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THE INFLUENCE OF CURCUMIN ON THE ACTION OF ETOPOSIDE IN A RAT ACUTE MYELOID LEUKEMIA CELL LINE

Abstract: Background: There are contradictory results from studies on the effect of curcumin, a plant phenolic compound with a well-established anticancer effect in vitro, on the action of etoposide in cancer cells.
Objective: The aim of this study was to evaluate the influence of curcumin on the genotoxic and cytostatic action of etoposide in the LT12 cell line derived from BN rat acute myeloid leukemia cells.
Material & Methods: The LT12 cells were treated with different doses of curcumin for 1–72 hours followed by application of etoposide. The amount of DNA damage was estimated via a comet assay. Viability, cell cycle and apoptosis were examined by using flow cytometry technique.
Results: The combined effect of curcumin and etoposide on viability was synergistic at low micromolar concentrations. In comparison to treatment with curcumin and etoposide alone, co-treatment with these compounds increased the extent of DNA damage, the percentage of cells arrested in the G2/M phase and the number of annexin-V-positive cells.
Conclusions: The interaction between etoposide and curcumin points to an enhanced antileukemic potential to be derived from the combination of these compounds.

Key words: curcumin, etoposide, apoptosis, cell cycle, DNA damage.

INTRODUCTION

Conventional chemotherapeutics used for treatment of leukemia stimulate apoptosis of leukemic cells but, due to defects in apoptotic signaling pathways, there are some cells which are resistant to cytostatic-induced apoptosis [1]. Acute myeloid leukemia (AML), a disease of myeloid progenitor cells, primarily affects the elderly, because the incidence of this leukemia increases significantly after 65 years of age. Paradoxically, it is the most difficult disease to treat in this age group [2]. Elderly patients have co-morbid conditions and do not tolerate the aggressive chemotherapy which is necessary to obtain positive therapeutic effects [2]. Younger patients, i.e. under 65 years of age, achieve initial remission in about 80% of cases, but most of them relapse [3]. Therefore, further research is being conducted into more effective and less aggressive treatments for this leukemia.

The research is focused in particular on natural ingredients with potential anticancer properties. Some of these ingredients are plant polyphenols. These compounds have more selective toxicity to cancer cells than to their normal counterparts and can reduce cytostatic side effects [4, 5]. Data have been published regarding the antiproliferative and proapoptotic effect on cancer cells of polyphenols, among them curcumin, the most active component of turmeric (*Curcuma longa*) [6]. Polyphenol curcumin can act selectively in cancer cells with high levels of free radicals, increasing their oxidative stress which leads to apoptosis, whereas it has a much lower toxicity to normal cells with reduced oxidative stress [7, 8].

One cytostatic frequently used as a first-line drug for treatment of a wide range of cancers such as various types of leukemia is etoposide. It is used together with other anticancer drugs and irradiation [9]. The anticancerous activity of etoposide depends on both DNA strand cleavage through stabilization of a DNA-topoisomerase II cleavable complex and direct or indirect DNA modification [10]. The DNA-topoisomerase II- etoposide complex disables the re-ligation reaction and relaxation. This results in permanent DNA breaks leading to induction of the apoptotic signaling pathway. Etoposide also inhibits G2 phase of the cell cycle and increases ROS level in leukemia cells [11, 12].

The effect of the prooxidative action of etoposide in myeloid leukemia cells could be enhanced by co-administration of curcumin, taking into account the fact that cancer cells are much more sensitive to oxidative DNA damage compared to normal cells [7]. The genotoxic and proapoptotic action of curcumin towards leukemia cells has been demonstrated by *in vitro* experiments [13]. Curcumin can induce apoptosis by caspase activation and cytochrome c release [14].

The combined effect of curcumin and etoposide on the LT12 cell line derived from Brown Norway rat acute myeloid leukemia (BNML) was tested in the present study. BNML leukemia cells have a similar sensitivity to chemotherapy as human AML cells [15]. Moreover, BNML leukemia is accompanied by oxidative stress [16] similar to human leukemia [17, 18]. Thus, rat leukemic cells are a good model for the study of compounds with potential anticancer activity.

The effect of curcumin on the cytostatic action of etoposide in leukemic cells is not very well known. The main goal of the present study was a determination of the effect of curcumin on the genotoxic and cytostatic action of etoposide in the LT12 cell line. The influence of curcumin on the DNA damage induced by etoposide was evaluated by comet assay. Cell cycles and the final effect of cytostatic action, apoptosis and necrosis were investigated using multicolor staining and flow cytometry.

MATERIAL AND METHODS

CHEMICALS

Curcumin, etoposide, normal and low melting point (LMPA) agarose, triton-X-100, ethylenediamine tetraacetc acid disodium salt (EDTA), propidium iodide (PI), ribonuclease A were acquired from Sigma-Aldrich Co. (USA) and dimethyl sulfoxide (DMSO) was from POCH (Poland). The medium (RPMI 1640), serum (FBS), phosphate-buffered saline (PBS) were purchased from PAA (Germany). Annexin V-FITC was from Roche (Germany).

CELL CULTURE AND TREATMENT

LT12 cells (supplied by Professor A.C.M. Martens, Utrecht) were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were incubated at 37˚C in a 5% CO_2 and 95% humidified atmosphere and kept in the logarithmic growth phase.

LT12 cells were seeded in 24-well culture plates at a density of 1×10^6 cells/ml and incubated in the presence of different concentrations of curcumin and/or etoposide for 1–48 hours. Polyphenol and etoposide were dissolved in 1% ethanol in PBS. Control cells were incubated only in the presence of vehicles.

MEASUREMENTS OF VIABILITY

To determine the viability after treatment, the cells were incubated for 48 hours in a concentration range of 1–40 µM and 1–100 µM for etoposide and curcumin, respectively. After incubation, the cells were washed twice in PBS. One million cells were resuspended in 100 µl of PBS with propidium iodide (PI) (2.5 µg/ml) and incubated for 10 minutes in the dark. Then, the cells were immediately analyzed by flow cytometry (Becton Dickinson, Biosciences Immunocytometry Systems, San Jose, CA, USA). Analysis was performed on at least 20 000 single cells. CalcuSyn (Biosoft, Cambridge, UK) software was used in the calculation of ED50 and the combination index (CI). The CI was evaluated based on the multiple drug-effect equation of Chou-Talalay [19]. CI values <1, = 1, and >1 indicate synergism, an additive effect, and antagonism, respectively.

MEASUREMENTS OF DNA DAMAGE USING AN ALKALINE COMET ASSAY

The cells were incubated for one hour in a concentration range of 0.25–2.0 µM and 1–20 µM for etoposide and curcumin, respectively. The extent of DNA damage was determined by alkaline single cell gel electrophoresis, according to Tice *et al.* [20]. Briefly, 5 µl of cells in PBS was mixed with 75 µl of 0.5% low melting point agarose. The mixture was pipetted into slides precoated with 0.5% normal

melting point agarose, covered with a coverglass and put on ice to allow the agarose to solidify. After 5 minutes, the slides were placed for 24 h at 4°C into lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0 and 1% Triton X-100, 10% DMSO) added freshly before use. The slides were then washed with Tris-HCL, pH 7.5 and placed in a horizontal electrophoresis tank containing fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) and incubated for 40 min at 4°C. Electrophoresis was carried out for 30 min in a cold room at 0.74 V/cm and 300 mA. The slides were then washed with Tris-HCL, pH 7.5, stained with propidium iodide (2.5 µg/ml) and analyzed using a fluorescence microscope (Olympus CX-40; Olympus, Tokyo, Japan). An image system (The Comet Score™ image analysis system, TriTek Corporation, Sumerduck, Virginia, USA) was used for the measurement of percentage of DNA in the tail of comets. Two slides per rat, with 100 randomly selected comets per slide, were analyzed.

CELL CYCLE ANALYSIS

The quantity of cells in different phases of the cell cycle and sub-G1 fraction were evaluated upon staining with propidium iodide. Briefly, cells were washed with PBS, fixed in cold 70% ethanol and stored at –20°C. After fixation, the cells (2×10^6 cells) were washed in PBS and incubated for 30 min in 0.5 ml of solution containing: 0.1% Triton X-100, 20 mg RNAse A and 2% PI. The cell cycle was analyzed via a flow cytometry using CellQuest software (Becton-Dickinson, BD Biosciences Immunocytometry Systems, San Jose, CA, USA).

QUANTIFICATION OF APOPTOTIC CELLS USING AN ANNEXIN V BINDING ASSAY

Apoptotic cells were detected using an Annexin V-FLUOS staining kit. The cells were resuspended in binding HEPES buffer at a concentration of 1×10^6 cells/100 µl. The cells were incubated in the dark with 2 µl Annexin V-FITC and 2 µl PI for 15 min and analyzed via a flow cytometry (Becton-Dickinson, BD Biosciences Immunocytometry Systems, San Jose, CA, USA) for green (FITC) and red (PI) fluorescence. Apoptosis was examined using CellQuest software. Early apoptotic cells were defined as annexin V-positive and PI-negative, while late apoptotic and/or necrotic cells were annexin V- and PI-positive. Results were expressed as the percentage of apoptotic cells (Annexin V-positive cells) in samples from treated cells versus the controls.

STATISTICAL ANALYSIS

Values were expressed as means ± SEM. A one-way analysis of variance ($p < 0.05$) and Tukey's post hoc test were used to determine significant differences between control and experimental samples.

RESULTS

Viability of LT12 cells after treatment with curcumin and/or etoposide. The viability of control LT12 cells was 97%. EC50 values were 8.3 μM and 47.7 μM for etoposide and curcumin, respectively. Analysis of CI revealed that lower concentrations of curcumin (15.8–67.7 μM) and etoposide (1.8–13 μM) exert synergistic interaction. Antagonistic interaction was observed at the higher concentrations of curcumin (above 70 μM) and etoposide (above 13 μM). The synergistic effect was observed for most of the combinations (Fig. 1).

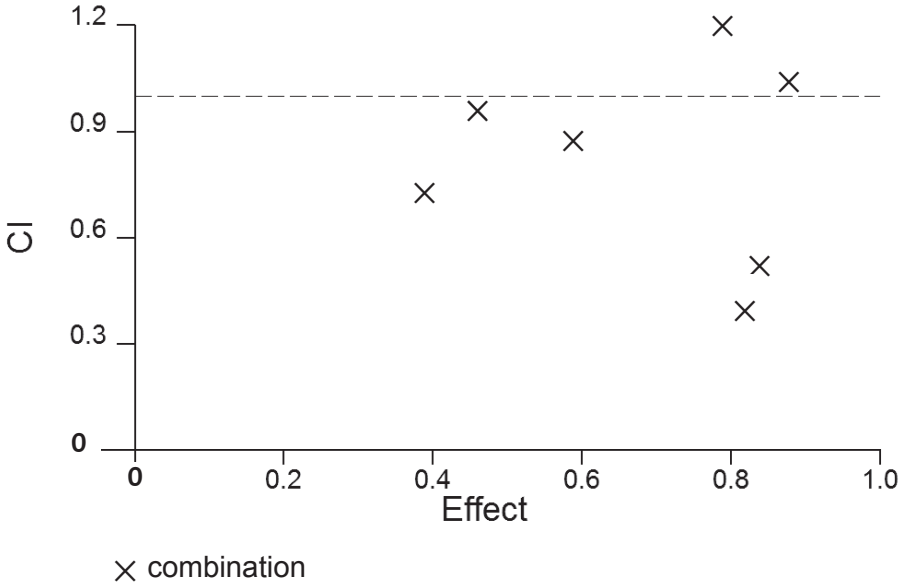

Fig. 1. The influence of combination of curcumin and etoposide on viability of LT-12 cells. The cells were incubated for 48 hours with curcumin (1–100 μM) and etoposide (1–40 μM). Then, the cells were stained with propidium iodide and the viability was measured by using a flow cytometry. CI — Combination index.

DNA damage. Etoposide induced a dose-dependent increase in DNA damage compared with the control (Fig. 2a). Curcumin did not induce significant DNA damage in the concentration range 1–10 μM, whereas incubation with the examined polyphenol at a dose of 20 μM led to a significant increase in DNA damage in comparison to the control cells (Fig. 2b). A significant increase in the DNA damage induced by etoposide was observed after pre-incubation with curcumin at doses of 10 and 20 μM (Fig. 2a). Pre-incubation with lower doses of curcumin (1–5 μM) did not significantly influence the extent of DNA damage induced by etoposide.

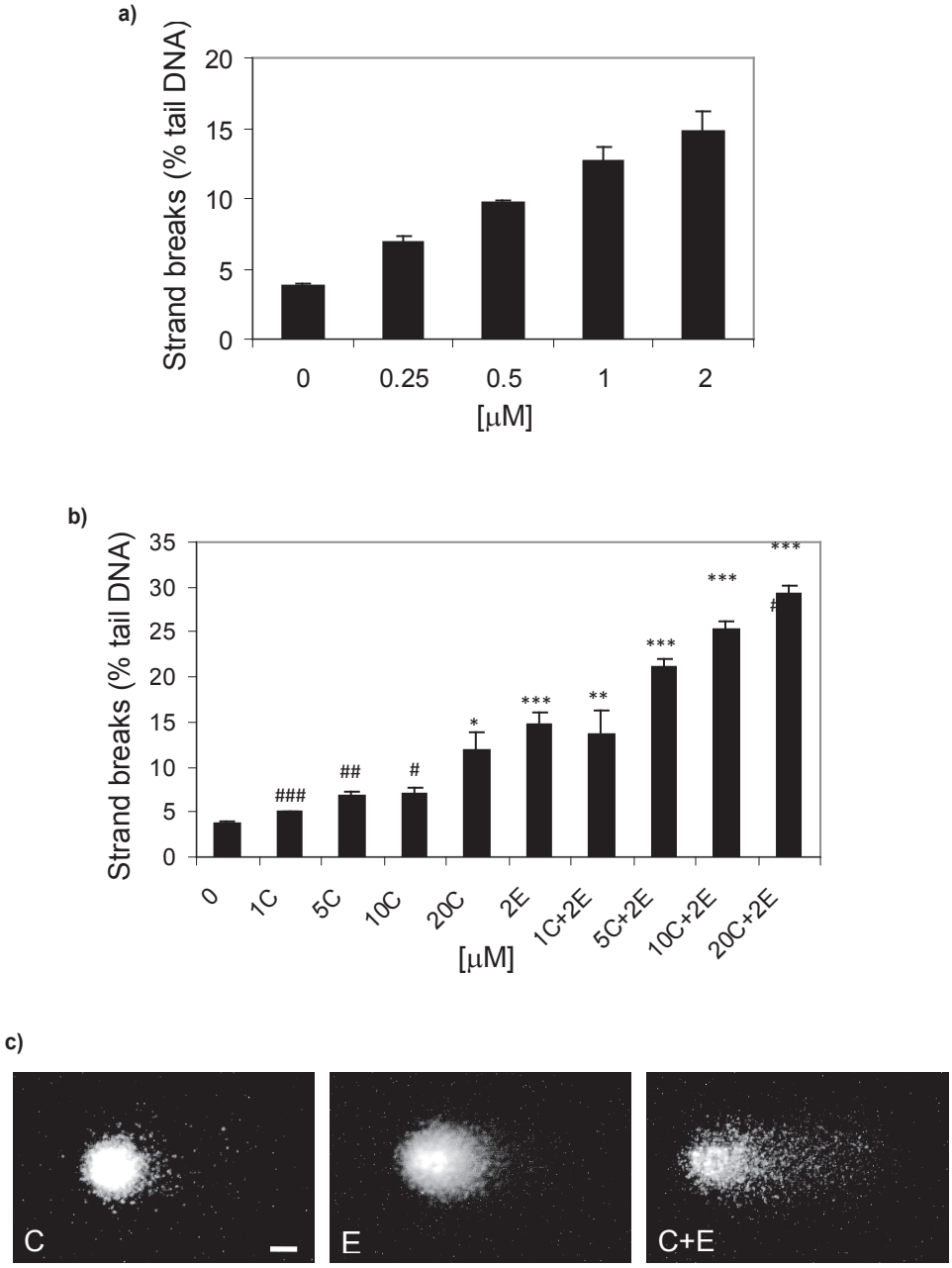

Fig. 2. The proportion of DNA strand breaks induced by etoposide (a), curcumin and combination of tested compounds (b) in LT-12 cells. DNA damage was measured by using comet assay. (c) Representative photographs show DNA strand breaks obtained by comet assay. Scale bar — 5 μM. C — curcumin, E — etoposide. p^{*} <0.05 *vs.* control, p^{**} <0.01 *vs.* control, p^{***} <0.001 *vs.* control, $p^{\#}$ <0.05 *vs.* etoposide, $p^{\#\#}$ <0.01 *vs.* etoposide, $p^{\#\#\#}$ <0.001 *vs.* etoposide.

Influence of curcumin on cell cycle. Curcumin administration significantly increased the number of cells in the G2/M at the dose of 10 and 20 μM after 72 hours in comparison to the control (Fig. 3a). A significant increase in the sub-G1 population (subdiploidal fraction) was observed 48 and 72 hours after curcumin administration at a dose of 20 μM (Fig. 3b).

The impact of etoposide at a dose of 2 μM on a cell cycle depended on the arrest of the cells in the G2/M phase after 72 hours (Fig. 3c). The sub-G1 fraction increased significantly after etoposide treatment of the cells with 48 and 72 hours of incubation (Fig. 3d). Pre-incubation of the cells with curcumin significantly increased the number of cells arrested by etoposide in the G2/M phase after 48 and 72 h compared to etoposide applied alone (Fig. 3c). Treatment with polyphenol and etoposide led to a significant enhancement of the sub-G1 fraction induced by the cytostatic after 72 hours of incubation in comparison to etoposide alone (Fig. 3d).

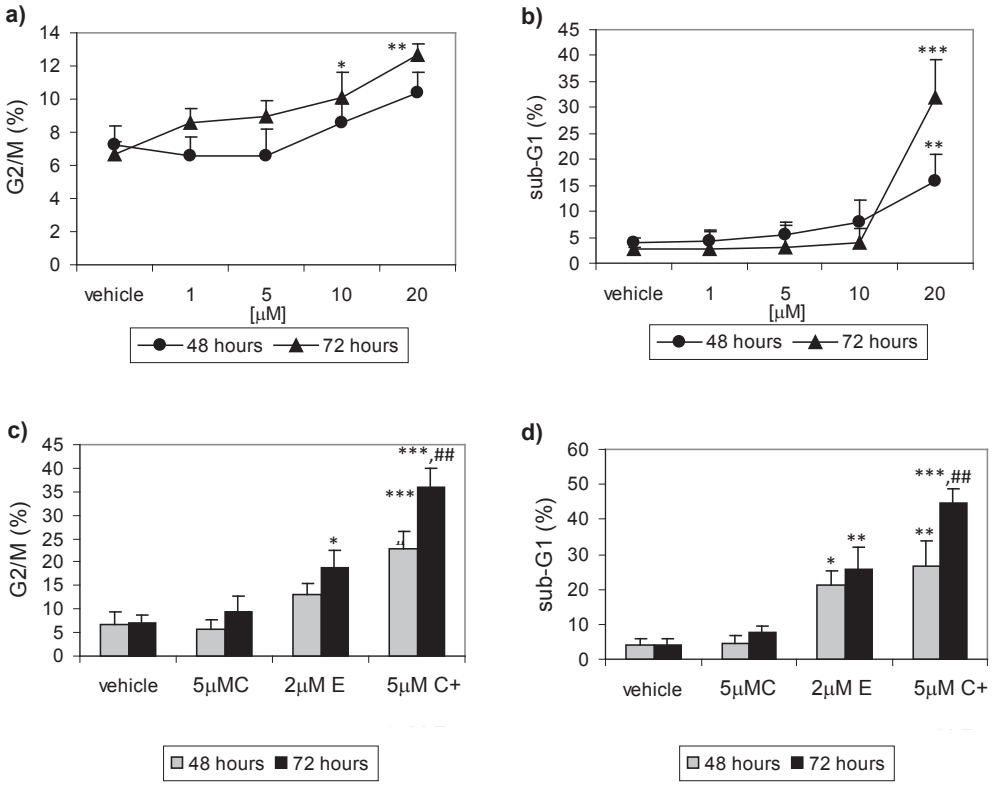

Fig. 3. The influence of curcumin and/or etoposide treatment on cell cycle of LT-2 cells. After incubation, the cells were stained with propidium iodide and DNA content was measured by using a flow cytometry. The percentage of cells in G2/M phase (a) and sub-G1 fraction (b) after curcumin treatment. C — curcumin, E — etoposide. p* <0.05 *vs.* control, p** <0.01 *vs.* control, p*** <0.001 *vs.* control, $p^{\#}$ <0.05 *vs.* etoposide, $p^{\#\#}$ <0.01 *vs.* etoposide.

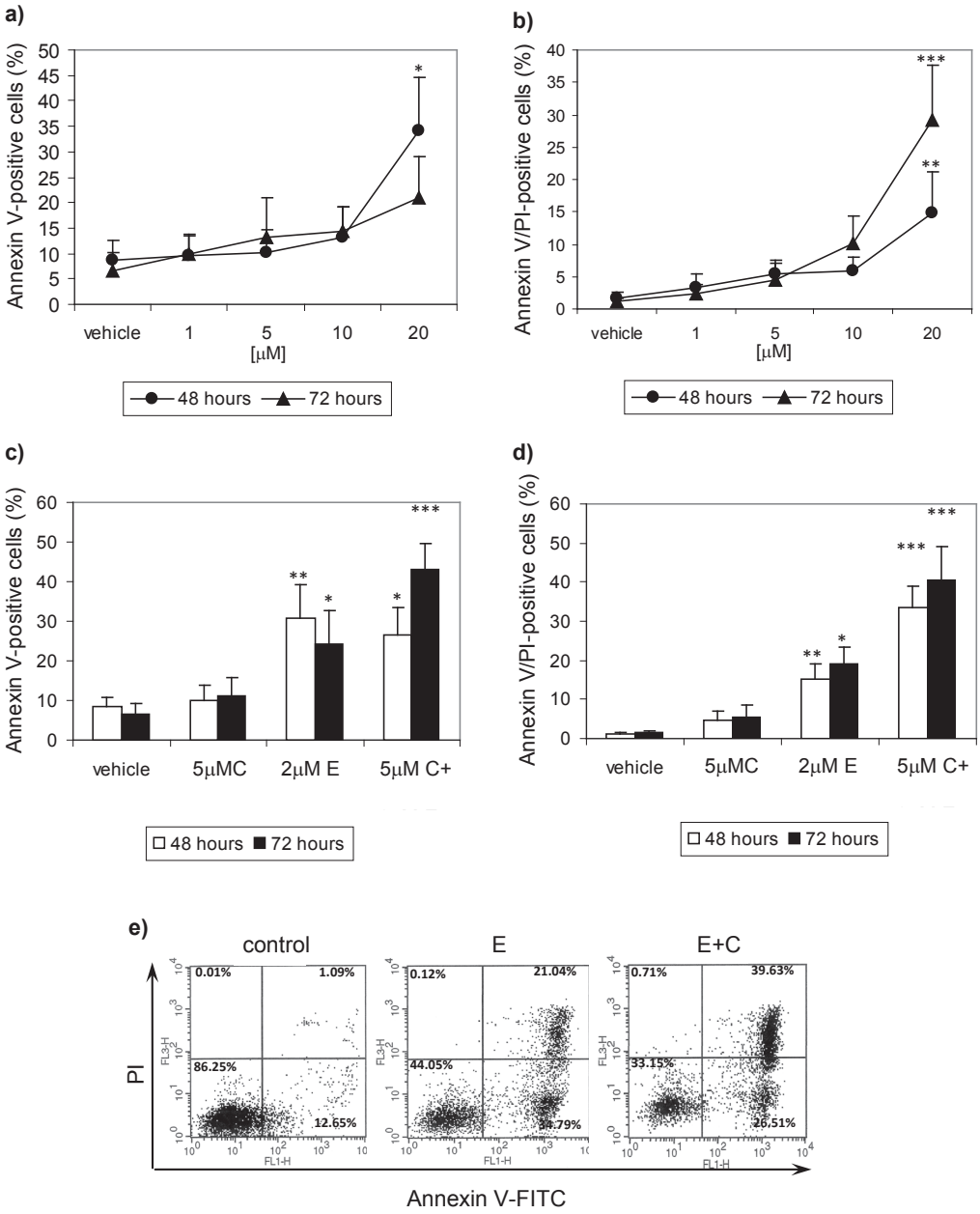

Fig. 4. The apoptosis of LT-12 cells induced by curcumin and/or etoposide after 48 and 72 hours of incubation. (a, b) Early and late apoptosis after incubation of LT-12 cells with curcumin. (c, d) Early and late apoptosis after coincubation with curcumin and etoposide. (e) Representative dot-plots of annexin V-FITC (FL1) *vs.* PI (FL3) are shown. C — curcumin, E — etoposide. p* <0.05 *vs.* control, p** <0.01 *vs.* control, p*** <0.001 *vs.* control, p$^{\#}$ <0.05 *vs.* etoposide, p$^{\#\#}$ <0.01 *vs.* etoposide.

Influence of curcumin on the apoptosis of LT12 cells. Curcumin applied at doses of 1–10 μM did not exert any significant proapoptotic effect in comparison with the control cells for 48–72 h, whereas incubation of LT12 cells with curcumin at a dose of 20 μM significantly increased the number of early apoptotic (An+) and/or late apoptotic (An+/PI+) cells compared to the control (Fig. 4a, b). Etoposide significantly induced apoptosis in each time series. Pre-incubation with curcumin at a dose of 5 μM for 48 h and 72 h significantly increased the proapoptotic effect of etoposide (Fig. 4c–e).

DISCUSSION

The effect of curcumin on the activity of etoposide is not yet well understood. There is evidence of a synergistic action between this polyphenol and etoposide in brain tumor cell lines [21]; however, there are some studies suggesting antagonism between curcumin and this chemotherapeutic in breast and uterus cancer cell lines [22]. Curcumin protected these last cancer cells against apoptosis caused by etoposide and induced DNA damages repair.

The results of the present study confirm that curcumin can enhance the effect of etoposide in acute myeloid leukemia cells leading to an increase in apoptosis. Moreover, the influence of a combination of the tested compounds on the viability of LT-12 cells was synergistic at lower concentrations. It may be supposed that the influence of curcumin on etoposide action can depend on the type of cancer cells.

Other research suggests that curcumin can cause damage to DNA and inhibit cell cycles of cancer cells using a variety of mechanisms depending on the type of cells [23]. The present study indicates that, at a concentration of 20 μM, curcumin induces significant DNA damage in LT12 cells after 1 hour of incubation. The concentrations of 10 and 20 μM of this polyphenol also significantly increased the extent of DNA strand breaks induced by etoposide. These results are consistent with studies by Lu *et al.* [24], who showed that curcumin has a genotoxic effect on colorectal carcinoma HCT116 cells at a concentration of 50 μM after 1 h incubation. However, in human hepatoma HepG2 cells, curcumin causes significant damage to the DNA in nuclear and mitochondrial genomes from concentrations of 10 μM after 1 hour incubation [25]. It can be assumed that the curcumin-generated ROS plays an important role in causing DNA damage. It is well documented that ROS can directly lead to double strand breaks [26].

DNA damage caused by curcumin may contribute to the observed cell cycle arrest in the G2/M phase and the increase in the percentage of the sub-G1 fraction. These results are consistent with other studies that have shown that curcumin arrests the cell cycle precisely in this phase in different cell lines [27]. Similar effects on the cell cycle are exerted by etoposide. Co-treatment of cells with curcumin and etoposide led to an increase in the level of G2/M arrest in

comparison to each compound alone. Treatment of the cells with curcumin and etoposide also resulted in an increase in the sub-G1 fraction, which may indicate the presence of apoptotic cells. Other studies have shown that the effect of curcumin on cell cycle arrest involves the use of various mechanisms, e.g. causation of spindle abnormalities, DNA damge, chomosomal alteration or topoisomerase poisoning [24, 27, 28].

A curcumin concentration of 20 μM was effective in inducing apoptosis of LT-12 cells. This polyphenol can exert apoptotic effect by inducing oxidative stress. The other studies showed that curcumin induced apoptosis and increased ROS level in HL-60 cells at concentrations of 10–20 μM and its â-diketone structure played a role in the prooxidative activity [29]. Curcumin reduced the transition metal ions which led to the activation of molecular oxygen.

In this study, curcumin increased the externalization of phosphatidylserine induced by etoposide. These results correlate with an increase in the sub-G1 fraction after simultaneous incubation with the test compounds, pointing to the complementary action of curcumin asserted together with this cytostatic. Other studies have demonstrated that curcumin can increase the effects of some cytostatics, e.g. cisplatin, paclitaxel, vincristine, doxorubicin, in various types of cancer cells [30–33].

Leukemia cells are characterized by abnormal proliferation and apoptosis, which predisposes them to prolonged survival [1, 34]. Curcumin hits the various molecular targets of cells of hematological malignancies, e.g. NFkB, STAT, p38 MAPK, bax, ornithine decarboxylase and mTOR, leading to inhibition of the proliferation and sensitization of the these cells to apoptosis [23]. Curcumin may therefore in many ways sensitize leukemia cells to the action of etoposide.

CONCLUSIONS

The present study indicates for the first time, that the combination of curcumin and etoposide can have a beneficial therapeutic effect on myeloid leukemia cells. The antineoplastic action of curcumin relied on DNA damage, arresting cell cycles and inducing apoptosis of impaired LT12 cells. Further studies are necessary to clarify the mechanism of action of the combination of curcumin and etoposide in different cell lines of acute myeloid leukemia.

CONFLICT OF INTEREST STATEMENT

None declared.

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