the nucleus the appearance of the head of a comet when visualized by fluorescence microscopy. The procedure of Singh et al. (1988) was used with minor changes suggested by Slameňová et al. (1997) and Gábelová et al. (1997). A base layer of 100 µL of 0.75% NMP agarose in PBS buffer (Ca^{2+}-and Mg^{2+} -free) was placed on microscope slides. HL-60 growing in suspension were treated with DNQC in concentrations

of 0.038, 0.38, 3.8, 19.07 and 38.14 μmol.L^{-1} for 24 h. Treated as well untreated (control) cells were suspended in 0.75% LMP agarose. 85 μL of LMP agarose, containing 2x10^4 cells was spread on the base layer. Triplicate slides were prepared per sample. The agarose was allowed to solidify and the slides were placed in a lysis mixture (2.5 mol.L^{-1} NaCl, 100 mmol.L^{-1} Na_2EDTA, 10 mmol.L^{-1} Tris-HCl, pH 10.0 and freshly added 1% Triton X-100) at 4°C. The slides were then transferred to an electrophoresis box containing an alkaline solution at pH >13 (300 mmol.L^{-1} NaOH, 1 mmol.L^{-1} Na_2EDTA, pH >13) and kept in this solution 40 min at 4°C for the DNA strands to unwind. A voltage of 25 V (300 mA current) was applied for 30 min. The slides were removed, neutralized by 2x10 min washing in Tris-HCl (0.4 mol.L^{-1}, pH 7.5), and stained with 20 μL EtBr (10 μg.m L^{-1}). EtBr stained nucleoids were evaluated with a Zeiss Jenalumar fluorescence microscope (magnification 200x).

Statistical analysis

Results obtained from antiproliferative assay, cell cycle analysis and comet assay are shown as the arithmetic means ±SD (standard deviation) of the mean of three separate experiments (each experiment was done with five parallels). Statistical analysis was performed with the Kruskal-Wallis one-way ANOVA test for nonparametric measurements ($H>7.20$, $P<0.05$ was considered statistically significant). The effect of time and concentration on cell number was analyzed by Friedman´s nonparametric test ($P<0.004$ was considered statistically significant). A multiple-comparison procedure by the post hoc test (Newman-Keuls test) was performed on all measurements. The collected data were analysed using a statistical software package (Statgrafics Plus 5.0).

The significance of differences between values acquired by comet assay (sample without DNQC or irradiation and with DNQC or irradiation) was evaluated by Student´s t-test statistically different from the control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results and Discussion

The growth of HL-60 cells exposed to non-photoactivated/photoactivated DNQC concentrations ranging from 0.038 to 190.07 μmol.L^{-1} was monitored within 72 h of culturing (Fig. 1). As shown in figure, the addition of non-photoactivated/photoactivated DNQC to medium reduced HL-60 viable cell number. After 24 h, the highest concentrations tested (190.07, 95.34 μmol.L^{-1} for non-photoactivated DNQC and 190.07, 95.34, 38.14, 19.07

μmol.L^{-1} for photoactivated DNQC) had acute cytotoxic/phototoxic effect manifested by degeneration (lysis-necrosis) of cell population or cessation of cell proliferation. Further concentrations of non-photoactivated/photoactivated DNQC (3.8, 0.38 and 0.038 μmol.L^{-1}) induced antiproliferative effect directly proportional to the concentration used. The changes in viable cell number were also observed when aliquots of the cultures were examined by light microscopy (Fig. 2). In the next 48 h interval, the cytotoxic/phototoxic effect of DNQC increased.

Figure 3 demonstrated the effect of non-photoactivated/photoactivated DNQC on the inhibition of cell proliferation. As it followed from the comparison of percentage of growth inhibition of non-irradiated/irradiated HL60 cells, UVA irradiation significantly increased the cytotoxicity of quinolone DNQC. Non-photoactivated/photoactivated DNCQ inhibited cell growth with an IC_{50} in the range from 0.2 to 14.1 μmol.L^{-1} and IC_{100} in the range from 3.8 to 95.0 μmol.L^{-1} (Tab. 1). The values indicate that irradiated HL-60 cells are 5.4 – 27.0 (IC_{50}) and 2.5 – 15.0 (IC_{100}) times sensitive in comparison to the non-irradiated cells.

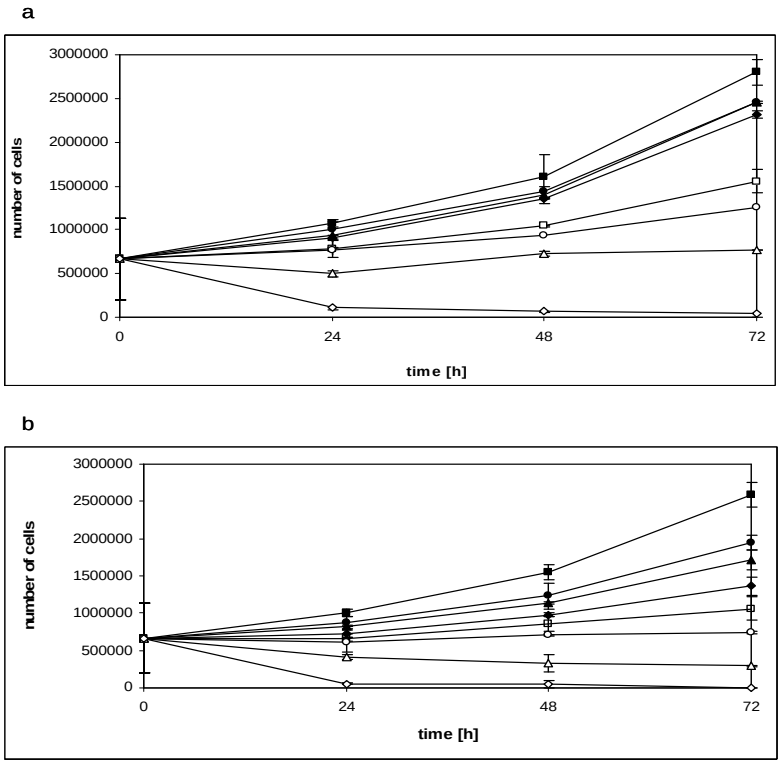


Fig. 1. Cell proliferation of HL-60 cells in response to quinolone DNQC in the course of 72 h to non-photoactivated (a) and photoactivated (b) cells. Concentration of DNQC (μmol.L^{-1}): control (■), 0.038 (●), 0.38 (▲), 3.8 (◆), (□), 38.14(○), 95.34 (△), 190.07 (◇).

Similar results were obtained when the effect of non-photoactivated /photoactivated DNQC on murine leukemia cells L1210 was studied (Jantová et al. 2008, Koňariková et al. 2009). Quinolone induced a different cytotoxic/phototoxic effect on murine L1210 and NIH-373 cells, which was concentration- and time-dependent. The four highest tested concentrations of non-photoactivated and photoactivated DNQC induced the immediate cytotoxic/phototoxic effect after 24h cultivation of both cell lines. This effect decreased with the time of treatment. The sensitivity of leukemia cell line L1210 and non-cancer cell line NIH-3T3 on the effect of non-photoactivated and photoactivated DNQC was in the same order. The irradiation increased the sensitivity of both cell lines on non-photoactivated and photoactivated DNQC, but the cell sensitivity decreased with time of influence (Jantová et al. 2008, Koňariková et. al. 2009).

By the influence of radiation many quinolones have demonstrated photosensitivity that can be not only desirable but also undesirable effect of these compounds. Photosensitive and phototoxic properties of fluoroquinolones that are commercially available as antibiotics are particularly known. Their photosensitivity is undesirable because it leads to genotoxicity, mutagenicity and cancerogenicity (Maziere et al. 1990, Satomi et al. 2006). On the other hand, the processes of photoinduced ROS generation by many of photosensitive compounds can be used in the therapeutic applications (phototherapy, photochemotherapy). Nowadays, phototherapy represents a special branch of medicine with major applications in dermatological diseases and in a new therapeutic approach for the treatment of chronic lymphocytic leukemia. Photochemotherapy of cancer diseases is often called “photodynamic therapy” (PDT) (Dougherty et al. 1998, Pandey, et al. 2006).

The cytotoxicity/phototoxicity of some quinolone derivatives on leukemia cells HL-60 and L1210 was also found by other authors (Viola et al. 2004, Barraja et al.2003, 2006, Rhee et al. 2007). Barraja et al. (2006) reported that the irradiation of human cancer cell lines HL-60, HT-1080 and LoVo treated with pyrrolo[2,3-*h*]quinolinones increased cytotoxicity of compounds. Viola et al. (2004) demonstrated phototoxicity of 6-aminoquinolones in two cell culture lines: human promyelocytic leukemia (HL-60) and human fibrosarcoma (HT-1080). Cytotoxicity/phototoxicity of some quinolone derivatives on further cancer cell lines was reported by many authors (Yamashita et al. 1992, Dogankoruznjak et al. 2002, Joseph et al. 2002).

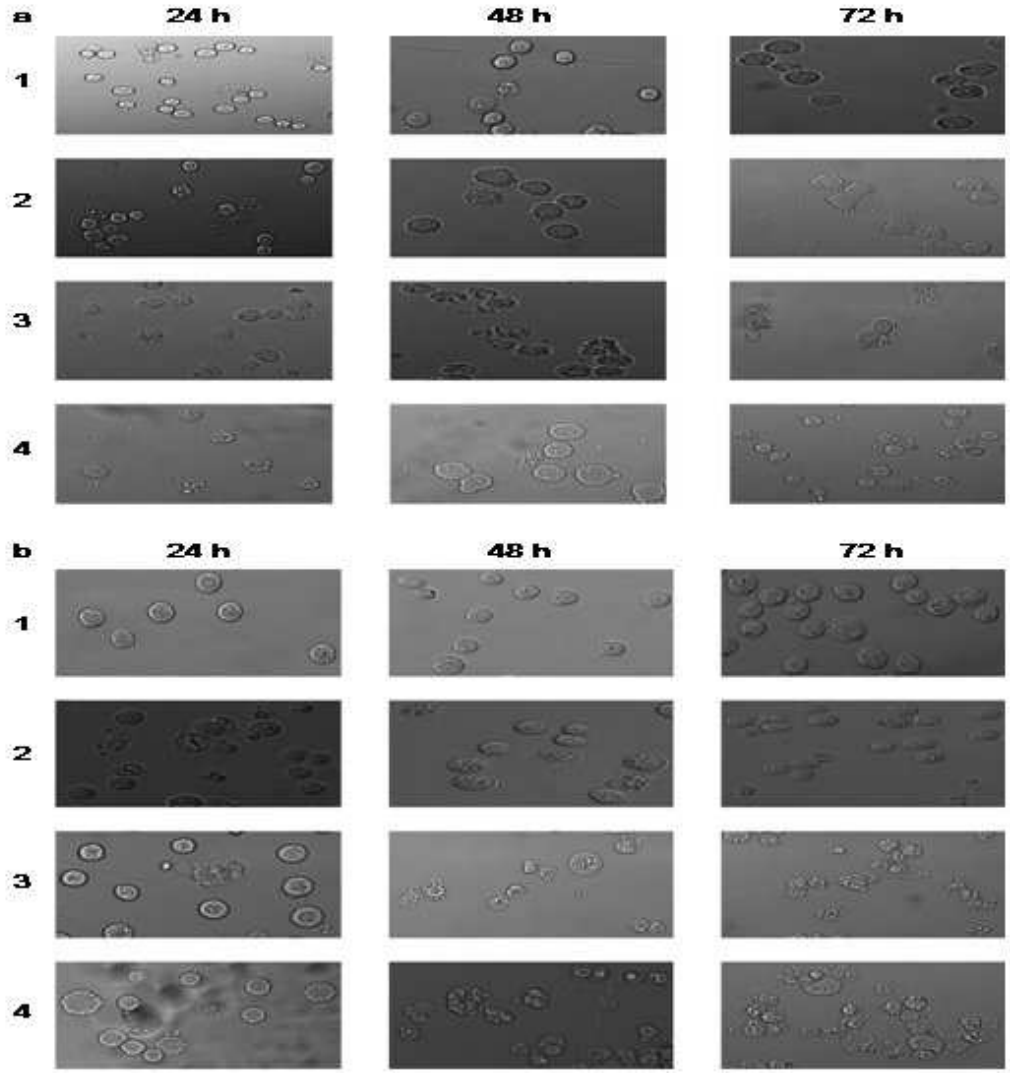


Fig. 2. The effect of DNQC on HL-60 Cells morphology during 72 h of quinolone-treatment without (a) and with (b) irradiation. Control cells (1), concentration of 38.14 µmol.L^{-1} (2), concentration of 95.34 µmol.L^{-1} (3), concentration of 190.07 µmol.L^{-1} (4). Magnification of 400x.

On the basis of our obtained results we can conclude that non-photoactivated / photoactivated DNQC acted cytotoxically on human leukemia cell line HL-60 and the activity was concentration- and time- dependent. During 72 h of influence, the UVA irradiation increased the DNQC effect on HL-60 cells ranging from 1.1 to 2.8 times.

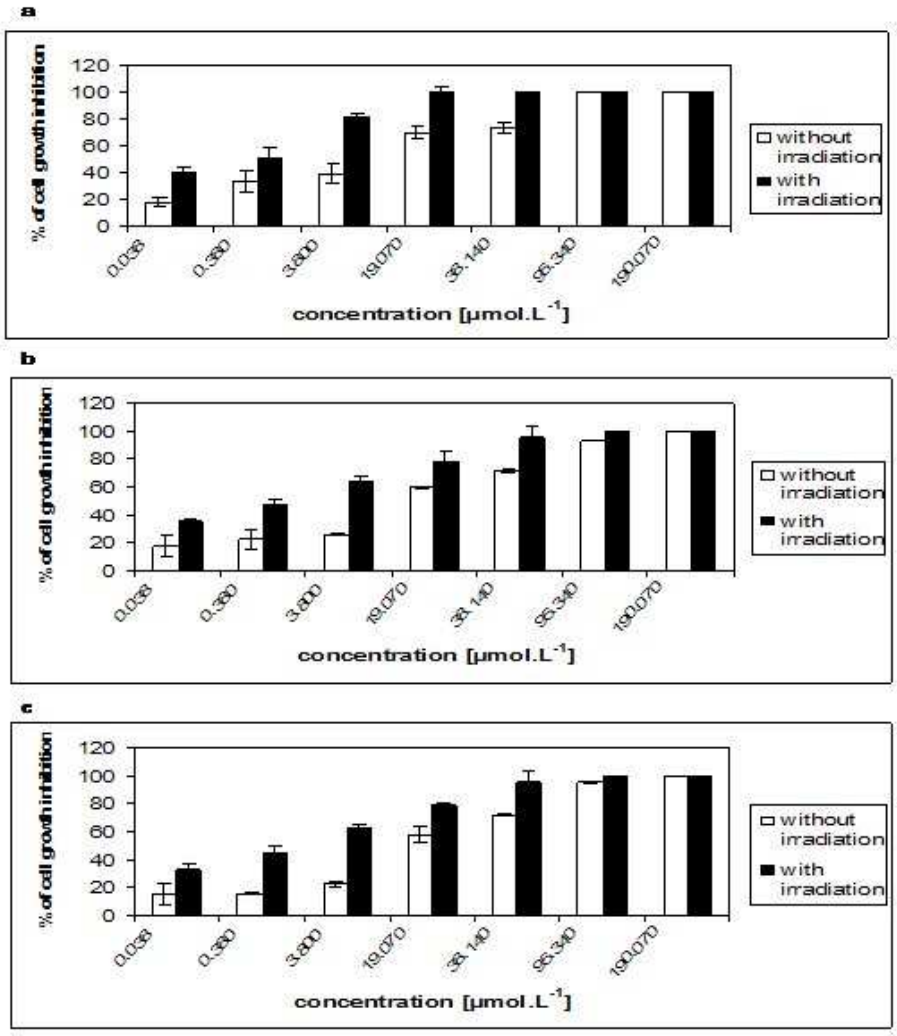


Fig. 3. Analysis of cell growth of HL-60 cels after 72 h treatment by non-photoactivated (□) and photoactivated (■) DNQC.

Table 1. The concentrations of non-photoactivated and photoactivated DNQC inducing 50% (IC_{50}*) and 100% (IC_{100}*) inhibition of growth of HL-60 cells.

Time (h)	without irradiation		with irradiation	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}
24	5.4 ± 0.39	95.34 ± 0.27	0.2 ± 0.02	95.34 ± 0.25
48	11.9 ± 0.57	190.07 ± 0.63	2.2 ± 0.10	95.34 ± 0.48
72	14.1 ± 0.45	190.07 ± 0.87	2.4 ± 0.14	95.34 ± 0.35

* The values are in µmol.L^{-1}.

Many compounds show cytotoxic effect by the interference with cell cycle, therefore, we further examined the ability of non-photoactivated/photoactivated DNQC to influence the cell cycle of HL-60 cells. The results are demonstrated in Table 2. The flow cytometric analysis showed that DNQC without/with presence of UVA irradiation significantly influenced the cell cycle of HL-60 cells. After 24, 48 and 72 h of influence, non-photoactivated DNQC caused a cell arrest in G_0/G_1 and block in S and G_2/M phase. On the other hand, while after 24 h photoactived DNQC induced a cell arrest in S and G_2/M phase and the block in G_0/G_1 phase, after next time intervals, a cell arrest in G_0/G_1 and the block in S and G_2/M phase was found. The ability of quinolone derivatives to influence cell cycle has been reported by many authors (Chou et al. 2009, Chang et al. 2009, Nadkartni et al. 2009). Chou et al. [2009] reported that 2-(2-fluorophenyl)-6,7-methylenedioxyquinolin-4-one acted against colorectal adenocarcinoma cells in vitro via G2/M arrest and apoptosis. Chang et al. [2009] demonstrated potential antitumor activity of novel 2-phenyl-4-quinolone CWC-8 which induced G_2/M arrest and apoptosis in human osteogenic sarcoma U-2 OS cells. Nadkarni et al. (2009) reported S-phase cell cycle arrest in human lung cancer cell lines treated with 1,3,4,8-tetrahydropyrrolo[4,3,2-de]quinolin-8(1H)-one alkaloid analog.

Table 2. Effect of non-photoactivated (a) and photoactivated (b) DNQC on cell cycle of HL-60 cells after 24, 48 and 72 h of treatment (* < 0.05).

a

Time (h)	**DNQC concentration ($\mu mol.L^{-1}$)**	**subG$_0$ (%)**	**G$_0$/G$_1$ (%)**	**S (%)**	**G$_2$/M (%)**
24	0	1.53 ± 0.01	20.57 ± 1.81	73.09 ± 4.64	6.24 ± 0.47
	19.07	1.19 ± 0.01	25.89 ± 2.24	69.03 ± 4.84	5.02 ± 0.41
	38.14	0.17 ± 0.001	28.84 ± 2.04 *	64.52 ± 4.88 *	6.60 ±0.39 *
	95.34	0.56 ± 0.003	36.58 ±3.52 *	58.76 ± 4.56 *	4.65 ± 0.44 *
	190.07	2.40 ± 0.02	42.76 ± 3.95	54.20 ± 4.58 *	3.04 ± 0.11
48	0	1.40 ± 0.08	29.81 ± 1.48	61.77 ± 4.98	8.41 ± 0.74
	19.07	0.02 ± 0.001	33.94 ± 2.02	59.23 ± 4.99	6.82 ± 0.18
	38.14	1.99 ± 0.09	38.24 ± 1.99	55.52 ± 4.82	5.90 ±0.059
	95.34	0.56 ± 0.01	43.03 ± 3.57 *	52.19 ± 5.13 *	4.34 ± 0.09 *
	190.07	5.10 ± 0.83	45.21 ± 4.51 *	50.95 ± 5.02 *	3.26 ± 0.08 *
72	0**	2.62 ± 0.04	40.84 ± 2.62	48.20 ± 4.08	10.94 ± 0.94
	19.07	0.31 ± 0.02	43.19 ± 3.14	45.43 ± 3.59	10.88 ± 0.49
	38.14	0.58 ± 0.01	46.98 ± 4.8	43.86 ± 3.69	8.46 ± 0.81
	95.34	0.86 ± 0.07	48.99 ± 3.86 *	42.84 ± 2.77 *	7.97 ± 0.47 *
	19007	2.82 ± 0.03	51.39 ± 4.82 *	41.47 ± 3.60 *	7.13 ± 0.61 *

b

Time (h)	DNQC concentration (μmol.L^{-1})	subG$_0$ (%)	G$_0$/G$_1$ (%)	S (%)	G$_2$/M (%)
24	0	0.79 ± 0.05	37.59 ± 3.60	55.63 ± 5.61	6.78 ± 0.33
	19.07	0.97 ± 0.06	37.51 ± 3.51	55.58 ± 5.39	6.91 ± 0.72
	38.14	0.27 ± 0.01	30.54 ± 2.97	58.73 ± 4.26	10.73 ± 0.84
	95.34	0.56 ± 0.03	28.91 ± 2.46 *	60.41 ± 4.86 *	10.68 ± 0.93 *
	190.07	0.73 ± 0.05	26.74 ± 2.27 *	62.11 ± 3.07 *	11.15 ± 0.99 *
48	0	0.50 ± 0.02	30.98 ± 3.02	61.15 ± 3.71	7.87 ± 0.39
	19.07	0.36 ± 0.03	35.09 ± 3.51	59.79 ± 3.67	5.02 ± 0.12
	38.14	0.11 ± 0.009	38.14 ± 3.76	56.71 ± 3.03	5.05 ± 0.39
	95.34	0.17 ± 0.01	43.22 ± 3.61 *	53.02 ± 3.81 *	3.66 ± 0.27 *
	190.07	2.23 ± 0.17	45.63 ± 4.30 *	51.82 ± 4.23 *	2.46 ± 0.11 *
72	0	0.40 ± 0.03	36.48 ± 3.63	47.09 ± 4.30	16.39 ± 1.21
	19.07	0.72 ± 0.05	37.57 ± 3.79	46.48 ± 3.65	15.91 ± 1.38
	38.14	0.73 ± 0.05	49.24 ± 3.97 *	40.73 ± 3.71 *	10.03 ± 0.94 *
	95.34	2.30 ± 0.11	49.72 ± 3.86 *	40.65 ± 3.55 *	9.63 ± 0.82 *
	190.07	2.48 ± 0.18	52.39 ± 4.91	39.12 ± 3.01	8.49 ± 0.66

In the next experiments, we evaluated pathway of death of HL-60 cells treated with cytotoxic/phototoxic concentrations of DNQC. Cell death can be divided into three classes, apoptosis, necrosis and autophagy. Apoptosis has been described as multiple pathways converging from numerous different initiating events and insults. Numerous studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents, too. Morphological changes of apoptosis are considered the results of complex cellular biochemical pathways. In mammals, apoptosis is a result of the proteolysis of various cellular components initiated by activated caspases (Jantová et al. 2006). Necrosis is the end result of a bioenergetic catastrophe resulting from ATP depletion to a level incompatible with cell survival and was thought to be initiated mainly by cellular “accidents” such as toxic insults or physical damage. Necrosis is morphologically characterized by swelling of the cell and organelles, vacuolation of the cytoplasm and results in disruption of the cell membrane and lysis (Grooten et al .1993). Along with proteolysis, necrosis is also accompanied by degradation of DNA. Degradation of DNA during necrosis usually occurs randomly, forming a “smear“ pattern on agarose gels, while apoptotic DNA fragmentation occurs to oligonucleosome fragments forming a remarkable “ladder“ pattern on the gels (Proskuryakov et al. 2003).

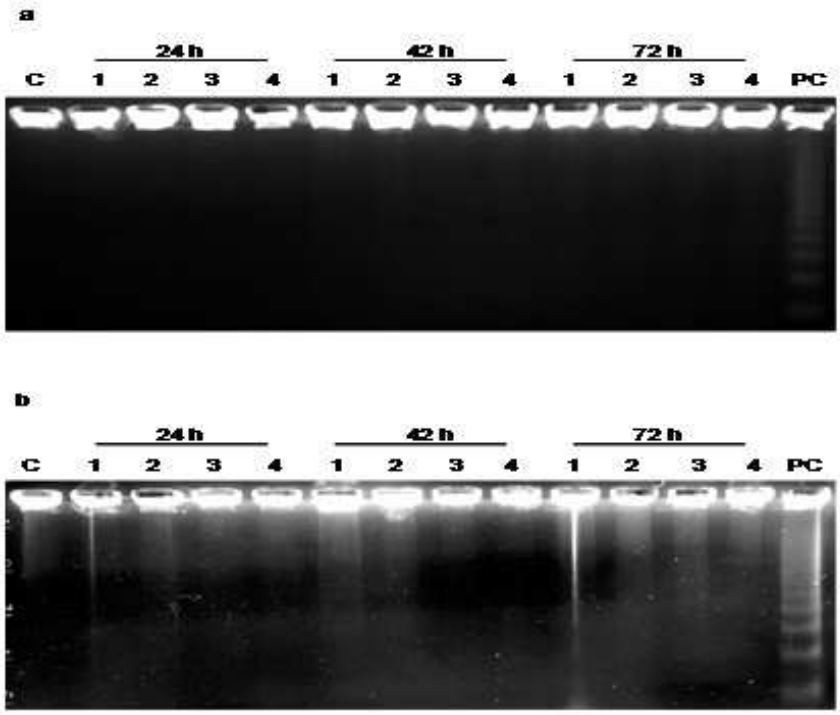


Fig. 4. Detection of apoptotic DNA fragmentation in non-irradiated (a) and irradiated (b), HL-60 cells treated with 19.07 (1) , 38.14 (2), 95.34 (3) and 190.07 (4) $\mu mol.L^{-1}$ of DNQC for 24, 48 and 72 h. C-control, PC-positive control (the cells treated with 6 μM cisplatin for 24 h).

In our experiments, the ability of cytotoxic/phototoxic concentrations of DNQC to induce apoptotic/necrotic cell death was evaluated using Trypan blue-exclusion assay, fluorescence microscopy, flow cytometry, agarose gel electrophoresis to assess apoptotic DNA fragmentation and by determination of LDH release from the cells with integrity damage of cytoplasmic membrane. The HL-60 cells treated with cytotoxic concentrations of non-photoactivated/photoactivated DNQC were stained by Trypan blue (data not shown), had changed integrity of cytoplasmic membrane (Fig. 2) and did not stain with Hoechst 33342 but were stained with propidium iodide (data not shown). The cell cycle profile analysis showed (Tab. 2) that sub-G_0 cell fraction was not detected. The results obtained from agarose gel electrophoresis (Fig. 4) demonstrated that the apoptotic DNA fragments in control (non-treated) HL-60 cells and cells treated with non-photoactivated/photoactivated DNQC were not formed. On the other hand, the apoptotic DNA fragments in the cells treated with cisplatin (6 $mol.L^{-1}$, positive control) were found. On the basis of these obtained results we supposed that HL-60 cells treated with cytotoxic/phototoxic concentrations of DNQC died by necrotic cell

death. In the next part of our work, we studied the ability of DNQC to induce the integrity damage of cytoplasmic membrane, to confirm the necrotic death accompanied by the leakage of cell content. Lactate dehydrogenase (LDH), a good marker of membrane integrity damage, is the first component of the cell content that leak from the cell. The ratio of released LDH (into the medium) and total LDH (in medium and cells) is the marker of cell membrane integrity and can be calculated according to Eq. 3. The effect of non-photoactivated /photoactivated DNQC on LDH level is demonstrated in the Fig. 5. As can be seen from graphs, percentage of LDH release from HL-60 cells increased with increasing quinolone concentration and time of influence. The highest proportion of LDH release from cells (100 %) was found at the highest concentration tested, that were cytotoxic/phototoxic. Radiation decreased the LDH release from cells. Single cell gel electrophoresis, also called comet assay, represents very simple, rapid and sensitive technique for detection of wide range of DNA damage, such as DNA strand breaks, oxidative DNA damage, alkali labile sites or DNA cross-links. This assay can be used to evaluate DNA damage in proliferating or non-proliferating cells and to define mechanisms of genotoxicity and determine sensitive cell types (Jantová et al. 2006). From the comet assay experiments (Fig. 6) we can conclude that non-photoactivated and photoactivated DNQC-induced direct DNA strand breaks which number increased with increasing concentrations of tested quinolone. The UVA irradiation decreased the amount of DNA damage in HL-60 cells.

DNA photodamage caused by 6-aminoquinolones in human promyelocytic HL-60 and sarcoma HT-1080 cells was also found by some other authors (Viola et al. 2004, Lhiaubet-Vallet et. al.2009). These quinolones induced direct DNA strand breaks. Verna et al. (2000) demonstrated oxidative DNA damage in retinal pigment epithelial (RPE) cell line treated with non-photoactivated and photoactivated sparfloxavin.

Our obtained results indicate that non-photoactivated/photoactivated DNQC acted cytotoxically on human leukemia cell line HL-60. The cytotoxic/phototoxic concentrations of DNQC induced necrotic death of leukemia cells HL-60. The dead cells had the integrity damage of cytoplasmic membrane and DNA damage. The DNA damage generated by DNQC alone or with combination of UVA irradiation induced cell cycle changes and led to necrosis.

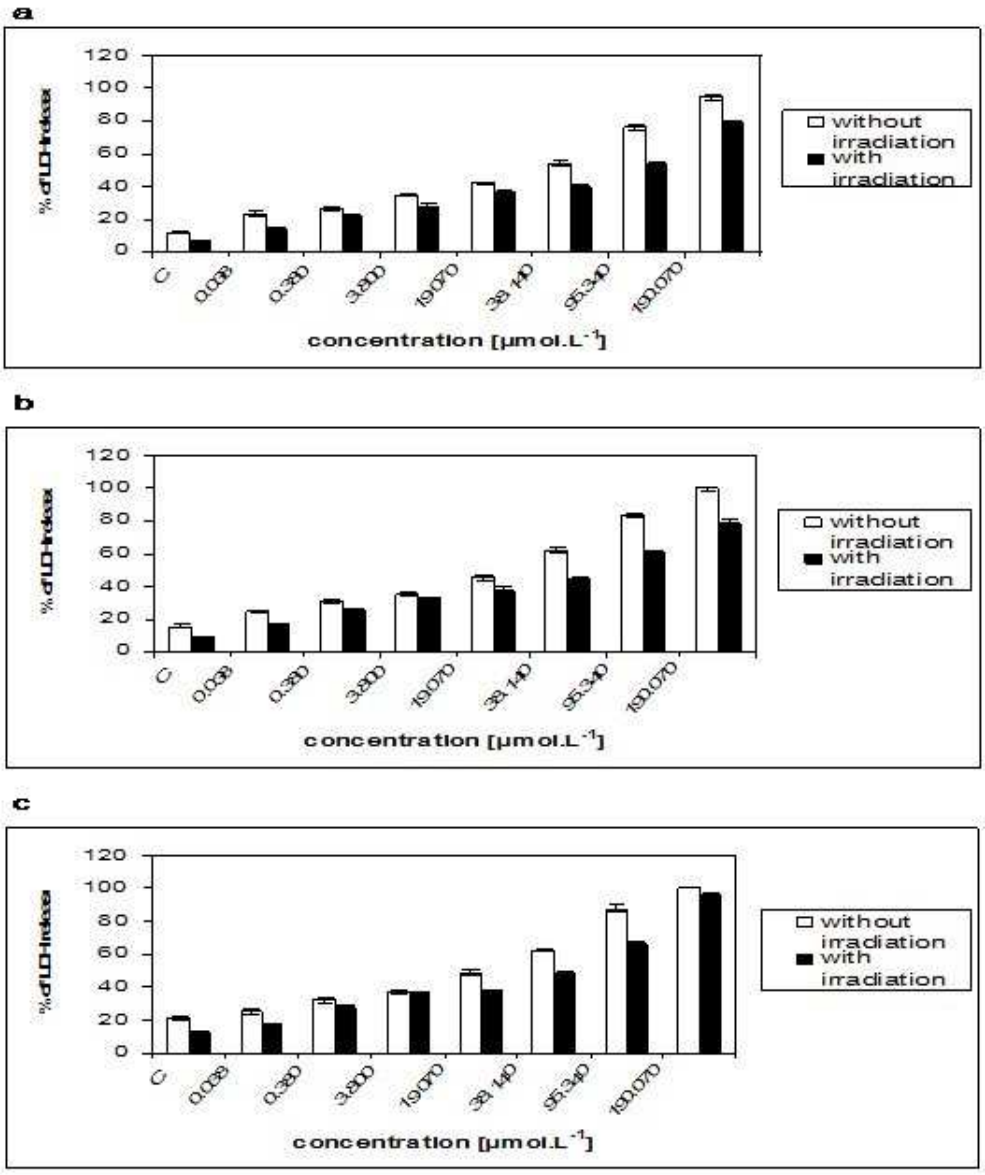


Fig. 5. Effect of non-photoactivated (□) and photoactivated (■) DNQC on LDH release from control and DNQC-treated HL-60 cells.

The concomitant effect of necrosis was the release of enzyme LDH from HL-60 cells with integrity damage of cytoplasmic membrane. Necrosis and DNA damage were also found in non-cancer murine NIH-3T3 cells treated with DNQC without/with presence of UVA irradiation (Konariková et al. 2008, 2009).

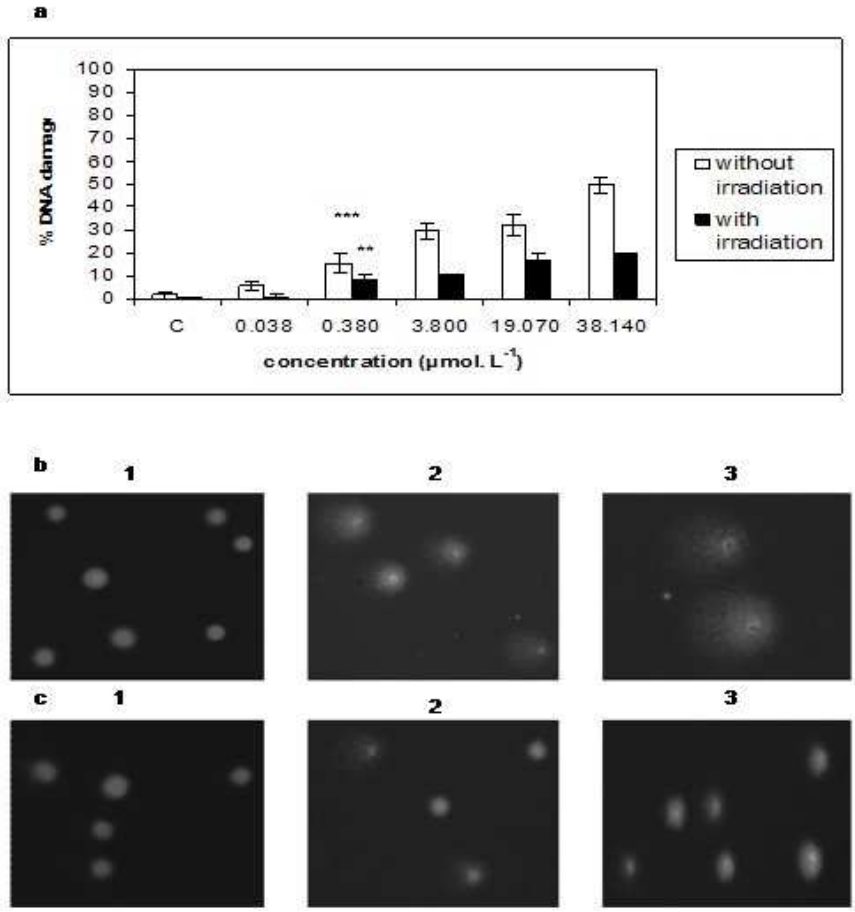


Fig. 6. Fluorescence analysis of DNA damage of HL-60 cells treated by non-phtoactivated (□) and photoactivated (■) DNQC (a) and the morphology of control (1) cells and damage HL-60 cells treated by non-photoactivated (b) and photoactivated (c) DNQC concentrations of 19.07 µmol.L^{-1} (2) and of 38.14 µmol.L^{-1} (3). ** $p<0.01$, ***<0.001.

Necrosis – lysis of cells treated with photoactivated quinolone derivatives was also demonstrated by Cardenas et al. (1991,1992), Trisciuoglio et al. (2002), Miolo et al. (2002), Ray et al. (2006). Authors presented that quinolones (nalidixic acid, oxolinic acid, rosoxacin,norfloxacin, ciprofloxacin, ofloxacin, enoxacin, lomefloxacin) induced red blood cell lysis after exposure with UVA irradiation. The photohaemolytic activity of quinolones was coupled with lipid peroxidation and DNA damage (degradation of deoxyguanosine).

Necrotic cell death (cell lysis) may be a normal physiological and regulated (programmed) event. Various stimuli (e.g. cytokines, ischemia, heat, irradiation, pathogens) can cause both necrosis and apoptosis in the same cell population. Necrosis and apoptosis appear to be a specific form of execution phase of programmed cell death, and there are

several examples of necrosis during embryogenesis, a normal tissue renewal, and immune response (Proskuryakov et al. 2003).

Recent studies have shed light on the mechanism by which programmed necrosis kills cells, highlighted the important features of programmed necrosis in patients treated with cancer chemotherapeutics and suggested an important physiologic role for programmed necrosis in response to viral infection (Edinger and Thompson 2004)

Programmed necrosis might also be important in protecting organisms from accumulating cells that have sustained DNA damage. Although DNA damage can initiate apoptosis, it appears that an apoptotic pathway is not required for the elimination of proliferating cells that acquire DNA damage. Thus, cells that have an impaired apoptotic response can still be removed by other form of cell death (Edinger and Thompson 2004) - cells deplete ATP followed by the poly (ADP-ribose) polymerase (PARP) activation and die by necrosis.

Programmed necrosis could help to explain how some chemotherapeutic agents induce caspase-independent cell death (Proskuryakov et al. 2003). This programmed cell death could bring new therapeutic targets for cancer treatment where apoptosis is blocked.

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