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(-)-Epigallocatechin-3-gallate Enhances Cytotoxic Effects of Epirubicin on U937 Cells

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(-)-Epigallocatechin-3-gallate (EGCG) represents natural polyphenolic compounds of the plant origin. Epirubicin (EPI) belongs to the second generation of anthracycline drugs. The present study was undertaken to assess a possible influence of EGCG and/or EPI on human histiocytic lymphoma U937 cells. The *in vitro* research was conducted using the Beckman Coulter method of cell sizing, as well as flow cytometry – FSC/SSC (forward scatter/side scatter) and FDA/PI (fluorescein diacetate/ propidium iodide) assays. The morphological and functional changes caused in U937 cells by each tested agent, EGCG and MAF, given alone or in combinations, were determined. The cytotoxic effects of (-)-epigallocatechin-3-gallate and epirubicin on human histiocytic lymphoma cells were shown. The combinations with EGCG enhanced the cytotoxic activity of epirubicin against U937 cells.

Key words: human histiocytic lymphoma U937 cells, (-)-epigallocatechin-3-gallate, epirubicin, combined action, morphological and functional changes, cytotoxic activity

INTRODUCTION

(-)-Epigallocatechin-3-gallate is the ester of epigallocatechin and gallic acid. EGCG is the most abundant and biologically active polyphenolic catechin in green tea *Camellia sinensis, Theaceae*. This polyphenol compound has gained significant attention among scientists as one of the leading plant-derived molecules studied for its health benefits (Chen et al., 2004; Gupta et al., 2008; Merelez and Hunstein, 2011; Bansal et al., 2012; Kanwar et al., 2012). (-)-Epigallocatechin-3-gallate is a potential therapeutic agent for cancer therapy (Farabegoli, 2005; Carlson et al., 2007; Shankar et al., 2007; Khan and Mukhtar, 2008; Blicharski et al., 2014). Elucidation of the action of EGCG, when applied alone or in combination with anticancer agents, can contribute to the development of more specific strategies against cancer.

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Epirubicin (EPI) is a semisynthetic derivative of doxorubicin obtained by axial – to – equatorial epimerization of the hydroxyl group at C-4' in daunosamine. EPI belongs to the second generation of anthracycline compounds with the improved anticancer properties. Epirubicin, an anthracycline drug, is primarily used for chemotherapy against breast and ovarian cancers, gastric cancer, lung cancer, and lymphomas (Bonfante, 1980; Hortobagyi, 1997; Minotti et al., 2004; Nadas and Sun, 2006; Khasraw et al., 2012). An increase in the effectiveness of epirubicin is of key importance in anticancer therapy.

The aim of the present study was to determine and compare possible cytotoxic activity of (-)-epigallocatechin-3-gallate and epirubicin, applied alone or in combinations, against human histiocytic lymphoma U937 cells. The morphological and functional changes occurring in U937 cells after their exposure to the action of EGCG and/or EPI, were analyzed.

MATERIALS AND METHODS

Cells

Human histiocytic lymphoma U937 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI 1640 medium (Gibco BRL Life Technologies), supplemented with 10% fetal calf serum (GIBCO BRL Life Technologies), 2 mM L-glutamine (Sigma Aldrich), and antibiotic antimycotic solution (AAS, Sigma Aldrich). AAS contained 20 units of penicillin, 20 µg streptomycin and 0.05 µg amphotericin B. Every third day the U937 cells were passaged. The cells grew at 37°C in an atmosphere of 5% CO_2 in air (HERAcell incubator, KendroLab). The cultures were periodically tested for *Mycoplasma infection*.

Chemicals

(-)-Epigallocatechin-3-gallate (EGCG, Sigma) and epirubicin hydrochloride (EPI, Bioepicin, Bioton S.A.) were used. EGCG and EPI were dissolved in 0.9% NaCl (Polpharma). All the solutions were freshly prepared directly before the treatment of the human histiocytic lymphoma U937 cells.

Agent doses and cell treatment

After diluting the cell suspension to a density of 15×10^4 cells/ml medium, the U-937 cells were exposed simultaneously to the action of EGCG and EPI. Based on the unpublished data, the doses of the tested compounds were chosen. (-)-Epigallocatechin-3-gallate was given at two single doses of 25 µg/ml and 50 µg/ml of the complete RPMI 1640 medium. Epirubicin was applied at a single dose of 0.3 µg/ml of the complete RPMI 1640 medium. Both EGCG and EPI were given alone and in combinations. The control material consisted of untreated U937 cells.

Analyses of U937 cells after EGCG and/or EPI application

Temporary changes occurring in the human histiocytic lymphoma U937 cells were analyzed at 24h and 48h after the application of (-)-epigallocatechin-3-gallate and/or epirubicin. At these two time intervals, the mean cell volume (Fig. 1), cell size and granularity (Fig. 2), as well as cell membrane integrity (Fig. 3), were assessed. The research was conducted using a Coulter counter and FACSCalibur flow cytometer.

Cell volume measurement

Samples of the U937 cell suspension were taken and immediately diluted in ISOTON II (Coulter filtered electrolyte solution based on 0.9% saline, Beckman Coulter). 500 µl of the cell suspension was added to 4.5 ml of ISOTON II. Immediately after the dilution of the cell suspension, individual U-937 cells were measured using the Z2 Coulter counter (Beckman Coulter). The instrument was calibrated using the Coulter CC size standard, 10 µm diameter latex beads (Beckman Coulter). Cell volume distributions of the U937 cells were obtained using the counter equipped with a 100 µm diameter orifice (Beckman Coulter). The flow rate was 500 µl/12.5 sec. The range for cell measurement was determined as 239.8-10230 fL. The cell volume was analyzed at 759.5-10230 fL. Using the Beckman Coulter method for cell sizing, the cell volume is detect-

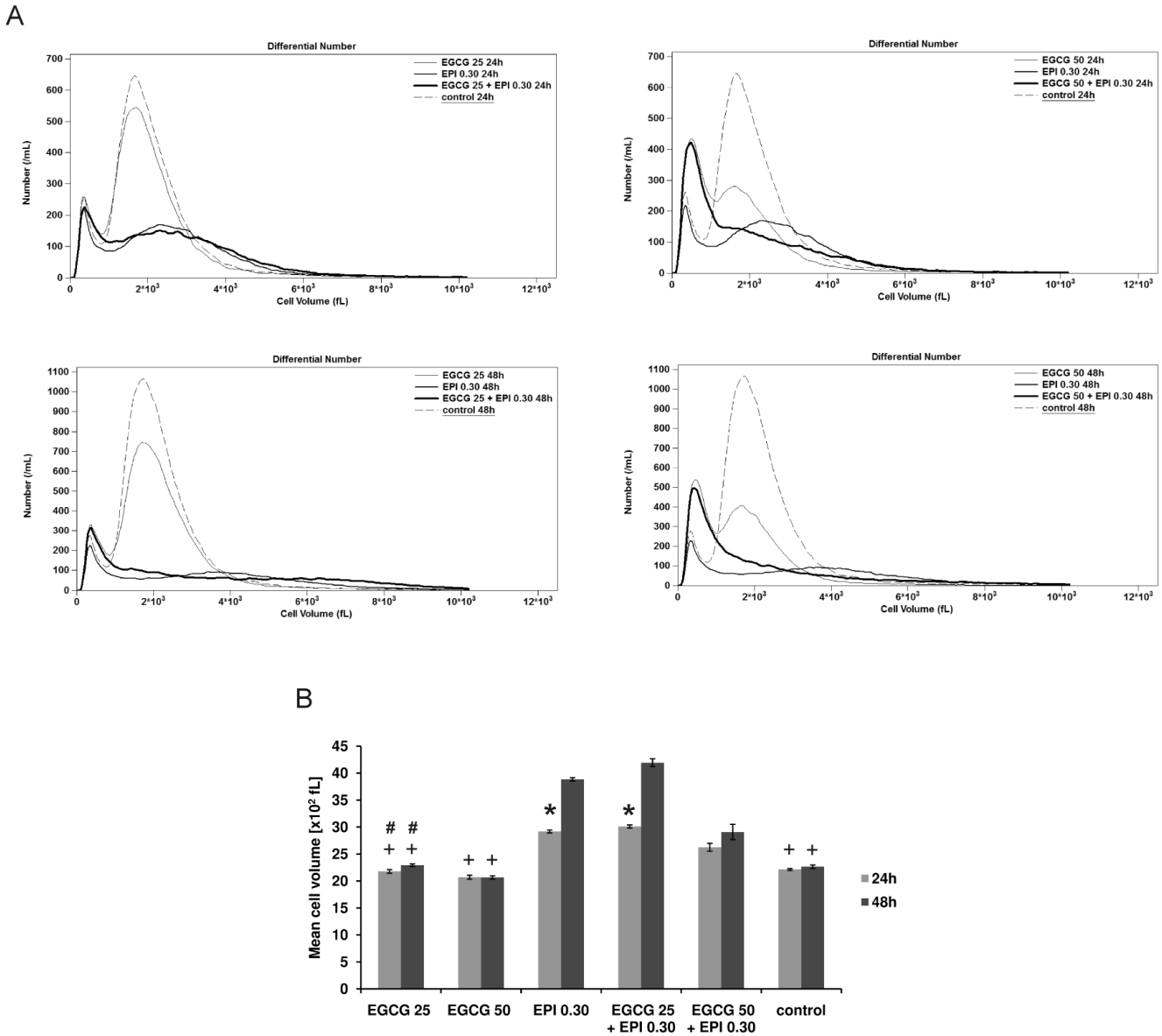

Fig. 1. Effects of EGCG and EPI on the U937 cell size. Representative curves for the volume distributions of U937 cells (A). The peaks on the left represent cellular debris, presumably apoptotic bodies and necrotic fragments, which were excluded from the cell volume analysis (A). The mean volume of U937 cells after their exposure to the action of EGCG and/or EPI (B). The obtained data are presented as mean values ± SD. Values not significantly different at $P<0.05$: * between the groups of U937 cells treated with the tested agent(s); # compared to controls; + between the time points. EGCG 25 – EGCG applied at a dose of 25 μg/ml medium; EGCG 50 – EGCG given at a dose of 50 μg/ml medium; EPI 0.3 – EPI applied at a dose of 0.3 μg/ml medium.

ed as the amplitude of the produced electrical pulse which results from the passage of each cell through the aperture. The mean cell volume was determined using the Z2 AccuComp software (Beckman Coulter).

Light scatter measurement

The cell suspension was centrifuged at 1000 rpm (MPW-351RH centrifuge, Med. Instruments) for 7 min at 4°C. The cell pellet was resuspended

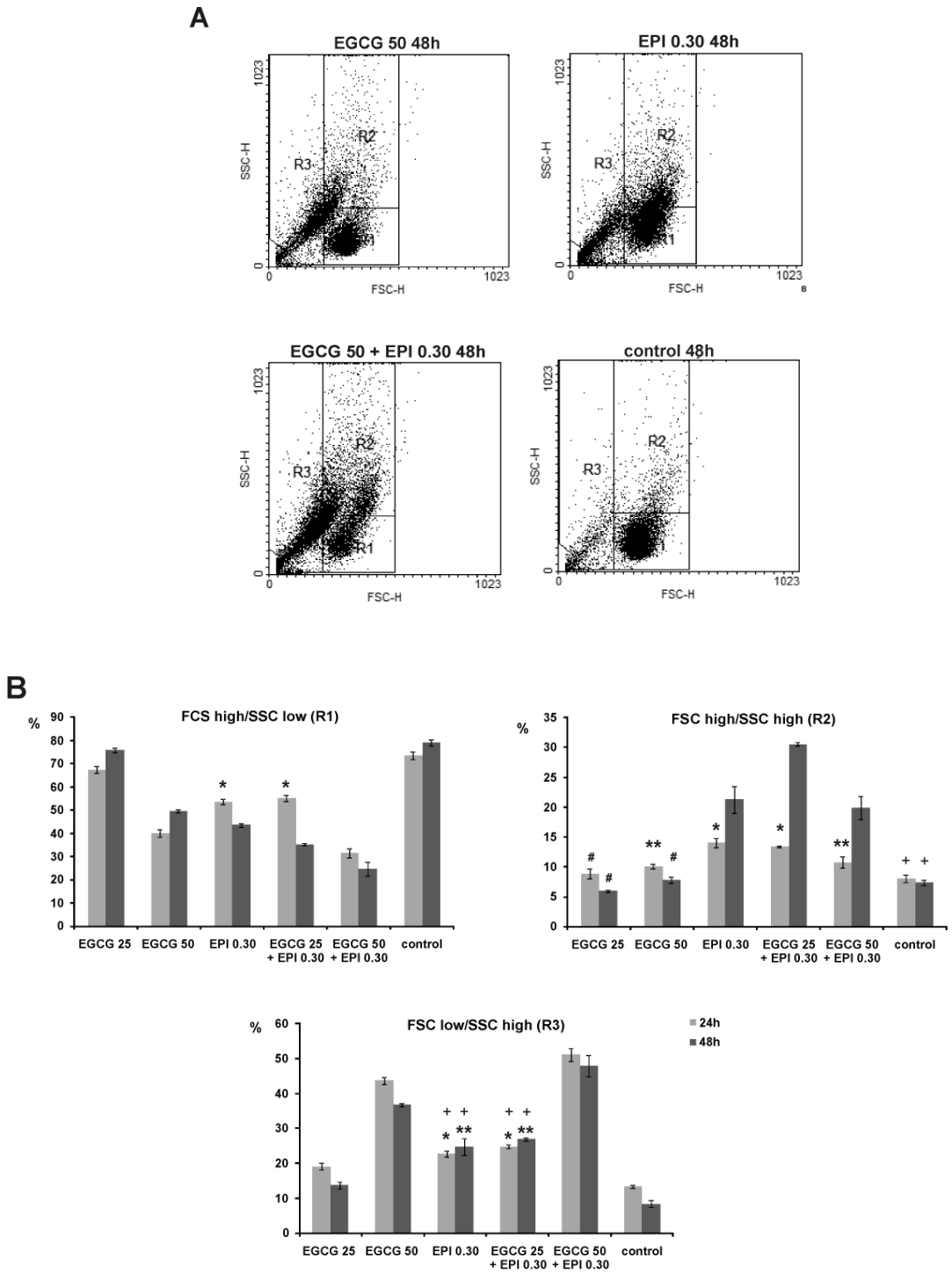

Fig. 2. Effects of EGCG and/or EPI on the light scattering properties of U937 cells. Flow cytometry analysis shows changes in the cell size (forward scatter, FSC on the x-axis) and granularity (side scatter, SSC on the Y-axis). Representative dot plots for U937 cells (A) and the values of FSC high/SSC low, FSC high/SSC high, and FSC low/SSC high (B). The obtained data (B) are presented as mean values ± SD. Values not significantly different at $P<0.05$: * ** between the groups of U937 cells treated with the tested agent(s); # compared to controls; + between the time points. EGCG 25 – EGCG applied at a dose of 25 µg/ml medium; EGCG 50 – EGCG given at a dose of 50 µg/ml medium; EPI 0.3 – EPI applied at a dose of 0.3 µg/ml medium.

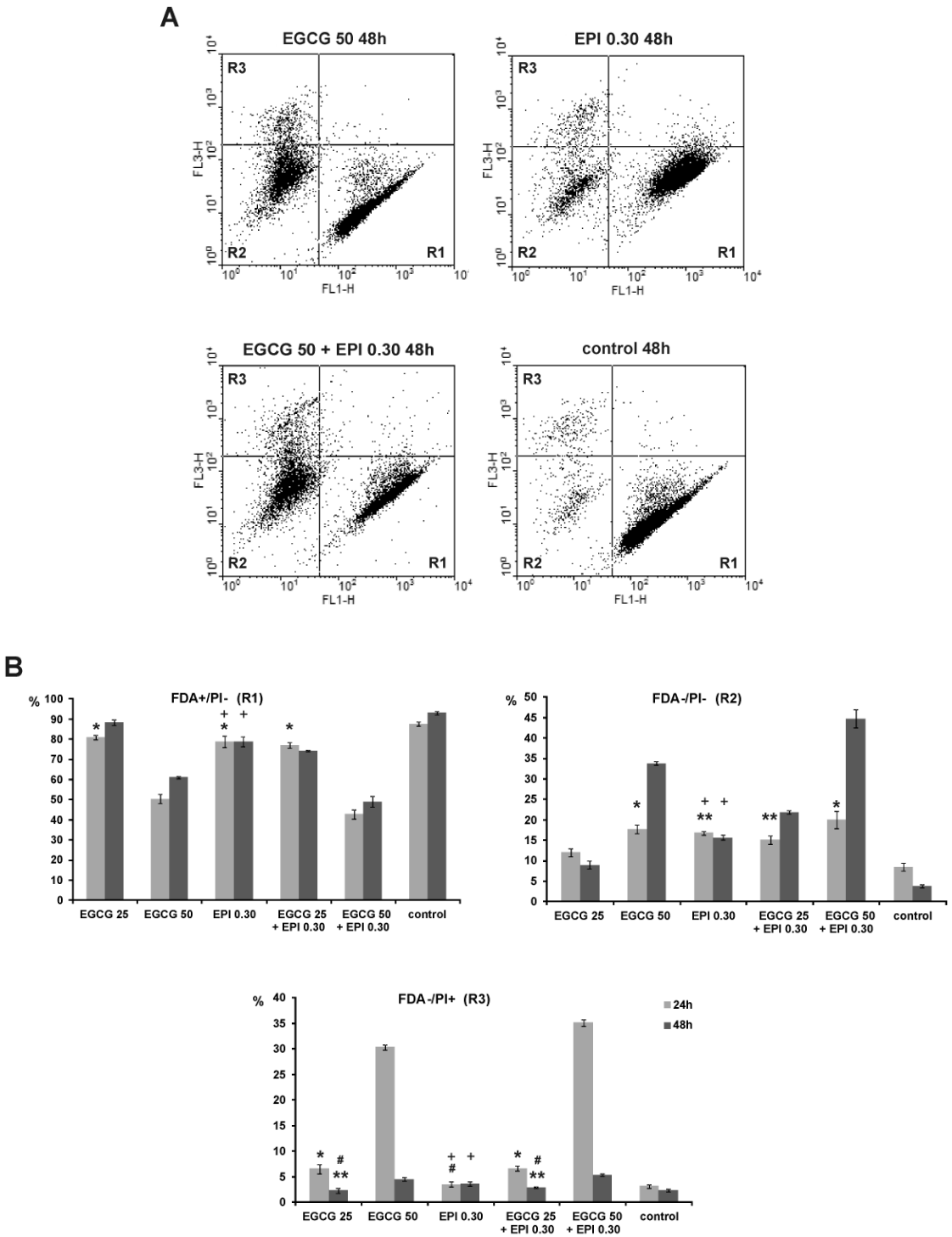

Fig. 3. Cytotoxic activity of EGCG and/or EPI against the U937 cells analyzed using the flow cytometry FDA/PI assay. Representative dot plots for U937 cells (A) stained with fluorescein diacetate FDA (FL1-H – fluorescence of FDA) and propidium iodide PI (FL3-H – fluorescence of PI), and the values of FDA+/PI-; FDA-/PI-; FDA-/PI+ (B). The obtained data (B) are presented as mean values ± SD. Values not significantly different at $P<0.05$: * ** between the groups of U937 cells treated with the tested agent(s); # compared to controls; + between the time points. EGCG 25 – EGCG applied at a dose of 25 μg/ml medium; EGCG 50 – EGCG given at a dose of 50 μg/ml medium; EPI 0.3 – EPI applied at a dose of 0.3 μg/ml medium.

in 500 µl of cold PBS, and forward scatter (FSC) and side scatter (SSC) were immediately measured using a FACSCalibur flow cytometer (Becton Dickinson) to detect changes in the cell size and granularity, respectively. The flow cytometry FSC/SSC assay shows that the intensity of light scattered in the forward direction (FSC) correlates with the cell size, and the intensity of scattered light measured at a right angle to the laser beam (SSC) correlates with the granularity/ presence of intracellular structures that can reflect the light. The data were analyzed using the CellQuest Pro software (Becton Dickinson). The frequencies of three cell populations, FSC high/ SSC low, FSC high/SSC high, and FSC low/SSC high, were determined.

FDA/PI assay

The cell suspension containing 5×10^5 cells was centrifuged at 1000 rpm (MPW-351RH centrifuge, Med. Instruments) for 7 min, and the cell pellet was resuspended in 1 ml of warm HBSS (Invitrogen). Then, 10 µl of fluorescein diacetate (FDA) working solution (at a concentration of 1 µg/ml of HBSS) were added and the cells were incubated in the dark for 15 min at 37°C. After incubation with FDA, 20 µl of propidium iodide (PI) working solution (at a concentration of 30 µg/ml of 0.9% NaCl) was added, and the cells were incubated for additional 5 min. Incubation of cells in the presence of both FDA and PI labels live cells green and dying cells red. Cell samples were placed on ice, away from light, and FDA and PI fluorescence was immediately measured using a FACS Calibur flow cytometer. The FDA/PI assay of membrane integrity, using fluorescein diacetate, shows that this substrate, after being taken up by live cells, is hydrolyzed by intracellular esterases. The product of the hydrolysis, fluorescein, is a highly fluorescent, charged molecule which becomes trapped in intact cells. Propidium iodide is excluded by an intact cell membrane. The data were analyzed using the CellQuest Pro software. The frequencies of three cell populations, FDA+/PI-, FDA-/PI-, and FDA-/PI+, were determined.

Statistical evaluation

Statistical significance of alterations in the analyzed parameters was evaluated by an analysis of variance and Duncan's new multiple range test. A difference was considered statistically significant if $P<0.05$.

RESULTS

The cytotoxic effects of (-)-epigallocatechin-3-gallate and/ or epirubicin on human histiocytic lymphoma U937 cells were determined using the Beckman Coulter method of cell sizing, as well as flow cytometry FSC/SSC and FDA/PI assays. The different patterns of temporary changes in the mean cell volume (Fig. 1), size and granularity (Fig. 2), and membrane integrity (Fig. 3) were observed at 24h and 48h after the application of EGCG, at two doses of 25 µg/ml and 50 µg/ml, and/or EPI at a dose of 0.3 µg/ml of medium.

The exposure of U937 cells to the action of EGCG and MAF, given alone and in combinations, distinctly affected the mean cell volume (Fig. 1). EGCG applied alone, at a higher dose, caused a decrease in the mean cell volume. EPI, given alone and in combinations with EGCG, caused an increase in the mean cell volume, especially at 48h after the agent(s) application. The influence of the combined action of catechin and anthracycline compouds on the U937 mean cell volume depended on the dose of EGCG.

Distinct alterations in the cell size and granularity of U937 cells were observed (Fig. 2). The exposure of U937 cells to the action of EGCG and EPI, applied alone and in combinations, resulted in a decrease in the frequency of the FSC high/ SSC low cell population, observed especially after the combined application of EPI and EGCG given at a higher dose. The application of catechin and anthracycline agents caused an increase in the frequency of FSC low/SSC high cell population, especially when EGCG was given alone at a higher dose, and when the combination with EPI was used. The increased percentage values of FSC high/SSC high cell population were found after the exposure of U937 cells to the action of EPI, and both EGCG and EPI, and after 24h when

EGCG was given alone at a higher dose. The distinctly higher frequency of FSC high/SSC high cell population was observed at 48h, compared with that found at 24h after the application of EPI alone and in combinations with EGCG.

The application of EGCG and EPI distinctly affected the membrane integrity of U937 cells (Fig. 3). After the exposure of U937 cells to the action of EGCG and EPI, given alone and in combinations, a decrease in the frequency of FDA+/PI- cell population, and an increase in the frequency FDA-/PI- population, were found. The highest values of FDA-/PI+ cell populations were observed at 24h after the application of EGCG alone, at a higher dose, and in combination with EPI. The highest values of FDA-/PI+ cell populations were found at 48h after the exposure of U937 cells to the action of EGCG given alone, at a higher dose, and in combination with EPI. The combined action of EPI and EGCG, given at a higher dose, caused the greatest reduction in the frequency of FDA+/PI- cell population.

DISCUSSION

In the present study, the cytotoxic activity of (-)-epigallocatechin-3-gallate and epirubicin against U937 cells was evaluated. The various patterns of temporary changes in the mean cell volume (Fig. 1), cell size, granularity (Fig. 2), and membrane integrity/ esterase activity (Fig. 3) were found at 24h and 48h after the exposure of U937 cells to the action of EGCG and/or EPI. The cytotoxic effects of EGCG and EPI on human histiocytic lymphoma cells were agent-, time-, and EGCG-dose-dependent. The effect of the combined action of EGCG and EPI on the morphological and functional alterations occurring in U937 cells was greater than the effect of each of the tested agents, polyphenolic catechin and anthracycline when applied alone.

The modes of action of EGCG and EPI on the cells, and the precise mechanisms responsible for the cytotoxic effects caused by these agents are not fully known yet (Minotti et al., 2004; Feng, 2006; Khan et al., 2006; Singh et al., 2011; Bansal et al., 2012; Kim et al., 2014). Nevertheless, multiple mechanisms have been proposed to explain the cytotoxic actions of polyphenolic catechin and anthracycline agents. The mechanisms of action of (-)-epigallocatechin-3-gallate can include the scavenging of reactive oxygen and nitrogen species and chelating of redox-active transition metal ions, as well as production of hydrogen peroxide and hydroxyl radicals. The anti-oxidant or pro-oxidant properties of EGCG depend on its dose and the cell type and status (Gupta et al., 2008; Kim et al., 2014). EGCG can modulate several key molecular signaling pathways at multiple levels, and it can regulate transcription factors, DNA methylation. This polyphenolic catechin can directly interact with a large set of proteins and phospholipids (Khan et al., 2006; Khan and Mukhar, 2008; Singh et al., 2011; Kanwar et al., 2012; Kim et al., 2014). The mechanisms of action of epirubicin, an anthracycline agent, include intercalation of DNA, inhibition of topoisomerase II activity, generation of free radicals with consequent interference with DNA, RNA and proteins (Minotti et al., 2004). The cytotoxic action of EGCG and EPI can alter the physiological responses of cells at molecular, biochemical and morphological levels (Minotti et al., 2004; Feng, 2006; Khan et al., 2006; Gupta et al., 2008; Kim et al., 2014).

To sum up, the findings of the present study are the first available data showing the anticancer potential of the combined action of EGCG and EPI on human histiocytic lymphoma cells. A better understanding of the exact mechanisms of action of (-)epigallocatechin-3-gallate and epirubicin on the pathological hematopoietic cells can offer an opportunity for the development of improved anticancer therapy.

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REFERENCES

Bansal, S., N. Syan, P. Mathur, and S. Choudhary. 2012. Pharmacological profile of green tea and its polyphenols: a review. *Med. Chem. Res.* 21: 3347–3360.

Blicharski, K., M. Łukawska, I. Oszczapowicz, M. Stojak, and L. Mazur. 2014. Effects of (-)-epigallocatechin-3-galate and

oxazolinodaunorubicin on human leukemia cells. *XXVIII Ogólnopolskie Seminarium „Mechanizmy służące utrzymaniu życia i regulacji fizjologicznych"* Kraków, 46–47.

Bonfante, V. 1980. Preliminary clinical experience with 4-epidoxorubicin in advanced human neoplasia. *Rec. Results Canc. Res.* 74: 192–199.

Carlson, J.R., B.A. Bauer, A. Vincent, P.J. Limburg, and T. Wilson. 2007. Reading the tea leaves: Anticarcinogenic properties of (-)-epigallocatechin-3-gallate. *Mayo Clin. Proc.* 82: 725–732.

Chen, D., K.G. Daniel, D.J. Kuhn, A. Kazi, M. Bhuiyan, L. Li, Z. Wang, S.B. Wan, W.H. Lam, T.H. Chan, and Q.P. Dou. 2004. Green tea and tea polyphenols in cancer prevention. *Front. Biosci.* 9: 2618–2631.

Farabegoli, F. 2005. Green tea in human cancer. *Minerva Biotecnol.* 17: 163–173.

Feng, Y.F. 2006. Metabolism of green tea catechins: an overview. *Curr. Drug Metab.* 7: 755–809.

Gupta, J., Y.H. Siddique, T. Beg, G. Ara, and M. Afzal. 2008. A review on the beneficial effects of tea polyphenols on human health. *Int. J. Pharmacol.* 4: 314–338.

Hortobagyi, G.N. 1997. Anthracyclines in the treatment of cancer. An overview. *Drugs* 54: 1–7.

Kanwar, J., M. Taskeen, I. Mohammad, C. Huo, T.H. Chan, and Q.P. Dou. 2012. Recent advances on tea polyphenols. *Front. Biosci.* 4: 111–131.

Khan, N., F. Afaq, M. Saleem, N. Ahmadn, and H. Mukhtar. 2006. Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res.* 66: 2500–2505.

Khan, N., and H. Mukhtar. 2008. Multitargeted therapy of cancer by green tea polyphenols. *Cancer Lett.* 269: 269–280.

Khasraw, M., R. Bell, and C. Dang. 2012. Epirubicin: Is it like doxorubicin in breast cancer? A clinical review. *Breast* 21: 142–149.

Kim, H.S., M.J. Quon, and J. Kim. 2014. New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from green tea polyphenol, epigallocatechin 3-gallate. *Redox Biol.* 2: 187–195.

Merelez, D., and W. Hunstein. 2011. Epigallocatechin-3-gallate (EGCG) for clinical trials: more pitfalls than promises? *Int. J. Mol. Sci.* 12: 5592–5603.

Minotti, G., P. Menna, E. Salvatorelli, G. Cairo, and A. Gianni. 2004. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* 56: 185–229.

Nadas, J., and D. Sun. 2006. Anthracyclines as effective anticancer agents. *Expert Opin. Drug. Discov.* 1: 539–548.

Shankar, S., S. Ganapathy, and R.K. Srivastava. 2007. Green tea polyphenols: Biology and therapeutic implications in cancer. *Front. Biosci.* 12**:** 4881–4899.

Singh, B.N., S. Shankar, and R.K. Srivastava. 2011. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem. Pharmacol. 82*: 1807–1821.