



## COMBINED ACTION OF (-)-EPIGALLOCATECHIN-3-GALLATE AND MAFOSFAMIDE ON HL-60 CELLS

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(-)-Epigallocatechin-3-gallate (EGCG) is a natural polyphenolic compound of the plant origin. Mafosfamide (MAF) belongs to the oxazaphosphorine agents. The aim of the present study was to determine *in vitro* antileukemic activity of (-)-epigallocatechin-3-gallate (EGCG) and mafosfamide (MAF). The experiments were performed on human promyelocytic leukemia HL-60 cells. The research was conducted using spectrophotometric and flow cytometric methods. The cell viability was analyzed using spectrophotometric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test, and flow cytometry FSC/SSC (forward scatter / side scatter) and FDA / PI (fluorescein diacetate / propidium iodide) assays. The influence of each tested agent, EGCG and MAF, given alone or in combination, on HL-60 cells was assessed. The cytotoxic effects of these two polyphenolic and oxazaphosphorine agents on human promyelocytic leukemia cells were dose- and time-dependent. EGCG and also MAF, applied alone, affected the leukemia cell viability. The combined action of (-)-epigallocatechin-3-gallate and mafosfamide enhanced the antileukemic activity against HL-60 cells.

**Key words:** human promyelocytic leukemia HL-60 cells, (-)-epigallocatechin-3-gallate, mafosfamide, combined action, cell viability assays, antileukemic activity

### INTRODUCTION

(-)-Epigallocatechin-3-gallate is the ester of gallic acid and epigallocatechin. EGCG is the most abundant and biologically active polyphenolic catechin in green tea *Camellia sinensis*. Among the green tea catechins, EGCG is accepted to be the

strongest chemopreventive and anticancer agent (CHEN et al., 2004; CARLSON et al., 2007; GUPTA, et al., 2008; MERELEZ and HUNSTEIN, 2011; SINGH et al., 2011; KANWAR et al., 2012). Mafosfamide is a chemically stable 4-thioethane sulfonic acid salt of 4-hydroxy-cyclophosphamide. MAF represents a new generation of oxazaphosphorine anticancer

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cer agents (LIANG et al., 2007; GIRAUD et al., 2010; MAZUR et al., 2012).

(-)-Epigallocatechin-3-gallate and mafosfamide are potential therapeutic compounds for cancer therapy (KHAN and MUKHTAR, 2008; GIRAUD et al., 2010; SINGH et al., 2011; MAZUR et al., 2012). EGCG as a natural compound could be useful either alone or in combination with other agents for the prevention of cancer progression and/or treatment of human malignancies. Elucidation of biological properties of EGCG and MAF can open up an opportunity for the development of improved anticancer therapy. Therefore, EGCG and MAF have gained significant attention among scientists, and these agents are still under investigation (BANSAL et al., 2012; MAZUR et al., 2012, 2013; OPYDO-CHANEK, 2013; Blicharski et al. 2014; KIM et al., 2014).

The cell viability is an important parameter characterizing potential antileukemic activity of the tested agents. The viability of cell population can be determined using spectrophotometric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test, and flow cytometry FSC/SSC (forward scatter / side scatter) and FDA / PI (fluorescein diacetate / propidium iodide) assays. Using the MTT assay, in viable, metabolically active cells, the tetrazolium ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide is cleaved, yielding formazan crystals. Changes in the metabolic activity and viability of cell populations result in a concomitant change in the amount of formazan formed. The other viability assay is based on the measurement of light scattered by the cells. A cell traversing through the focus of the laser beam in a flow cytometer scatters the laser light. Analysis of the scattered light provides information about the cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with the cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter, SSC) correlates with granularity and the presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death. The FDA/PI assay of membrane integrity employs the non-fluorescent esterase substrate, fluorescein diacetate and propidium iodide. Fluorescein diacetate, 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 membrane (DARZYŃKIEWICZ et al., 1997).

The present study was undertaken to assess the antileukemic activity of (-)-epigallocatechin-3-gallate and mafosfamide, applied alone or in combination, against human promyelocytic leukemia cells. The cell viability of HL-60 cells exposed to the action of EGCG and/or MAF was analyzed using spectrophotometry and flow cytometry assays.

## MATERIALS AND METHODS

### Cells

Human promyelocytic leukemia HL-60 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, HL-60 cells were passaged. The cells grew at 37°C in an atmosphere of 5% CO<sub>2</sub> in air (HERAcell incubator, KendroLab).

### Chemicals

(-)-Epigallocatechin-3-gallate (EGCG, Sigma) and mafosfamide cyclohexylamine salt (MAF, NIOMECH, Bielefeld, Germany) were used. EGCG and MAF were dissolved in 0.9 % NaCl (Polpharma). All the solutions were freshly prepared directly before the treatment of the leukemia HL-60 cells.

### Agent doses and cell treatment

After diluting the cell suspension to a density of  $15 \times 10^4$  cells/ml medium, HL-60 cells were exposed simultaneously to the action of EGCG and

MAF. 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. Mafosfamide cyclohexylamine salt was applied at a single dose of 15 µg/ml of the complete RPMI 1640 medium. EGCG and also MAF were given alone, as well as both these agents were applied in combination. The control material consisted of untreated HL-60 cells.

#### Analyses of HL-60 cell viability after EGCG and/or MAF application

The cell viability of HL-60 cells was analyzed at 24 h and 48 h after the EGCG and/or MAF application. The research was conducted using the spectrophotometry and flow cytometry assays.

#### Spectrophotometric MTT assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich) was dissolved in RPMI 1640 medium, at a concentration of 5 mg/ml and filtered through a 0.2 µm filter. Subsequently, 100 µl of the yellow MTT solution was added to each well of a 24-well plate, containing 1 ml of the cell suspension. The cells were then incubated at 37°C with 5% CO<sub>2</sub>. A blank solution was prepared according to the above procedure using complete medium without cells. After a three-hour incubation period, the resulting formazan crystals were dissolved using 1 ml of acidified isopropanol (0.05 N HCL in absolute isopropanol), and the absorbance of the obtained solution was measured at a wavelength of 570 nm using a Pharmacia Ultrospec III spectrophotometer (Pharmacia LKB Biotechnology).

#### Cell viability assay by 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 in 500 µl of cold PBS, and forward scatter and side scatter were immediately measured using

a FACSCalibur flow cytometer (Becton Dickinson) to detect changes in the cell size and granularity, respectively. Data were analyzed using CellQuest Pro software (Becton Dickinson). The frequency of three cell populations, FSC high/SSC low, FSC low/SSC high, and FSC high/SSC high, was determined.

#### Flow cytometry FDA/PI assay

The cell suspension containing 5×10<sup>5</sup> 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 FDA working solution (at a concentration of 1 µg/ml of HBSS) was added and the cells were incubated in the dark for 15 min at 37°C. After incubation with FDA, 20 µl of 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. Data were analyzed using CellQuest Pro software. The frequency of three cell populations, FDA+/PI-, FDA-/PI-, and FDA-/PI+, was 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 with P<0.05 was considered statistically significant.

## RESULTS

The effects of (-)-epigallocatechin-3-gallate and/or mafosfamide on the viability of human promyelocytic leukemia HL-60 cells were determined using spectrophotometric MTT test, and FSC/SSC and FDA/PI flow cytometry assays. The different patterns of temporary changes in the cell viability rate (Fig.1), size and granula-

urity (Fig. 2) and membrane integrity (Fig.3) were observed at 24 h and 48 h after the application of EGCG, at two doses of 25  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$ , and/or MAF at a dose of 15  $\mu\text{g/ml}$  of medium.

The exposure of HL-60 cells to the action of EGCG and MAF, applied only, and also in combination, distinctly decreased the viability rate of HL-60 cells (Fig.1). The combined application of EGCG and MAF affected the leukemia cell viability to a higher degree than did each of the tested agent given alone. The influence of the combined action of the catechin and oxazaphosphorine agents on the HL-60 cell viability rate was dependent on the dose of EGCG.

Distinct changes in the cell size and granularity of HL-60 cells were found (Fig.2). The exposure of HL-60 cells to the action of EGCG and MAF, applied only and in combination, resulted in a decrease in the frequency of the FSC high/SSC low cell population and an increase in the percentage values of two cell populations, FSC low/SSC high and FSC high/SSC high.

The application of EGCG and MAF distinctly affected the membrane integrity of HL-60 cells (Fig.3). After exposure of HL-60 cells to the action of EGCG and MAF, applied alone and in combination, significant changes in the frequency of FDA+/PI-, FDA-/PI-, and FDA-/PI+ cell populations were assessed. The distinctly lower frequency of FDA+/PI- cell population and the

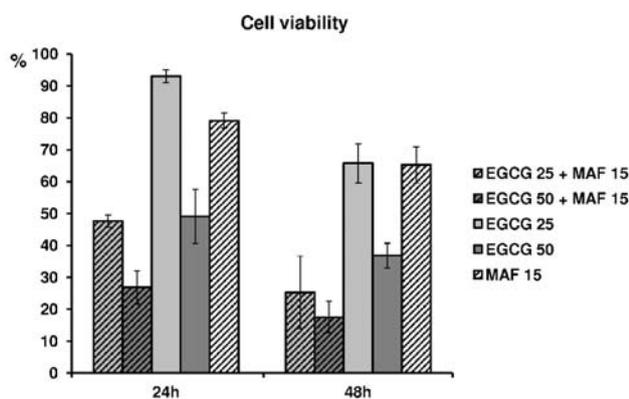
significantly higher frequency of two cell populations, FDA-/PI- and FDA-/PI+, were observed after the combined action of EGCG and MAF as compared with the percentage values found after the application of each of the tested agent only.

## DISCUSSION

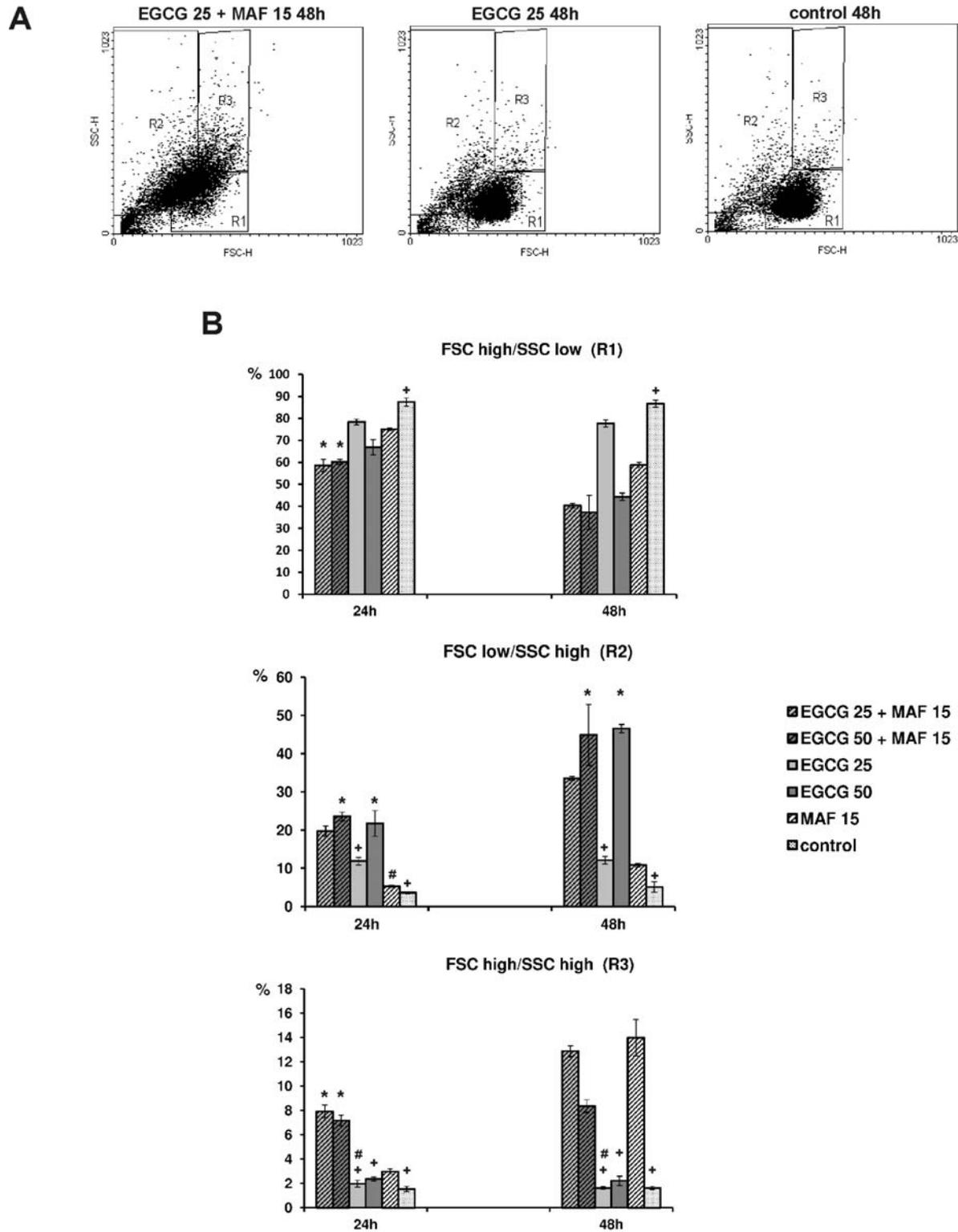
The results of the present study have shown various *in vitro* antileukemic activities of (-)-epigallocatechin-3-gallate and mafosfamide against HL-60 cells. The cytotoxic effects of EGCG and MAF on human promyelocytic leukemia cells were dose- and time-dependent. Moreover, it should be underlined that the combined action of EGCG and MAF affected the HL-60 cell viability to a greater degree than did each of the tested agent, polyphenolic catechin and oxazaphosphorine.

The mechanisms of action of (-)-epigallocatechin-3-gallate and mafosfamide have been extensively investigated in recent years. The cell response to the action of EGCG and MAF was determined at the molecular, biochemical, and cellular levels. Nevertheless, the modes of their action on the cells have not been fully explained yet (KHAN et al., 2006, 2008; SINGH et al., 2011; BANSAL et al., 2012; MAZUR et al., 2012, 2013; OPYDO-CHANEK et al., 2013; BLICHARSKI et al., 2014; KIM et al., 2014).

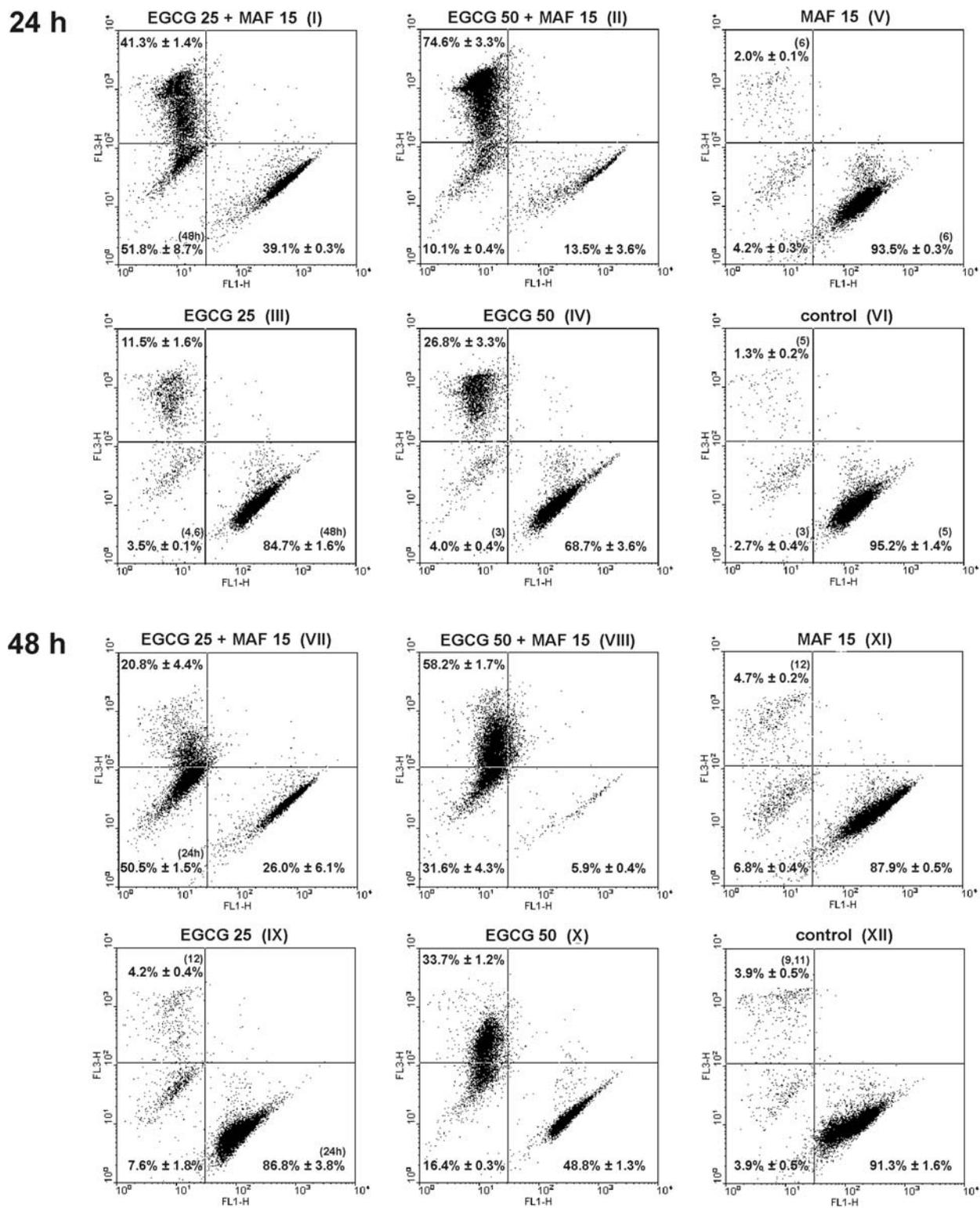
(-)-Epigallocatechin-3-gallate, a polyphenolic agent, is often classified as an anti-oxidant acting by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions. However, EGCG also functions as a pro-oxidant producing hydrogen peroxide and hydroxyl radicals. The anti-oxidant or pro-oxidant action of EGCG depends on its dose and the cell type and status (GUPTA et al., 2008; KIM et al., 2014). Recent studies have revealed many other direct actions of EGCG which alter the physiological responses of cells (FENG, 2006; GUPTA et al., 2008; KIM et al., 2014). Catechin modulates several key molecular signaling pathways at multiple levels, and regulates transcription factors, DNA methylation. EGCG can directly interact with a large set of proteins and phospholipids in the plasma membrane, and affects mitochondrial function. The action of this polyphenolic agent results in the cell cycle arrest and induction of programmed



**Fig. 1.** Effects of EGCG and MAF on the HL-60 cell viability rate. The extent of MTT conversion in HL-60 cells expressed as a percentage of the control value. The data are presented as mean values  $\pm$  SD. Values not significantly different at  $P < 0.05$ : \* between the groups of leukemia cells treated with the tested agent(s); # compared to controls; + between the time points. EGCG 25 – EGCG applied at a dose of 25  $\mu\text{g/ml}$  medium; EGCG 50 – EGCG given at a dose of 50  $\mu\text{g/ml}$  medium; MAF 15 – MAF applied at a dose of 15  $\mu\text{g/ml}$  medium.



**Fig. 2.** Effects of EGCG and MAF on the light scattering properties of HL-60 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 HL-60 cells (A) and the values of FSC high/SSC low, FSC low/SSC high and FSC high/SSC high (B). The obtained data (B) are presented as mean values  $\pm$  SD. Values not significantly different at  $P < 0.05$ : \* between the groups of leukemia cells treated with the tested agent(s); # compared to controls; + between the time points. EGCG 25, 50; MAF 15 – see description Fig.1.



**Fig. 3.** Effects of EGCG and MAF on the HL-60 cell viability analyzed using the flow cytometry FDA/PI assay. Representative dot plots for HL-60 cells stained with fluorescein diacetate FDA (FL1-H – fluorescence of FDA) and propidium iodide PI (FL3-H – fluorescence of PI). The data are presented as mean values  $\pm$ SD. No statistically significant differences ( $P < 0.05$ ) between the values shown on dot plots : III – (3); IV – (4); V – (5); VI – (6); IX – (9); XI – (11); XII – (12). The value not significantly different from 24h – 24h; 48h - 48h. EGCG 25, 50, MAF 15 – see description Fig.1.

cell death (KHAN et al., 2006, 2008; SINGH et al., 2011; KANWAR et al., 2012; Blicharski et al., 2014; KIM et al., 2014).

Mafosfamide, an oxazaphosphorine agent, is a pre-activated cyclophosphamide analog. The effects of MAF on cells are accepted as being dependent mainly on its metabolites, phosphoramidate mustard and acrolein. These reactive alkylating agents can bind to a variety of molecules and react with available groups of amino acids, proteins and peptides, but the DNA binding site is most important. MAF caused the disruption of DNA function, the cell cycle disturbance and induced mitotic catastrophe and programmed cell death (ZHANG et al., 2005; LIANG et al., 2007; GIRAUD et al., 2010; MAZUR et al., 2012, 2013; Opydo-Chanek et al., 2013).

A better understanding of the exact mechanisms of action of (-)epigallocatechin-3-gallate and mafosfamide, given only and in combination, on the pathological hematopoietic cells is of great importance in experimental hematology. Elucidation of the modes of the combined action of EGCG and MAF can contribute to the development of more specific strategies against hematological malignancies.

To summarize, the research has revealed the effects of (-)epigallocatechin-3-gallate and mafosfamide, applied alone or in combination, on temporary changes in the viability of human promyelocytic leukemia HL-60 cells. These are the first data showing the antileukemic potential of the combined action of EGCG and MAF.

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