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Original article

Effect of sodium dichloroacetate on apoptotic gene expression in human leukemia cell lines

Jagoda Abramek[a], Jacek Bogucki[b], Marta Ziaja-Sołtys[a], Andrzej Stępniewski[c], Anna Bogucka-Kocka[a,*]

[a] Chair and Department of Biology and Genetics, Medical University of Lublin, Lublin, Poland
[b] Department of Clinical Genetics, Medical University of Lublin, Lublin, Poland
[c] ECOTECH-COMPLEX Centre, Lublin, Poland

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ABSTRACT

Background: Sodium dichloroacetate (DCA) is an agent with anticancer properties against solid tumors. DCA also seems to have antileukemic activity. In order to affirm it we investigate the effect of DCA on cell viability and apoptotic gene expression profiles in leukemia cell lines: CEM/C1, CCRF/CEM, HL-60, HL-60/MX2.

Methods: Cell viability was assessed by trypan blue staining. The expression of 93 genes involved in the process of apoptosis was determined by real-time PCR method using Taqman Low Density Array (TLDA).

Results: CEM/C1, CCRF/CEM, HL-60, HL-60/MX2 cells were exposed to DCA for 24 h. The sensitivity of each cell line to DCA is different and depends on the concentration. CEM/C1 was the most sensitive with an half-maximal inhibitory concentration (IC50) value of 30 mM, while HL-60/MX2 was the most resistant with an IC50 value of 75 mM. Exposure of leukemia cells to DCA causes differences in gene expression profiles which cannot indicate that any particular pathway of apoptosis is initiated. However, the presence of 388 statistically significant correlations between expression pattern of gens was determined.

Conclusion: We showed that DCA causes a decrease in viability of leukemia cells. The decline depends on DCA concentration. The induction of any particular apoptosis pathway is not shown in cells after DCA treatment. For that reason, studies on the molecular mechanism of cell death after exposure to DCA should be continued.

Introduction

Leukemia is a type of cancer originating from the hematopoietic system and is responsible for 2.8% of deaths among men and 3% of deaths among women in Poland [1] and the mortality rate has been growing. It is a challenge to develop anticancer treatment which selectively target cancer cells in contrast to conventional chemotherapy.

DCA (sodium dichloroacetate) is a chemical compound with antitumour activity confirmed in experiments on cell lines of different origins such as: oral squamous cell carcinoma HSC 2 and HSC 3 [2], prostate cancer cells PC-3-Bcl-2 and PC-3-Neo [3], multiple myeloma cells RPMII8226-TGL and JJN-3 [4], glioblastoma [5] and glioma cells [6], animals' cells [7–9] and in numerous clinical trials [10–15].

DCA is an incompetitive inhibitor of PDK (pyruvate dehydrogenase kinase) that increases the rate of oxidative phosphorylation and decreases the rate of glycolysis [16]. It targets especially cancer cells whose metabolism is switched from oxidative phosphorylation to glycolysis. Such an alteration in metabolism can be observed even in aerobic conditions and is referred to as the Warburg effect [8]. Although DCA has promising cancer treatment effect, the molecular mechanism of its action is not fully understood. It is suggested that DCA acts by inducing apoptosis in cancer cells [3,17,18].

The purpose of our study was to investigate the effect of DCA on cell viability and the expression changes of 93 genes involved in the process of apoptosis in leukemia cell lines: HL-60, HL-60/MX2, CCRF/CEM and CEM/C1 after the exposure to DCA. We used TaqMan low-density arrays (TLDA) based on real-time PCR in order to provide profiles of gene expression changes. To the best of our

* Corresponding author.
E-mail addresses: jagoda.abramek@umlub.pl (J. Abramek), jacek.bogucki@umlub.pl (J. Bogucki), marta.ziaja-soltys@umlub.pl (M. Ziaja-Sołtys), andrzej.stepniewski@poczta.umcs.lublin.pl (A. Stępniewski), anna.bogucka-kocka@umlub.pl (A. Bogucka-Kocka).

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knowledge, it is the first extensive expression analysis of apoptotic genes in cells following the exposure to DCA.

Materials and methods

Materials

Human leukemia cell lines derived from peripherial blood: HL-60, HL-60/MX2 (cell lines of Acute myeloid leukaemia origin), CCRF/CEM and CEM/C1 (cell lines of Acute lymphoblastic leukaemia origin) were purchased from the ECACC. RPMI 1640 medium was bought from Biomed Lublin and Sigma-Aldrich. DCA stock solution was prepared in PBS at a concentration of 1 M and was sterilised by filtration and stored at 4 °C.

Cell cultures

HL-60, HL-60/MX2, CCRF/CEM and CEM/C1 cells were maintained in RPMI 1640 medium supplemented with 10% (or 20% in the case of HL60) fetal bovine serum (v/v) and antibiotics: 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

In vitro cytotoxicity assay

The effect of DCA on HL-60, HL-60/MX2, CCRF/CEM and CEM/C1 cell lines was measured using the trypan blue assay. HL-60, HL-60/MX2, CCRF/CEM and CEM/C1 cells were seeded in 12-well plates at a density of $3–5 \times 10^5$ cells per well. After 24 h, cells were treated with DCA at concentrations ranging from 1 mM to 50 mM (100 mM in the case of HL-60/MX2) and incubated for 24 h. Then, cells were centrifugated (800 rpm for 5 min), washed with PBS and centrifugated. Cells were stained with a 0.4% solution of trypan blue (Bio-Rad) and counted with TC 10 T M Automated Cell Counter (Bio-Rad). All measurements were performed in triplicate. Dose response curves were made, due to which the determination of IC50 values was feasible.

RNA isolation

Total RNA was isolated from cells after exposure to IC50 value for 24, 48 and 72 h and from adequate control cells according to the protocol of the Single-step modified method of RNA isolation [19] using both TRI Reagent® Solution (Ambion) and 1-Bromo-3-chloropropane (Sigma Aldrich) according to the manufacturer's protocol (Thermo Scientific). The concentration and quality of RNA was determined using NanoDrop 2000c spectrophotometer. A260/A280 ratio was between 1.8 and 2.0. Isolated RNA was stored at -80 °C until used. cDNA was synthetised using *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems) according to the manufacturer's protocol.

Gene expression analysis

TaqMan low-density array (TLDA) cards enable simultaneous real-time PCR detection of multiple gene expression (Applied Biosystems). This research was carried out by means of 384-well Taqman Low Density Array: Human Apoptosis cards. Each card contains primers and probes for 93 tested genes involved in apoptosis and 3 genes used as endogenous control: 18S-Hs99999901_s1, ACTB-Hs99999903_m1 and GAPDH-Hs99999905_m1. In our research GAPDH-Hs99999905_m1 was chosen as endogenous control. TLDA cards were run on a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems). Gene expression values were calculated using the comparative quantification method $\Delta\Delta C_T$ with Expression Suite Software v 1.0.

The number of cDNA molecules in specimen was calculated using Ct values (threshold cycle) - a cycle number at which the fluorescence exceed the threshold. Ct values for both: a) the gene of interest in test (treated) as well as control sample (calibrator) sample are normalised using Ct of endogenous control gene – GAPDH according to the formulas:

$$Ct_{\text{gene of interest}}{}^{s} - Ct_{\text{endogenous control}}{}^{s} = \Delta Ct_{\text{sample}} \quad \text{a)}$$

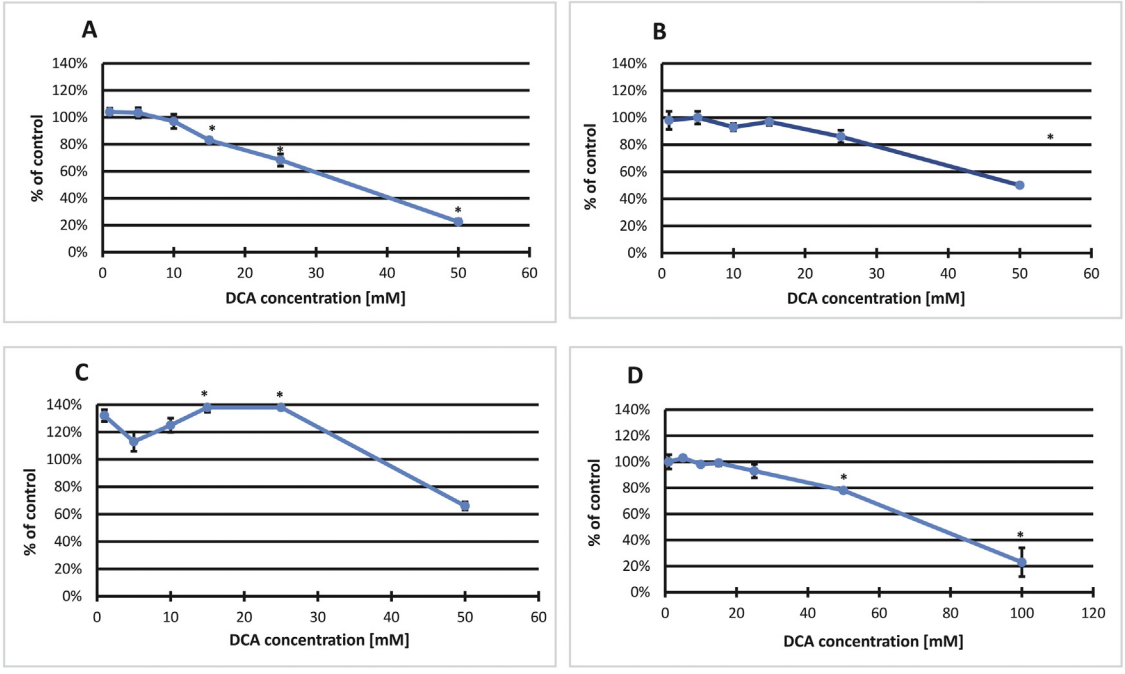


Fig. 1. Effect of sodium dichloroacetate (DCA) concentrations (1, 5, 10, 25, 50 mM) on CEM/C1 (A), CCRF/CEM (B), HL-60 (C), HL-60/MX2 (D) cells viability after 24-hour exposure. Results are shown as a percentage of a control set to 100%.

$$Ct_{\text{gene of interest}}{}^{c} - Ct_{\text{endogenous control}}{}^{c} = \Delta Ct_{\text{calibrator}} \quad b)$$

Normalising ΔCt of test sample to the ΔCt of the calibrator we get a formula:

$$\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}} = \Delta\Delta Ct$$

Then relative expression (RQ) of examined genes was calculated using formula:

$$RQ = 2^{-\Delta\Delta Ct}$$

Table 1
IC50 values after 24-hour exposure of CEM/C1, CCRF/CEM, HL-60, HL-60/MX2 cell lines to DCA.

	CEM/C1	CCRF/CEM	HL-60	HL-60/MX2
IC50/24	30 mM	50 mM	higher than 50 mM	75 mM

RQ values were converted to logarithm of RQ (log RQ) and analysis [20].

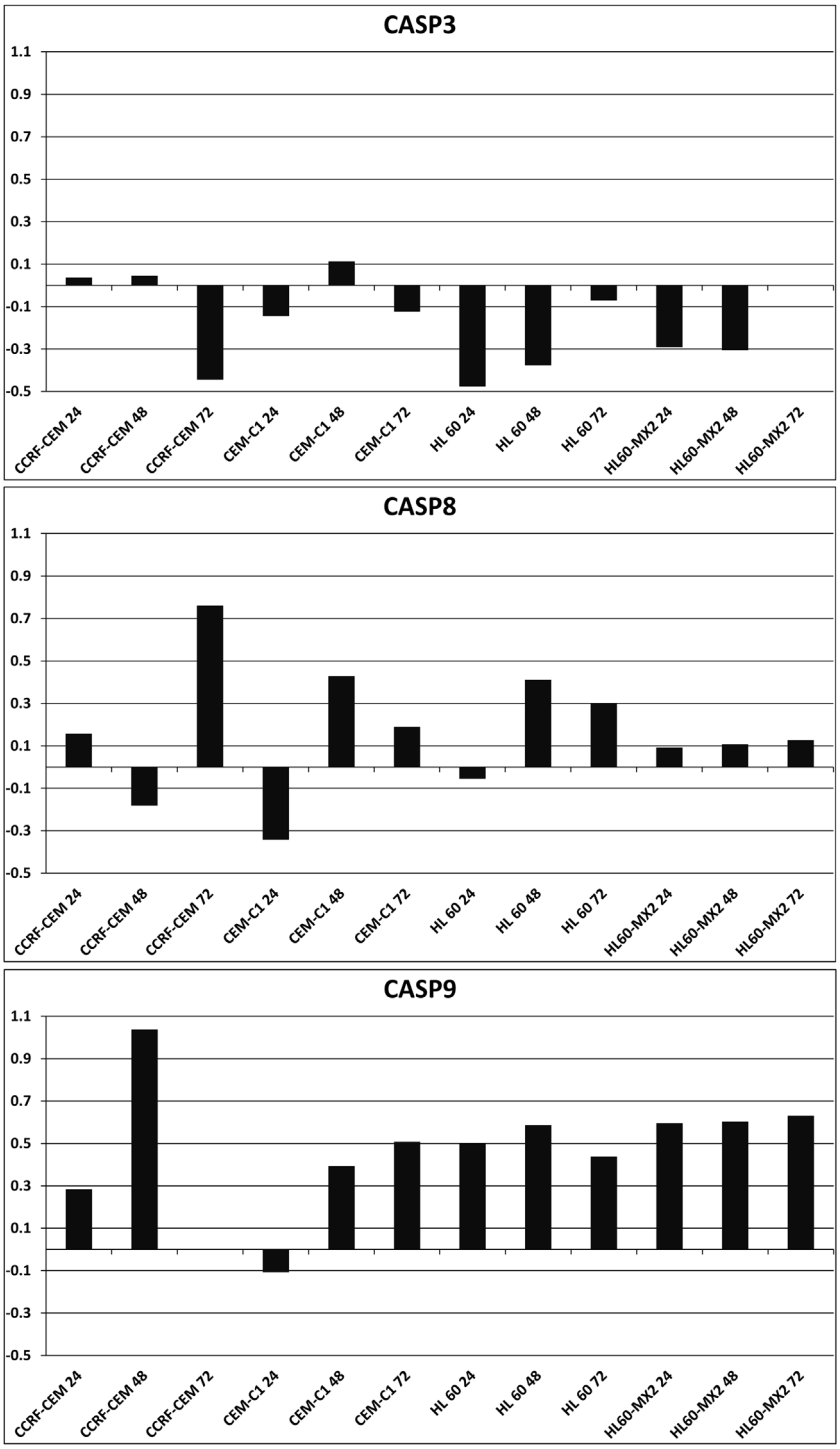


Fig. 2. Effect of DCA on changes of CASP3, CASP8 and CASP9 genes expression.

Results

Effect of DCA on CEM/C1, CCRF/CEM, HL-60, HL-60/MX2 cell viability

CEM/C1, CCRF/CEM, HL-60, HL-60/MX2 cell lines were treated with dichloroacetate at concentrations ranged from 1 to 50 mM and stained with trypan blue. On this basis, the dose response curves were made (Fig. 1) and IC10 and IC50 values were obtained (Table 1).

We demonstrated a fall in cell viability after exposure to DCA dependent on DCA concentration. CEM/C1 cells having IC50 value of 30 mM were the most sensitive to dichloroacetate.

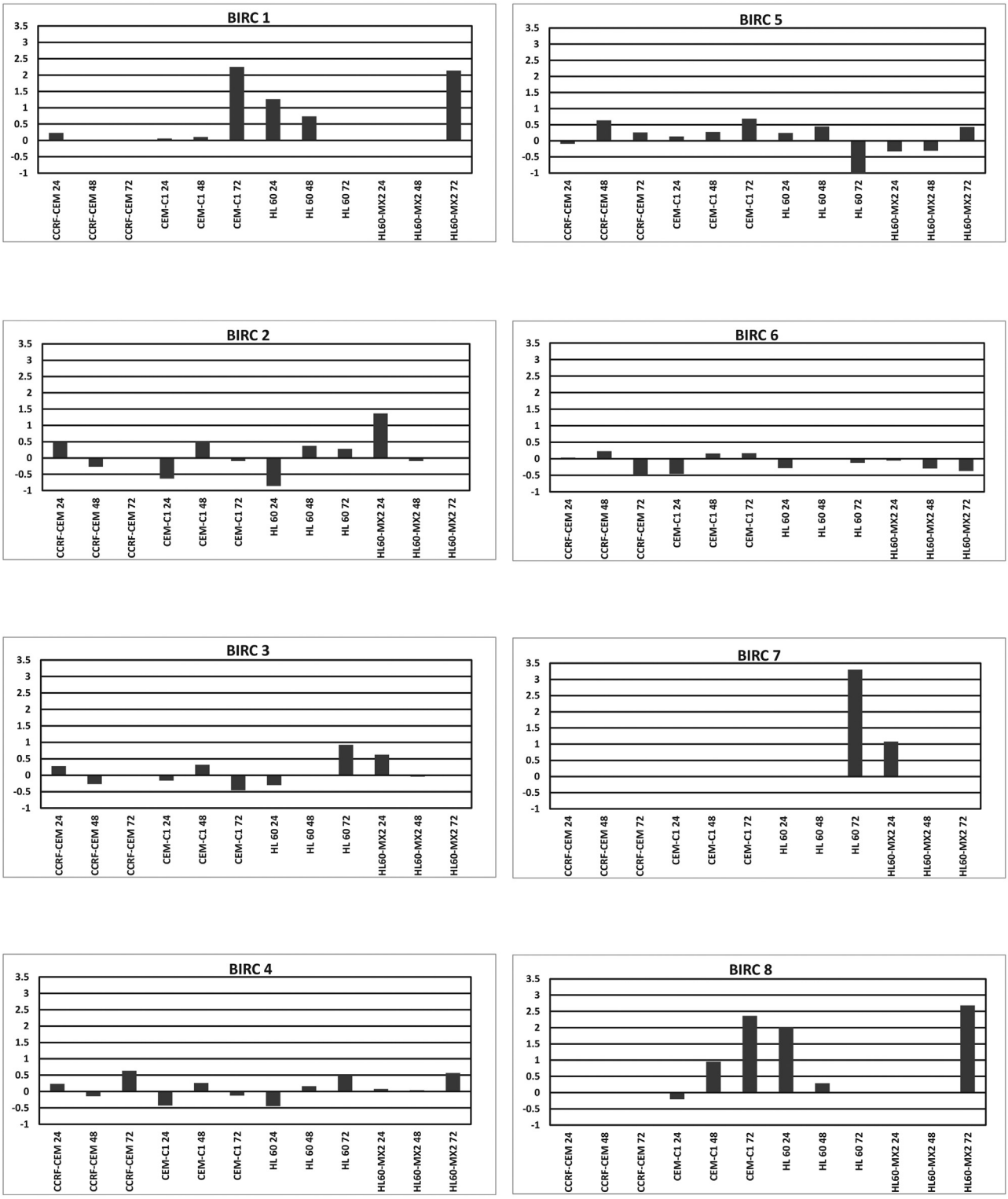
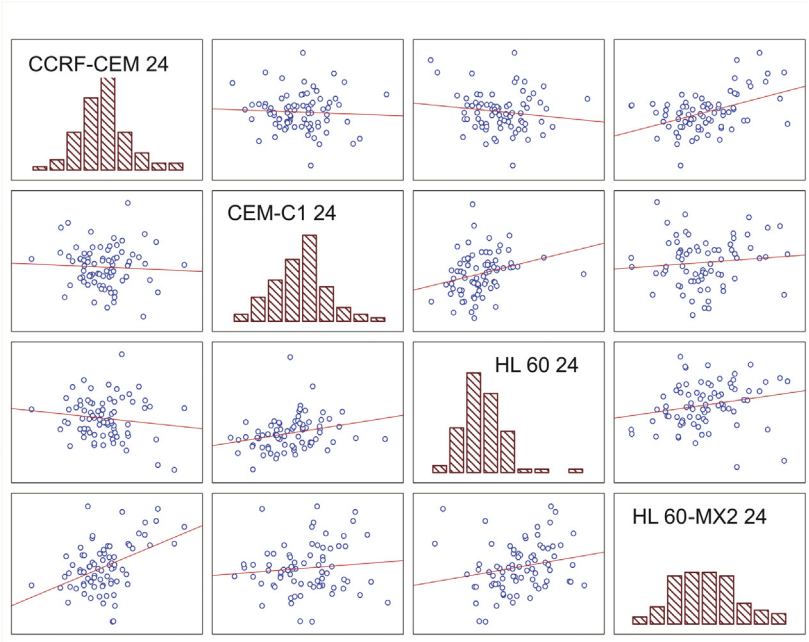


Fig. 3. Effect of DCA on changes of BIRC genes expression.

A

Variable	Spearman's Rank Correlations coefficient (TLDA 24h) In red are statistically significant p <0.05			
	CCRF-CEM 24	CEM-C1 24	HL 60 24	HL60-MX2 24
CCRF-CEM 24		-0.035772	-0.095917	0.360959
CEM-C1 24	-0.035772		0.335791	0.060391
HL 60 24	-0.095917	0.335791		0.308404
HL60-MX2 24	0.360959	0.060391	0.308404	

B



C

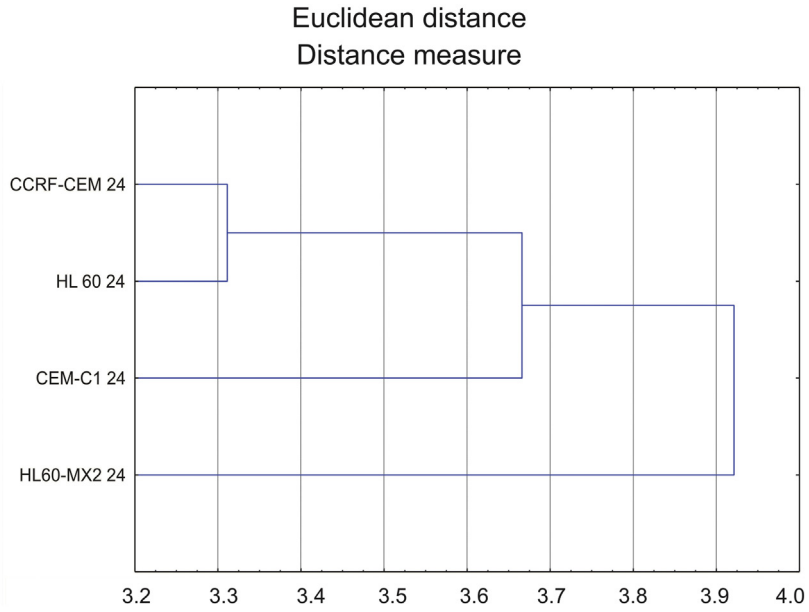


Fig. 4. Spearman's Rank Correlation Coefficients of each cell line after 24-hour exposure to DCA of IC50/24 value calculated on the basis of results of gene expression analysis **(A)**, graphs of correlations between molecular response of CCRF/CEM, CEM/C1, HL-60, HL-60/MX2 after 24-hour exposure to DCA of IC50/24 value **(B)**, dendrogram obtained as a result of Cluster Analysis on the basis of expression values (variables) of 93 genes in four cell lines after exposure to IC50/24 of sodium dichloroacetate **(C)**.

The HL60/MX2 cell line having IC50 value of 75 mM, was the most resistant to DCA was (Fig. 1).

The effect of DCA on gene expression in human leukemia cell lines

To provide the insight into the effect of DCA on the genomic level, we performed TLDA microarrays to identify gene expression differences in four leukemia cell lines treated with DCA of IC50/24 for 24, 48 and 72 h. HL-60 cell line was treated with 50 mM concentration of DCA because of lack of IC50 value. There was no certain group of genes (connected with intrinsic, extrinsic or other apoptosis pathways) in which an increase in expression level was significantly higher than in others in each cell line. We observed 40%–60% increase in the level of caspase 9 gene expression in most cases. Only in case of CCRF/CEM 72, CEM/C1 24 and CCRF/CEM 24 there was no increase or the increase was below 30% while in case of CCRF/CEM cell line after 48 h of exposure to DCA we observed the increase in caspase 9 level higher than 100%. We also observed the decrease (CCRF/CEM 72, HL60 24 – below 40%; HL60 48, HL60-MX2 24, HL60-MX2 48 was about 30%) or no significant changes in the caspase 3 gene expression (less than 20%) after the exposure of cells on DCA. The level of caspase 8 expression is different in different cell lines. The highest increase in caspase 8 gene expression level was in CCRF/CEM 72 – nearly 80%; CEM/C1 48, HL60 48, HL60 72 – increase between 20–40%; CEM/C1 24 – decrease below 30%. In other cases there were no significant changes of caspase 8 gene expression (Fig. 2).

Only BIRC genes were upregulated in different cell lines after 24, 48 and 72 h of exposure to DCA. However, the highest – more than 200% – increase in gene expression was observed after 72 h of exposure to DCA. Such an increase was present in BIRC1, BIRC8 expression (CEM/C1 after exposure to IC50/24), BIRC7 (HL60 after exposure to IC50/24), BIRC1, BIRC8 (HL60/MX2 after exposure to IC50/24) (Fig. 3). As BIRC genes encode inhibitors of apoptosis (IAP), the increase in their experssion level can indicate the inhibition of apoptosis by inhibiting caspase activity.

In order to compare the molecular response of CEM/C1, CCRF/CEM, HL-60 and HL-60/MX2 cell lines, an analysis of correlations and cluster analysis was performed. Correlation analysis of 93 genes expression changes was conducted using Spearman's Rank Correlation Coefficients as a distance measure between cell lines. Cluster Analysis was conducted and as a result dendrograms were obtained using the Euclidean distance as a distance measure. Correlation and cluster analyses show similarity between gene expression in CCRF/CEM, CEM/C1, HL60 cell lines and lack of similarity to HL60/MX2 after exposure to IC50/24 of DCA for 24, 48 and 72 h (Fig. 4).

In order to estimate relationship between variables (genes), an analysis of correlations was conducted calculating Spearman's Rank Correlation Coefficients between gene expression in groups created from cell lines exposed to DCA at a concentration of IC50 after 24, 48 and 72 h. The presence of 388 statistically significant correlations of the examined genes was ascertained ($p < 0.01$; n=4, n – the numer of examined cell lines) where 241 of which were positive correlations and 147 were negative ones. The highest number of correlations – 241 – was observed after 24-hour exposure of the examined cells to DCA and the lowest number equalling 39 after 72 h-exposure. Groups of genes that create the highest number of correlations with other genes are depicted on graphs (Figs. 5–7)

Discussion

Tumor cells usually exhibit metabolic alterations such as reliance on glycolysis rather than oxidative phosphorylation. It is a difference between tumor and normal cells which can be used in cancer treatment. Inhibitors of cancer metabolism such as DCA

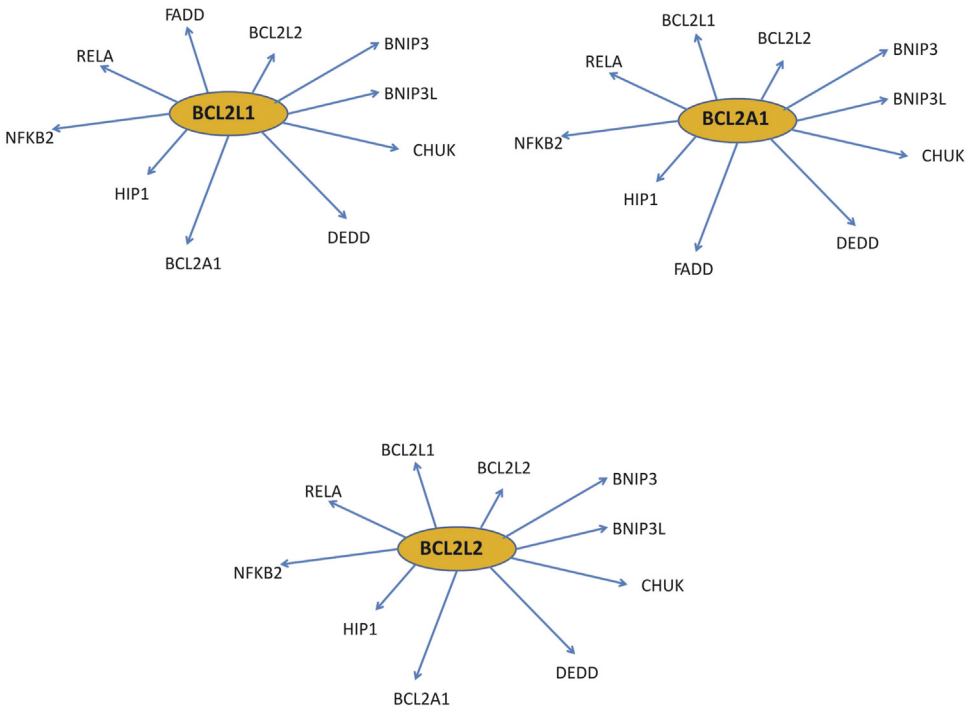


Fig. 5. Groups of genes, which expression changes are positively correlated with one another in CCRF/CEM, CEM/C1, HL-60, HL-60/MX2 cell lines after 24-hour exposure to the IC50/24 value of DCA. The expression of BCL2L1, BCL2L2, BCL2 A1 (antiapoptotic genes of BCL-2 subfamily) is highly correlated with the expression of 8 other genes: BNIP3, BNIP3L (proapoptotic BH3-only genes); FADD, DEDD (genes involved in extrinsic pathway of apoptosis induction); CHUK, NFKB2, RELA (genes involved in NFκB pathway); HIP1 (gene encoding a protein which interacts with huntingtin).

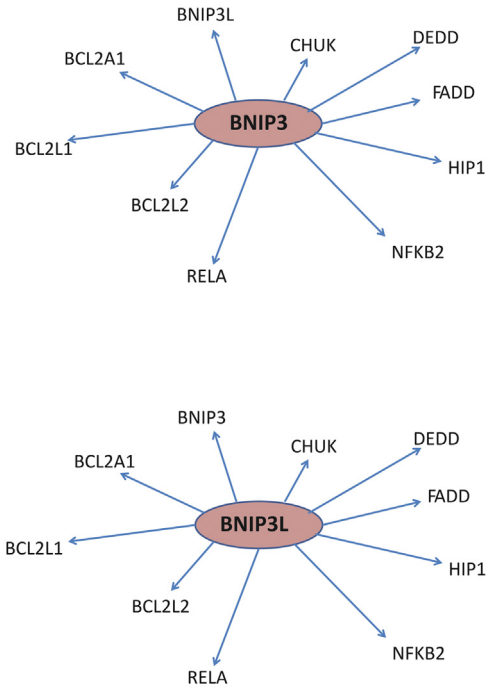


Fig. 6. Groups of genes, which expression changes are positively correlated with one another in CCRF/CEM, CEM/C1, HL-60, HL-60/MX2 cell lines after 24-hour exposure to the IC50/24 value of DCA. The expression of BNIP3 and BNIP3L (proapoptotic BH3-only genes) is highly correlated with the expression of 9 other genes: BCL2L1, BCL2L2, BCL2 A1 (antiapoptotic genes of BCL-2 subfamily), RELA, NFKB2, CHUK (genes involved in NFκB pathway), DEDD, FADD (genes involved in extrinsic pathway of apoptosis induction), HIP1 (gene encoding a protein which interacts with huntingtin).

have been shown to cause cancer cell death. The anticancer effect of DCA was reported on various cell lines such as: HSC2, HSC3 [2], U87 [21], MCF 7, 13,762 MA, V14 [9], AN3CA, SKUT1B, KLE, Ishikawa [22], RPMII8226-TGL, JJN-3 [4]. A few human leukemia cell lines such as: MOLM-14, MV4-11, MonoMac6, THP-1 [23], HL60 [8] were also investigated.

In this study we investigated the effect of DCA on cell viability and gene expression of four leukemia cell lines: CEM/C1, CCRF/CEM, HL-60, HL-60/MX2. The cytotoxicity of DCA for 3 of them has never been investigated before. As a result of cell incubation with low concentrations of DCA ranging from 1 to 5 mM, a slight increase in viability of cells was observed in comparison to the control. This observation is compatible with the results of Feuerecker who showed that DCA concentrations of 0.5–5 mM cause an increase in proliferation of SKBr3 breast cancer cells and Neuro 2 A and Kell cell lines [24]. Incubation with high concentrations of DCA, however, leads to a decrease in viability of cells. CCRF/CEM and CEM/C1 cell lines (originated from acute limfoblastic leukemia) were observed to be more sensitive to DCA than HL60 and HL60/MX2 (originated from acute promyelocytic leukemia). CEM/C1 cells (camptotecin resistant) were the most sensitive to sodium dichloroacetate. The IC50/24 value was 30 mM. The HL60/MX2 cell line (multidrug resistant) was the most resistant to DCA. In this case IC50/24 was 75 mM. These findings were similar to the results of Wong on endometrial cancer cell lines who showed that drug resistant HEC1A and HEC1B cell lines were insensitive to DCA [22]. Xu et al. reported that HL-60 and drug resistant HL60/AR have similar sensitivity to the glycolysis inhibitor 3BrPA. However, cells were cultured in different conditions (hipoxy) [25]. In HL-60 cell line, the IC50 value was impossible to establish within concentrations of DCA ranged from 1 to 50 mM while Agnoletto reported IC50 value for this cell line equalling 43 mM. IC50 value of MAVER, MEC-1, MEC-2 cell line was 27.9; 16.2 and 33.7 mM, respectively.

The current study focused on the analysis of the expression level of 93 genes involved in regulation of the process of apoptosis in human leukemia cell lines using TLDA microarray method, which has been the first research of this type. Our aim was to reveal the mechanism of cell death following DCA treatment common to all examined cell lines. Our results have not provided us with a clear answer. The gene expression profile does not indicate a certain pathway of apoptosis induction. It seems to be a sort of mixed mechanism as genes with the highest number of correlations are involved in different pathways of apoptosis induction. There are also some reports suggesting that apoptosis induction is not the only way of DCA action. It can also reduce proliferation [5], cause cell cycle arrest [4,26] and trigger autophagy in cancer cells [27,28].

It seems that there are also differences between molecular response to DCA of the examined cell lines. We also demonstrated an increase in expression of some BIRC genes that encode apoptosis inhibitors (IAP) after exposure to DCA with the highest increase after 72 h observed for: BIRC1, BIRC8 (CEM/C1 after 72-hour exposure to IC50/24), BIRC7 (HL60 after 72-hour exposure to IC50/24), BIRC1, BIRC8 (HL60/MX2 after 72-hour exposure to IC50/24).

Due to the correlation analysis of gene expression levels after 24, 48, 72-hour exposure to DCA, the presence of 388 statistically significant correlations of the examined genes was ascertained in total ($p < 0.01$) obtaining 241 positive correlations and 147 negative ones. As a result of Cluster Analysis, the similarity of molecular response between CEM/C1, CCRF/CEM and HL60 cell lines and the minor similarity between them and HL60/MX2, which may be connected with MDR (Multi Drug Resistance) phenotype of HL60/MX2 cell line. The highest number of correlations – 241 - was observed after 24-hour exposure of the examined cells to DCA and the lowest number of 39 after 72-hour exposure. The highest number of correlations was observed in the case of BCL2L1, BCL2L2, BCL2A2, BNIP3, BNIP3L, DEDD, FADD genes, which create a group of genes correlated with

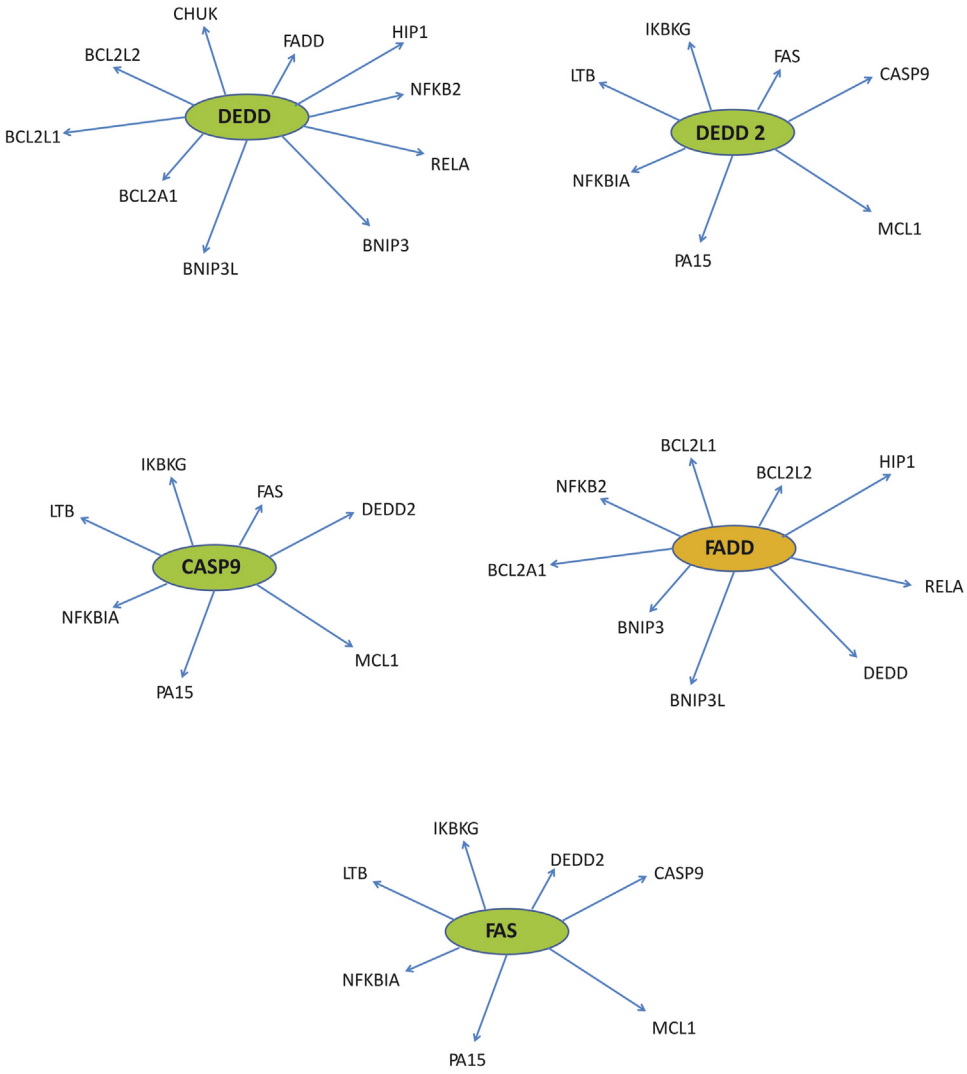


Fig. 7. Groups of genes, which expression changes are positively correlated with one another in CCRF/CEM, CEM/C1, HL-60, HL-60/MX2 cell lines after 24-hour exposure to the IC50/24 value of DCA. The expression of FADD and DEDD genes highly correlates with the expression of 9 other genes: BCL2L1, BCL2L2, BCL2 A1 (antiapoptotic genes of BCL-2 subfamily); BNIP3, BNIP3L (proapoptotic BH3-only genes); RELA, NFKB2, CHUK (genes involved in NFκB pathway); HIP1 (gene encoding a protein which interacts with huntingtin). The expression of FAS, DEDD and CASP9 highly correlates with the expression of 9 other genes: MCL1 (antiapoptotic genes of BCL-2 subfamily), LTB (lymphotoxin beta–ligand of TNF family receptors), NFKBIA (NFκB inhibitor), PEA 15 (apoptosis negative regulator).

one another, and with *RELA, HIP1* and *NFKB2. BCL2L1, BCL2L2, BCL2A1* genes which are connected with intrinsic apoptosis pathway, *FADD, DEDD* that connected with extrinsic pathway and *NFKB2, CHUK, RELA* that are connected with *NFκB* factor.

DCA can be concluded to possess anticancer activity assessed on cells obtained from different cancer types which has been confirmed by the results of our studies on leukemia cell lines. DCA causes a decrease in viability of CEM/C1, CCRF/CEM, HL-60, HL-60/MX2 cells. The expression analysis of genes involved in the process of apoptosis does not confirm that induction of apoptosis is the main mechanism of DCA effect on leukemia cells. There is some evidence that DCA may also act by influencing proliferation [18,29,30] or aurophagy. The molecular mechanism of antitumor properties of DCA needs further investigation.

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