Original Article

Benfluron Induces Cell Cycle Arrest, Apoptosis and Activation of p53 Pathway in MOLT-4 Leukemic Cells

(benfluron / apoptosis / cell cycle / MOLT-4 / p53)

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Abstract. The aim of our study was to determine the effect of potential anti-tumour agent benfluron on human leukemic cells MOLT-4 and elucidate the molecular mechanisms of response of tumour cells to this chemotherapeutic agent. It has been shown that the mechanisms of action of benfluron are complex, but the molecular pathways of the cytostatic effect have remained unknown and the present study contributes to their elucidation. In this work, benfluron reduced viability of the treated cells and induced caspase-mediated apoptosis. The programmed cell death was associated with activation of caspases 8, 9 and 3/7. Moreover, exposure of cells to benfluron resulted in accumulation of the cells primarily in late S and G2/M phases. The changes in the levels of key proteins show that benfluron provoked activation of p53 and induced phosphorylation of p53 on serine 15 and serine 392. The application of benfluron led to phosphorylation of Chk1 on serine 345 and phosphorylation of Chk2 on threonine 68 in the treated cells. Higher doses of benfluron caused phosphorylation of ERK1/2 on threonine 202 and tyrosine 204, whereas JNK and p38 kinases were not activated. In conclusion, benfluron induces apoptosis, cell cycle arrest in late S and G2/M phases, and activates various signalling pathways of the DNA damage response.

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Abbreviations: BF – benfluron, MAPK – mitogen-activated protein kinases, MTX – mitoxantrone, PC – positive control, PI – propidium iodide, PVDF – polyvinylidene fluoride.

Introduction

Benfluron (BF) is an antineoplastic agent that can be used in the treatment of numerous types of cancer. Benfluron was prepared in the Research Institute for Pharmacy and Biochemistry in Prague in the 1980s. The mechanism of action of benfluron was extensively studied in the past, but the molecular pathways of its cytostatic and cytotoxic effects have not been completely elucidated (Horáková et al., 1987).

It was found that BF induced the cytotoxic reaction in dependence on the concentration applied. The decrease in cell proliferation was found in a range of micromolar concentrations (Horáková et al., 1988). Benfluron exhibits effects against several tumours such as murine tumours, Crocker sarcoma 180, sarcoma Sa 37, Krebs tumours, and rat Yoshida tumours (Horáková et al., 1987) and against murine leukaemias La and L1210 (Pujman and Černochová, 1981). The antineoplastic effect of benfluron positively influenced the survival of leukemic mice, in which the life span increased in dependence on the dose and the time of its application (Pujman and Černochová, 1981).

The cytostatic and cytotoxic effects of BF were also proved in the studies performed *in vitro*. Inhibition of cell proliferation was proved in HeLa and V/79 cells (Horáková et al., 1988), Ehrlich carcinoma cells and P388 leukaemia cells (Miko et al., 1989). As well, Soucek et al. (1987) showed that BF had a significant cytostatic effect on nine human leukemic cell lines *in vitro*. In their study BF exhibited higher cytotoxicity in lymphoblastoid cell lines than in the myeloid ones.

The mechanisms of the cytostatic effect of BF are complex and currently not fully understood. Early studies assessed the influence of BF on the cell metabolism, proliferation and cell membrane integrity. It was established that benfluron may influence cell membrane processes, as well as synthesis of proteins and nucleic acids (Miko et al., 1989). The final cytotoxic effect of BF is concentration-dependent and differs in various cell types. The treatment of some cell types with BF resulted

in cell cycle arrest in S and G2 phases, whereas higher concentrations of BF induced cell lysis documented by changes in morphology and cell membrane permeability (Horáková et al., 1988). In the study of Jantová et al. (1992), the application of cytotoxic concentrations of BF was accompanied by unbalanced metabolism, BF caused gluconeogenesis, and the influenced cells utilized glutamine as the main source of energy. The highest tested concentration induced perforations of the cytoplasmic membrane. In the study of Horáková et al. (2001), the mechanisms of cytotoxic action of BF was assumed to be a consequence of oxidative reactions.

It is unknown whether BF affects DNA, which is a primary target for most of the commonly used anticancer compounds. In the present work, the underlying mechanism of the anticancer action of benfluron was elucidated by evaluating cell processes and signalling pathways that are triggered in response to DNA damage caused by this chemotherapeutic agent.

Material and Methods

Cell cultures and culture conditions

The experiments were carried out in the MOLT-4 cell line (human leukemic T lymphocytes) from the American Type Culture Collection ATCC (Manassas, VA). MOLT-4 cells were cultured in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO), supplemented with 20% foetal calf serum (Sigma-Aldrich), L-glutamine, penicillin and streptomycin (all from Sigma-Aldrich) in a humidified incubator at 37 °C and a controlled 5% CO_2 atmosphere. The culture was divided every 2nd day by dilution to a concentration of 2×10^5 cells/ml. The cell lines in the maximal range of up to 20 passages were used for this study.

Chemotherapeutic agents

The stock solution of benfluron was prepared by dissolving 1.77 mg of the substance in 5 ml distilled sterile water (Roche, Basel, Switzerland) to reach 1 mmol/l concentration. The stock solution was further diluted to reach a concentration of 0.1 mmol/l of a working solution prior to the experiments. The final concentrations to which the cells were exposed were 0.2–1 μmol/l BF.

Mitoxantrone (MTX) (Sigma-Aldrich) was prepared by dissolving 1.3 mg of the substance in 2.5 ml of distilled sterile water (final concentration c = 1 mmol/l). For the experiments, the stock solution was diluted to final concentration 5 nmol/l.

Proliferation and viability

Cell proliferation and viability were determined 24, 48 and 72 h after the treatment with 0.2, 0.4, 0.6, 0.8 and 1 μmol/l benfluron. The experiment was performed using the trypan blue exclusion staining technique. The number of cells and the percentage of cells that excluded trypan blue were counted using Bürker chamber.

Activity of caspases

Induction of apoptosis was determined by monitoring the activities of caspase 3/7, caspase 8, and caspase 9 by Caspase-Glo Assays (Promega, Madison, WI). The assays provide proluminogenic substrates in an optimized buffer system. The addition of Caspase-Glo reagents results in cell lysis, followed by caspase cleavage of the substrate and generation of luminescent signal. The signal generated is proportional to the amount of caspase activity present. A total of 1×10^4 cells were seeded per well using a 96-well-plate format (Sigma-Aldrich). The cells treated with 0.4, 0.5 and 0.6 μmol/l BF and 5 nmol/l mitoxantrone were cultivated in 50 μl culture medium and incubated 24 and 48 h in 5% CO_2 at 37 °C. After that, Caspase-Glo assay reagents were added to each well (50 μl/well) and incubated for 30 min before measuring luminescence using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland).

Electrophoresis and Western blotting

The cells were harvested for preparation of whole-cell lysates using cell lysis buffer (Cell Signaling Technology, Danvers, MA) 4 and 24 h after the application of 0.4 and 0.6 μmol/l benfluron and 5 nmol/l mitoxantrone. The protein content was quantified using the bicinchoninic acid (BCA) assay (Sigma-Aldrich). The lysates containing an equal amount of protein (20 μg) were loaded into each lane of polyacrylamide gel. After electrophoretic separation, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Prague, Czech Republic). The membranes were blocked in Tris-buffered saline containing 0.05 % Tween 20 and 5% non-fat dry milk and then incubated with primary antibody (p53, p53_Ser392 – Exbio, Vestec, Czech Republic; β-actin, $p21^{WAF1/Cip1}$, p38_Thr180/Tyr182 – Sigma-Aldrich; p53_Ser15 – Calbiochem-Merck, Prague, Czech Republic; Erk, Erk_Thr202/Tyr204, JNK, JNK_Thr183/Tyr185, p38, Chk1, Chk1_Ser345, Chk2, Chk2_Thr68 – Cell Signaling Technology) at 4 °C overnight. After washing, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody, Polyclonal Goat Anti-Mouse Immunoglobulins or Polyclonal Swine Anti-Rabbit Immunoglobulins (DakoCytomation, Brno, Czech Republic), for one hour at room temperature. Antigen-antibody complexes were detected with a chemiluminescence detection kit (Roche, Prague, Czech Republic). Chemiluminescence detection reagents represent a two-component system, the kit contains Solution A (horseradish peroxidase substrate buffer and H_2O_2) and Solution B (stabilized chemiluminescent luminol and enhancers). In the presence of peroxidase, the reagents emit chemiluminescent light. The signal was quantitatively detected via autoradiographic film (Foma, Hradec Králové, Czech Republic). To confirm equal protein loading, each membrane was re-probed and re-incubated to detect β-actin.

Analysis of apoptosis

Apoptosis was determined by flow cytometry using an APOPTEST™-FITC kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The APOPTEST™-FITC kit employs the property of fluorescein isothiocyanate (FITC) conjugated to Annexin V (Ann-FITC) to bind to phosphatidylserine in the presence of Ca^{2+}, and the property of propidium iodide (PI) to enter cells with damaged cell membranes and to bind to DNA. The measurement was performed 48 h after application of 0.4 and 0.6 μmol/l benfluron and 5 nmol/l MTX using a CyAn™ ADP (Beckman Coulter, Indianapolis, IN) flow cytometer. Listmode data were analysed using Summit v4.3 software (Beckman Coulter).

Cell cycle analysis

For analysis of cell cycle distribution, the cells were treated with 0.4 and 0.6 μmol/l BF. After 16 h of incubation, the cells were washed with ice-cold PBS and fixed with 70% ethanol. For detection of low-molecular-weight fragments of DNA, the cells were incubated for 5 min at room temperature in a buffer (192 ml 0.2 M Na_2HPO_4 + 8 ml of 0.1 M citric acid, pH 7.8) and then labelled with PI in Vindelov's solution for 1 h at 37 °C. The DNA content was determined using the flow cytometer CyAn™ ADP (Beckman Coulter) with an excitation wavelength of 488 nm. The data were analysed using Multicycle AV software (Phoenix Flow Systems, San Diego, CA).

Light scatter analysis

The light-scattering characteristics of the cells associated with cell death were determined using flow cytometry. This measurement allows analysis of morphologically different subpopulations within a heterogeneous cell suspension. The treated cells and non-treated controls were washed two times with PBS. After washing, cells were resuspended in 500 μl of PBS buffer and immediately acquired in a flow cytometer CyAn™ ADP (Beckman Coulter). Forward scatter channel versus side scatter channel double-parameter histograms were made for gating cells and to identify any changes in the light scatter properties of the cells. Data were analysed using Summit v4.3 software (Beckman Coulter).

Statistical analysis

The descriptive statistics of the results were calculated and the charts were made in Microsoft Office Excel 2003 or GraphPad Prism 5 biostatistics (GraphPad, La Jolla, CA). In this study, all the values were expressed as arithmetic means with SD of triplicates unless otherwise noted. The significant differences between the groups were analysed using the Student's *t*-test and P values ≤ 0.05 were considered statistically significant.

Results

The effect of benfluron on proliferation and viability

The proliferation and viability of the cells were measured by trypan blue exclusion staining during three days after the exposure of cells to BF in concentrations of 0.2, 0.4, 0.6, 0.8 and 1 μmol/l. The effect of 0.2 μmol/l BF was comparable with the non-treated control group of cells. Exposure of cells to 0.4 μmol/l BF after 72 h resulted in a slight increase in percentage of dead cells to 16 % when compared with the control (5 %) (Fig. 1B). However, the total number of cells still increased after 48 and 72 h (Fig. 1A). Application of 0.6 μmol/l BF led to a decrease in viability of MOLT-4 cells, the percentage of dead cells increased to 51 % after 48 h and 67 % after 72 h (Fig. 1B) and the proliferation was almost completely inhibited (Fig. 1A). Exposure of cells to 0.8 and 1 μmol/l BF resulted in complete elimination of cells after 48 and 72 h (Fig. 1A, B).

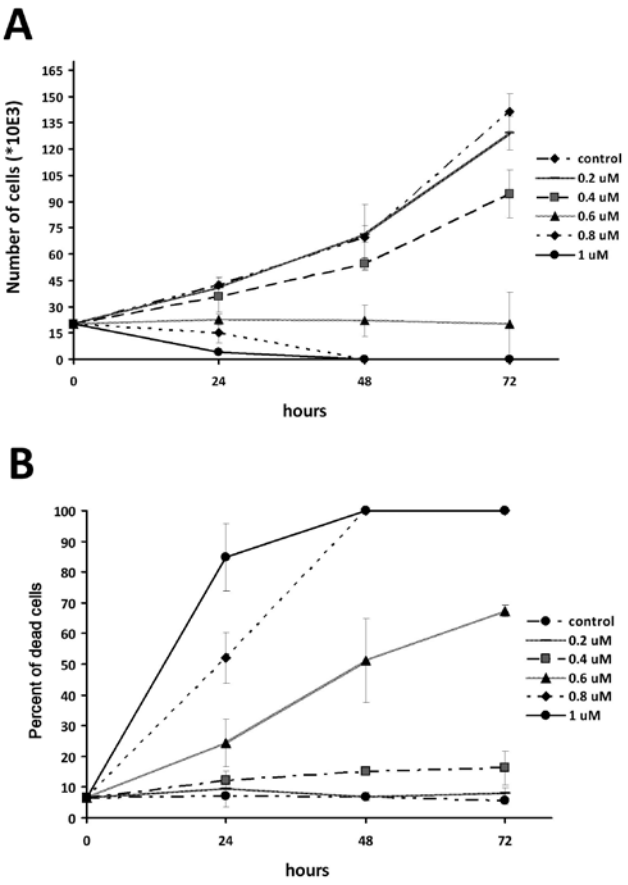

Fig. 1. The effect of benfluron on the proliferation (**A**) and viability (**B**) of MOLT-4 cells
Cells were treated with 0.2, 0.4, 0.6, 0.8 and 1 μmol/l benfluron and the effect was measured after 24, 48 and 72 h following befluron application. The analysis was performed using trypan blue exclusion staining. Results are shown as mean ± SD from three experiments.

Apoptosis induction

Induction of apoptosis was analysed using Annexin V-FITC and PI staining 48 h after the application of 0.4 and 0.6 μmol/l BF. The cells treated with 5 nmol/l MTX served as a positive control for this assay. The number of apoptotic cells increased in all treated groups in comparison with the control group (in which 7 % of apoptotic cells were observed). The amount of apoptotic cells after BF application increased in a dose-dependent manner. The application of 0.4 μmol/l BF led to an increase in apoptotic cells, 7 % of affected cells were in the early

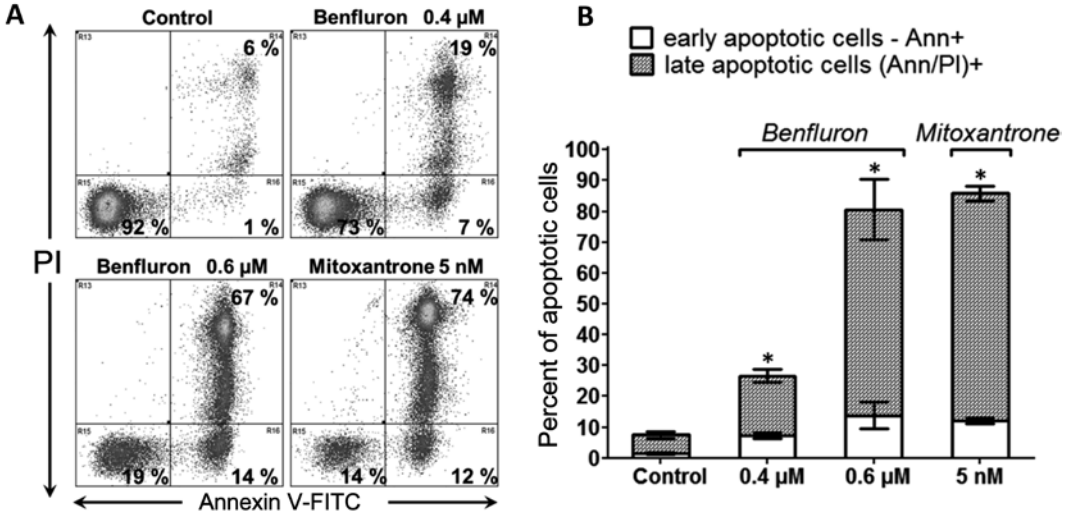

Fig. 2. The effect of benfluron on the induction of apoptosis in MOLT-4 cells
Apoptosis was detected by flow cytometry using Annexin V-FITC and propidium iodide staining 48 h after the application of 0.4 and 0.6 μmol/l benfluron. Cells treated with 5 nmol/l mitoxantrone were used as a positive control. (**A**) The double parameter histograms represent the percentage of three groups of cells; the percentage of negative cells (cells considered viable), Annexin V-positive/PI-negative cells (cells that are in early apoptosis) and annexinV/PI both positive (cells that are in late apoptosis or already dead). Representative results of one of three independent measurements are shown. (**B**) The bar graph represents the percentage of early and late apoptotic cells detected by flow cytometry (mean ± SD, N = 3). * – significantly different from the control for early and late apoptotic cells ($P \leq 0.05$)

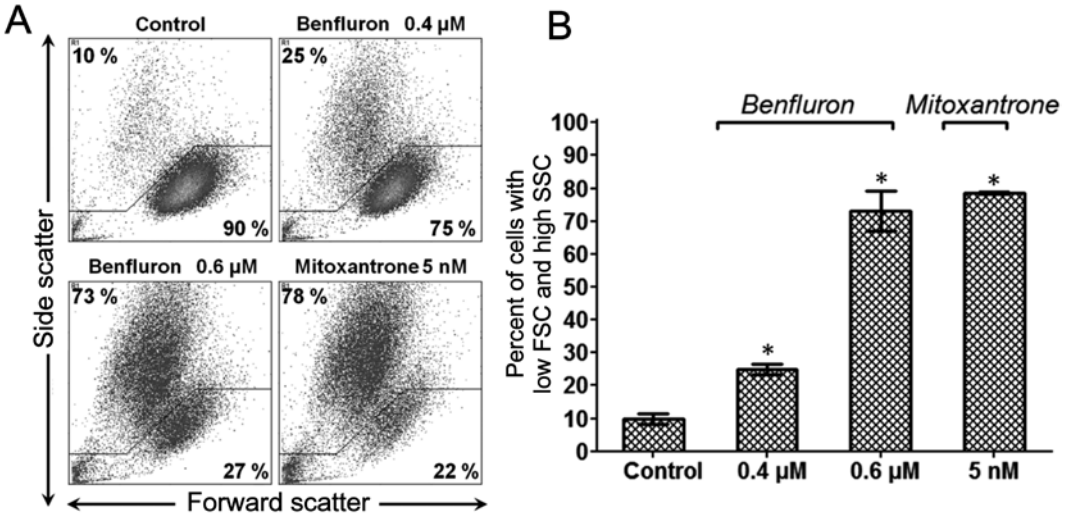

Fig. 3. Changes in light scatter properties of MOLT-4 cells upon treatment with benfluron
Morphological hallmarks of cells associated with apoptosis were analysed by flow cytometry 48 h after the treatment with 0.4 and 0.6 μmol/l benfluron. Cells treated with 5 nmol/l mitoxantrone were used as a positive control. (**A**) Representative histograms show forward scatter channel FSC (x axis) and side scatter channel SSC (y axis). Two populations with distinct light-scattering properties in bivariate histograms of FSC vs. SSC can be identified. Note the appearance of an apoptotic population (upper gate) with increased side scatter and reduced forward scatter relative to the viable cell population (lower gate). (**B**) The bar graph represents the percentage of cells undergoing apoptosis with low FSC and high SSC (mean ± SD, N = 3). * – significantly different from the control ($P \leq 0.05$)

phase and 19 % in the late phase of apoptosis. The treatment with 0.6 μmol/l BF caused more intensive apoptosis, 14 % of affected cells were in the early phase and 67 % in the late phase of apoptosis. The treatment with 0.6 μmol/l BF or MTX influenced the apoptosis in a very similar manner. After MTX treatment, 12 % of cells were in the early phase and 74 % in the late phase of apoptosis (Fig. 2A, B).

BF also induced changes in cell morphology (Fig. 3), which was evaluated through changes in the light scatter properties by flow cytometry. Forward scattering of light is a measure of cell size, whereas side scattering of light is a measure of cell complexity. Shrinkage is one of the morphological characteristics of apoptosis and manifests itself as reduced cell size, while apoptosis-associated plasma membrane blebbing increases the cell surface. This analysis showed very similar results as the analysis using annexin V-FITC and PI staining. The total amount of apoptotic cells increased after the treatment with 0.4 μmol/l BF (25 %), 0.6 μmol/l BF (73 %), 5 nmol/l MTX (78 %) in comparison with the control group (10 %) (Fig. 3A, B).

As the concentrations of 0.4 and 0.6 μmol/l led to induction of apoptosis, we evaluated the activity of effector caspases 3/7 and initiator caspases 8 and 9 as a further marker of apoptosis. The analysis was performed after application of 0.4, 0.5 and 0.6 μmol/l BF in the intervals 24 and 48 h. The activity of caspases 3/7, 9 as well as caspase 8 significantly increased ($P \leq 0.05$) in BF-treated cells in comparison to the control and the increase was dose-dependent (Fig. 4A, B, C).

Cell cycle analysis

The possible cell cycle arrest was evaluated by flow cytometry 16 h after the application of 0.4 and 0.6 μmol/l BF. MOLT-4 cells treated with 5 nmol/l MTX were used as a positive control. The cell cycle arrest in late S and G2/M phases increased in BF-treated cells in a dose-dependent manner. The treatment of cells with 0.4 μmol/l BF resulted in a small increase in the percentage of cells arrested in G2/M phase (10 %) in comparison to control (7 %). After the application of 0.6 μmol/l BF, 13 % of cells were arrested in G2/M phase and accumulation of cells in late S phase was also observed; 34 % of cells were accumulated in S phase in comparison to the control (31 %). MTX itself induced the most apparent cell cycle arrest in comparison to the control; 19 % of treated cells were accumulated in G2/M phase and 38 % in late S phase (Fig. 5).

Changes in protein expression

Changes in the levels of protein p53 and its phosphorylated forms were analysed after the treatment with 0.4 and 0.6 μmol/l BF in intervals 4 and 24 h. The amount of protein p53 increased in a dose-dependent manner and it reached maximum after the treatment with 0.6 μmol/l BF and 5 nmol/l MTX. The application of BF also led to phosphorylation of p53 on serine 15 and serine 392 and the increase in the amount of phosphorylated forms was dose-dependent. The phosphorylations of p53 also occurred after the incubation with 5 nmol/l MTX (positive control) (Fig. 6).

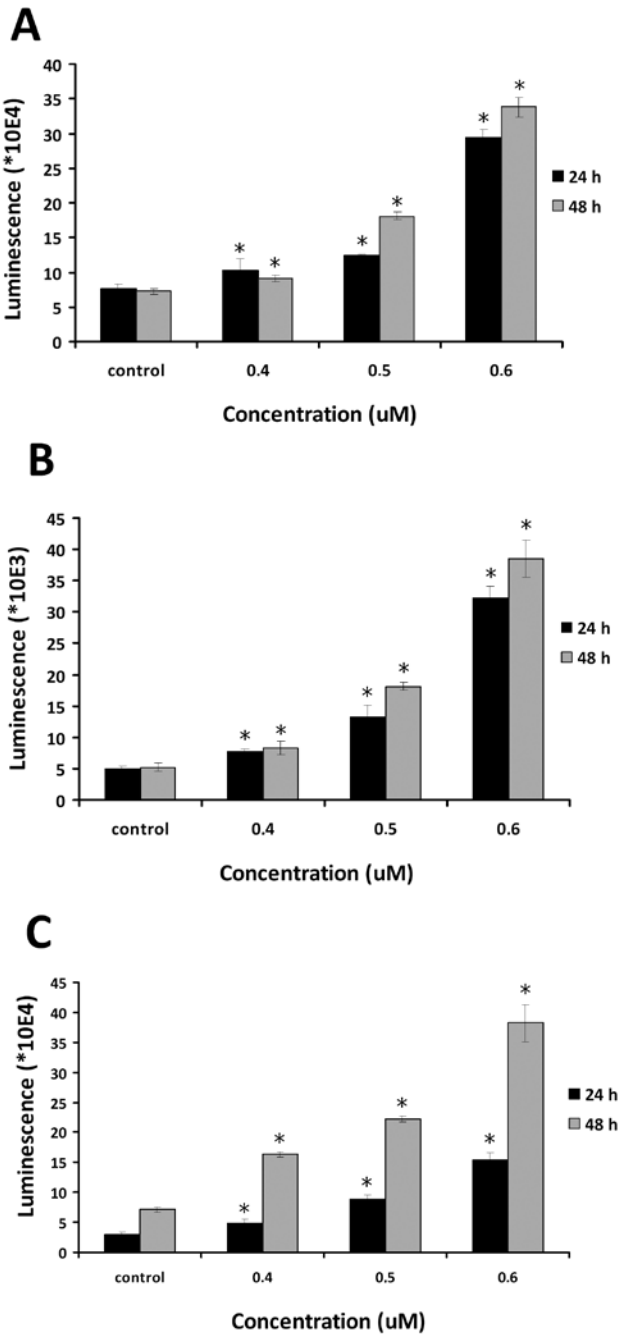

Fig. 4. Activity of caspase 9 (**A**), caspase 8 (**B**) and caspases 3/7 (**C**) in benfluron-treated MOLT-4 cells
The activity of caspases was determined 24 and 48 h after application of 0.4, 0.5, 0.6 μmol/l benfluron in MOLT-4. Results are shown as mean ± SD from three experiments. * – significantly different from the control ($P \leq 0.05$)

The levels of check-point kinases Chk1, Chk2 and their phosphorylated forms were analysed after the treatment with 0.4 and 0.6 μmol/l BF in 4 and 24 h intervals. The amount of proteins Chk1 and Chk2 after BF treatment remained constant during the entire experiment. The application of 0.4 and 0.6 μmol/l BF led to phosphorylation of Chk1 on serine 345 in both intervals

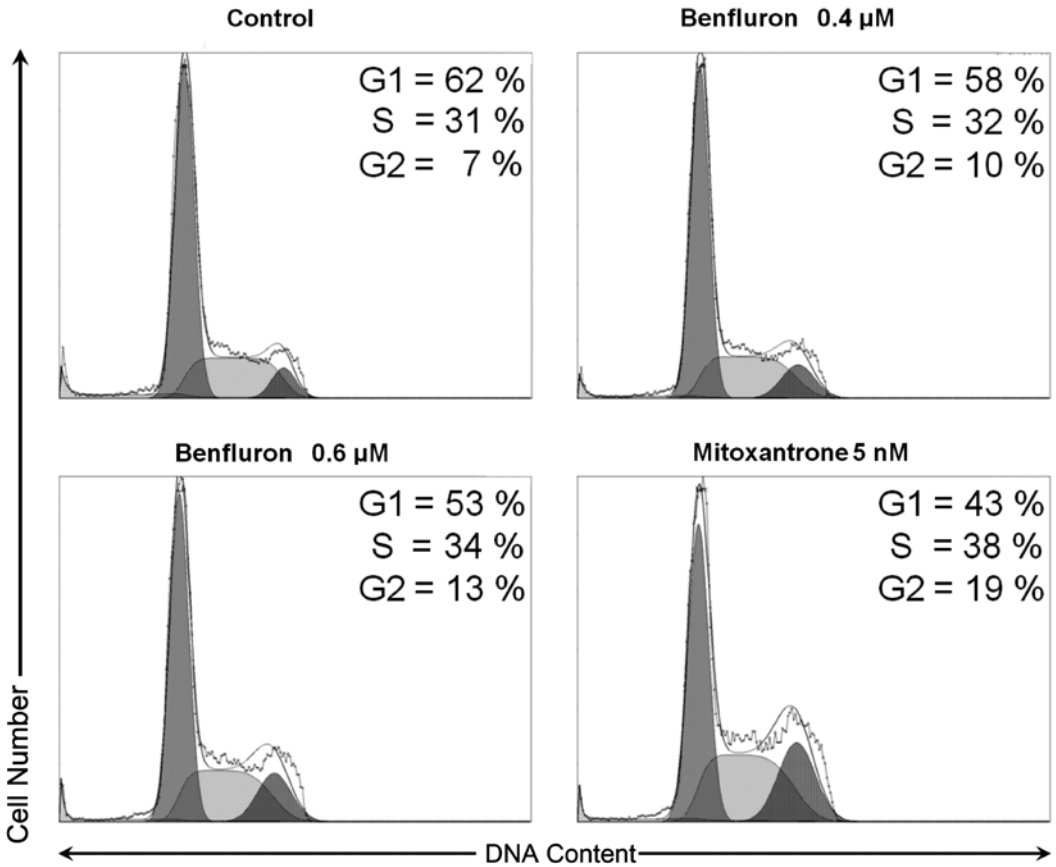

Fig. 5. Analysis of the cell cycle of MOLT-4 cells after the application of benfluron
Cell cycle distributions were measured using flow cytometric detection of DNA content in the cell 16 h after the treatment with 0.4 and 0.6 µmol/l benfluron. Cells treated with 5 nmol/l mitoxantrone were used as a positive control. The pooled results of one of three independent experiments are shown. The histograms show the percentage of cycling cells in G1, S, and G2/M phases of the cell cycle.

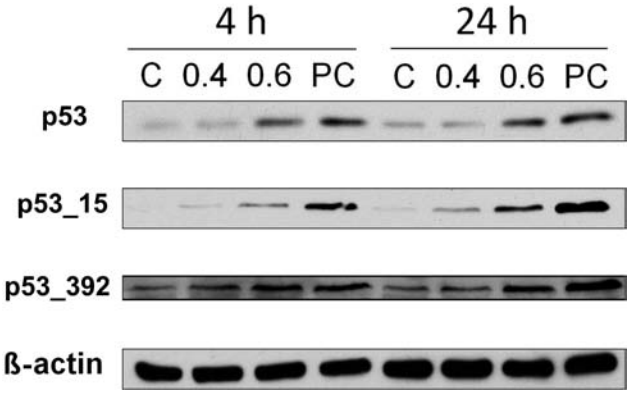

Fig. 6. Activation of protein p53 in MOLT-4 cells treated with benfluron
Upregulation of p53, p53 phosphorylated on serine 15 (p53_15) and serine 392 (p53_392) in MOLT-4 cells exposed to 0.4 and 0.6 µmol/l benfluron 4 and 24 h after the drug application. Cells treated with 5 nmol/l mitoxantrone were used as a positive control (PC). Protein expression was detected by electrophoresis and Western blotting as described in Material and Methods. To confirm equal protein loading, membranes were re-incubated with β-actin. Representative results of one of three experiments (C – untreated control)

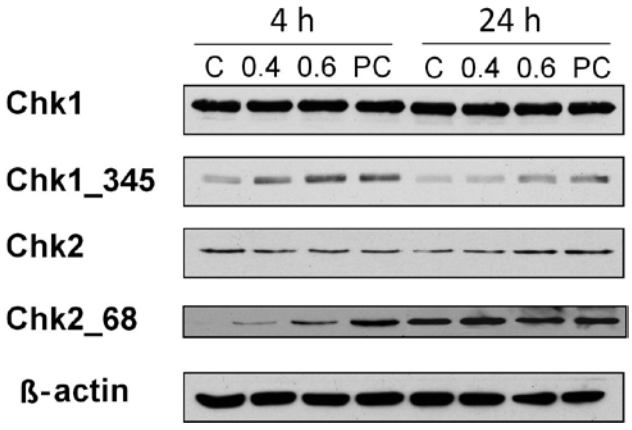

Fig. 7. Activation of check-point kinases in MOLT-4 cells treated with benfluron
Activation of Chk1 by its phosphorylation on serine 345 (Chk1_345), Chk2 and its phosphorylation on threonine 68 (Chk2_68) in MOLT-4 cells exposed to 0.4 and 0.6 µmol/l benfluron 4 and 24 h after the drug application. Cells treated with 5 nmol/l mitoxantrone were used as a positive control (PC). Protein expression was detected by electrophoresis and Western blotting as described in Material and Methods. To confirm equal protein loading, membranes were re-incubated with β-actin. Representative results of one of three experiments. (C – untreated control)

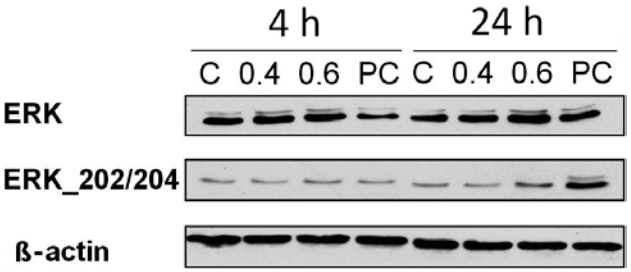

Fig. 8. MAPK pathways in MOLT-4 cells treated with benfluron

Activation of ERK1/2 and its phosphorylation on threonine 202 and tyrosine 204 in MOLT-4 exposed to 0.4 and 0.6 μmol/l benfluron 4 and 24 h after the drug application. Cells treated with 5 nmol/l mitoxantrone were used as a positive control (PC). Protein expression was detected by electrophoresis and Western blotting as described in Material and Methods. To confirm equal protein loading, membranes were re-incubated with β-actin. Representative results of one of three experiments (C – untreated control)

and the levels of phosphorylated form increased in a dose-dependent manner. The amount of Chk2 phosphorylated on threonine 68 increased only at 4 h; at later intervals, the levels of phosphorylated Chk2 did not change (Fig. 7).

Activation of three main MAPK families – extracellular signal-regulated kinases (ERK1/2), c-Jun-N-terminal kinase (JNK) and p38 kinases – was evaluated after the treatment with 0.4 and 0.6 μmol/l BF in intervals 4 and 24 h. BF did not trigger activation of JNK and p38 (data not shown), but caused activation of ERK1/2. The application of BF led to phosphorylation of ERK1/2 on threonine 202 and tyrosine 204 only after treatment with 0.6 μmol/l in interval 24 h. The most apparent increase in the amount of the phosphorylated form of ERK1/2 was detected in positive control after the treatment with mitoxantrone at 24 h (Fig. 8).

Discussion

Benfluron as a potential chemotherapeutic agent shows cytostatic and cytotoxic effects in the MOLT-4 leukemic cell line. BF induced inhibition of proliferation and a decrease in cell viability in a concentration-dependent way. The highest tested BF concentration (1 μmol/l) caused total elimination of the treated cells. The cytotoxic effect of benfluron was directly proportional to the concentrations used. Concentrations effective against leukemic MOLT-4 cells correspond with the results of Horáková et al. (2001), who studied cytotoxicity of BF in the concentration range of 0.156–5 μmol/l in cervical HeLa cells. Total inhibition of cell proliferation was observed at the highest tested concentrations of BF. In another study (Horáková et al., 1988), benfluron at concentrations ranging from 0.26–0.52 μmol/l inhibited formation of V79 (hamster lung) colonies in a dose-dependent manner; the inhibition of proliferation was accompanied with a decrease of cell size, decrease of protein content per BF-treated cell and increase in metabolic activity. Higher concentrations of BF (> 1 μmol/l) induced lysis of V79 cells (Horáková et al., 1988). Using electron microscopy, perforations of the plasma membrane were also observed at highest tested concentrations of BF (4.05 μmol/l) in HMB-2 cells (Jantová et al., 1992). In the study of Horáková et al. (2001), the mechanisms of cytotoxic action of BF were assumed as a consequence of oxidative reactions. In the treated cells, lysosomal membrane damage may occur due to free radicals. This results in the leakage of a variety of hydrolytic enzymes into the cytosol, which in turn may affect nuclear DNA and cause perforations of the cell plasma membrane.

In our study, we observed induction of apoptosis in BF-treated MOLT-4 cells that was accompanied by changes in the cell structures. The characteristic markers of apoptosis such as shrinkage of cells and plasma membrane blebbing were proved by flow cytometry. The apoptotic process was accompanied by activation of caspases 8, 9 and 3/7, which are considered as major determinants in the effectiveness of anticancer agents. Activation of the apoptotic process following treatment with chemotherapeutic drugs has been shown to lead to activation of the mitochondrial (intrinsic) pathway as well as to activation of the extrinsic pathway through death receptors (Debatin, 2004). Primarily, caspases 9 and 3/7 are activated following treatment with chemotherapeutic agents (MacKenzie and Clark, 2008). Despite this fact, the activation of caspase 8 was also described after the treatment of leukaemia cells with doxorubicin, methotrexate and cytarabine (Kim et al., 2001). Treatment of MOLT-4 cells with BF resulted in the activation of all three tested caspases, which indicates that the intrinsic and extrinsic pathways of apoptosis were activated simultaneously.

In our work, MOLT-4 cells were preferentially arrested in late S and G2/M phases of the cell cycle. In agreement with our study, benfluron caused cell cycle arrest in S and G2/M phases in V79 cells (Horáková et al., 1988). The treatment of V79 cells with BF in combination with disodium cromoglycate resulted in cell cycle arrest in late S and G2/M phases as well (Horáková et al., 1993). Our finding is also in agreement with the results of Seifrtová et al. (2011), who detected mainly G2/M or S phase cell cycle arrest in MOLT-4 and Jurkat cells after the treatment with mitoxantrone. Further, BF triggered activation of check-point kinases, which play a major role in the regulation of the cell cycle. We observed phosphorylation of Chk1 on serine 345 and Chk2 on threonine 68. Both these kinases are involved in cell cycle regulation and control the checkpoints in G1/S and G2/M transition by the inhibition of Cdc25A and Cdc25C (Bartek and Lukas, 2003; Wang et al., 2012). The Chk1-mediated signalling pathway is required for the G2/M arrest in response to DNA-damaging agents. At present, many studies are focused on Chk1 inhibition to enhance tumour cell sensitivity to chemotherapy (Dai et al., 2013). The phosphorylation of Chk1 correlates

with the detected increase of BF-treated cells in late S and G2/M phases.

It is well known that induction of DNA damage by chemotherapeutic agents in turn leads to the activation of p53 pathways, which can promote cell cycle arrest, senescence or apoptosis (Levine and Oren, 2009). Since the discovery of a major role of p53 in cell death induced by DNA-damaging agents, the activation of the p53 pathways after exposure to many cytostatics was already detected (Vousden and Prives, 2009). For example, the amount of protein p53 and its phosphorylation on serine 15 and 392 increased as a response to DNA damage caused by mitoxantrone in MOLT-4 cells (Seifrtová et al., 2011). The increase in p53 phosphorylated on serine 15 is proportional to sustained DNA damage after γ irradiation and can serve as a biodosimetric marker (Tichý et al., 2009). In our study, BF provoked an increase in the amount of p53 and its phosphorylation on serine 15 and serine 392. Our findings support the hypothesis postulated by Horáková et al. (2001) that BF caused DNA damage in tumour cells.

Further, we studied the possible activation of mitogen-activated protein kinases (MAPK). We observed an increase in the amount of ERK1/2 kinases phosphorylated at threonine 202 and tyrosine 204 after BF application, while the amount of phosphorylated JNK and p38 did not change after the treatment of MOLT-4 cells with BF. It is known that the activation of individual members of MAPK depends on the cell type and type of DNA damage. Some studies demonstrated that activation of ERK1/2 is associated with G2/M cell cycle arrest (Tang et al., 2002). In agreement with our results, simultaneous ERK1/2 activation and G2/M cell cycle arrest occurred in response to topoisomerase II poisons (doxorubicin and etoposide) in MCF-7 and T47D breast cancer cells (Kolb et al., 2012). Similarly, ionizing irradiation exposure of MCF-7 cells resulted in activation of ERK1/2 and induction of G2/M cell cycle arrest (Yan et al., 2007). Also, in our experiments both activation of ERK1/2 and G2/M cell cycle arrest was observed in BF-treated leukemic cells MOLT-4.

In conclusion, our study reveals that the molecular mechanisms of action of BF involve the response to DNA damage in MOLT-4 cells. Our data indicate that MOLT-4 leukemic cells react to BF by induction of apoptosis and accumulation of cells in late S and G2/M phases. BF activates the DNA damage response pathway and downstream signalling pathways involved in the process of programmed cell death. In response to DNA damage, BF activates p53 pathways, Chk1, Chk2 and ERK1/2 kinases.

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