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Effect of Dichloroacetate on Inducing Apoptosis in HeLa Cells

The Warburg effect-functioning primarily with aerobic glycolysis is a unique metabolic feature of cancer cells, and a potential target for therapy because shifting metabolism towards glucose oxidation might induce apoptosis. Recently, it was shown that dichloroacetate (DCA) may reverse the glycolytic phenotype, trigger apoptosis and produce significant cytotoxic effects in certain tumor cells. However, direct preclinical evidence of anticancer effects of DCA has been published only with non-small cell lung cancer, glioblastoma and breast, endometrial and prostate cancer. In the present work, we exposed HeLa cells to increasing concentration of DCA (0.5, 1, 5, and 10, 20 mM) to study in vitro *cytotoxicity of DCA and its effect on cell-induced apoptosis. The MTT viability test and the crystal violet assay were applied to access the effects of DCA on the viability of HeLa cells. The MTT assay results showed that DCA increased the cell--induced apoptosis in HeLa cells in a dose-dependent manner after 24 h and 48 h treatment at a concentration of above 1 mM. The crystal violet assay supported these results, showing that the cell death was caused by apoptosis and not necrosis. The results supported the hypothesis that DCA may reverse the suppressed mitochondrial apoptosis and could lead, at least in some extend, to less tumor growth.*

Introduction

According to Michelakis *et al*. (2008), the biggest challenge in cancer therapy is the selective induction of cell death (mainly apoptosis) in cancer cells but not normal ones. A potential approach to therapy is utilizing the knowledge that the unique metabolism of most solid tumors results in a remodeling of mitochondria (where the regulation of energy production and apoptosis converge), to produce a glycolytic phenotype and a strong resistance to apoptosis. Michelakis *et al*. (2008) note that the glycolytic phenotype is a "common denominator" for a vast range of cancer types, and may be linked to a potentially reversible suppression of mitochondrial function.

The Warburg effect is this use of glycolysis for energy in cancer cells, despite the presence of oxygen, although glucose oxidation is far more efficient. Because glycolysis is less efficient in ATP generation, cancer cells increase their glucose uptake to "catch up". However, glycolysis causes lactic acidosis, which might cause toxicity to the surrounding tissues and cancer cells; yet, Gatenby and Gillies (2004) also show lactic acidosis can aid tumor growth by breaking down the extracellular matrix allowing tumor expansion and increased metastatic potential. Kim and Dang (2006) have additionally shown that several of the enzymes involved in glycolysis are also important regulators of apoptosis and gene transcription. Shifting metabolism away from glucose oxidation and towards the glycolysis, might suppress apoptosis, because this type of cell death is dependent on energy production. Hence, the mitochondria might be essential in suppressing the cell death of cancer cells, and thus should be primary targets in cancer therapeutics.

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Dichloroacetic acid, often abbreviated DCA, is the chemical compound with formula $CHCl_2$-COOH. The salts and esters of dichloroacetic acid are called dichloroacetates. This generic drug is orally available and inhibits the pyruvate dehydrogenase kinase, thus increasing the entry of pyruvate into the mitochondria, promoting glucose oxidation over glycolysis in the tumor cells. This, at least to some extent, would reverse the suppressed mitochondrial apoptosis and result in less tumor growth. Bonnet *et al*. (2007) and Pan and Mak (2007) have shown DCA, a small molecule, penetrates most tissues after oral administration, which would increase its efficacy.

DCA has often been modelled as a "cheap, safe drug" that kills cancer cells, as in New Scientist. Still, clinical evidence is rare and limited, as Michelakis and Mackey point out. Realizing that majority of cancer tissue have hyperpolarized or remodeled mitochondria, and most tumors have increased glucose uptake, DCA might be effective in a wide range of cancer types. However, direct preclinical evidence of anticancer effects of DCA has been published only with non-small cell lung cancer, glioblastoma and breast, endometrial and prostate cancer.

HeLa cells are the first immortalized cell line, obtained from the aggressive glandular cervical cancer from 30-year-old Henrietta Lacks in1951. Since then, they've been widely utilized for research involving cell culture, particularly *in vivo* cancer studies; the use of HeLa cells ranges from cancer research to studies of biochemical pathways in both normal and diseased human tissue. HeLa would be suited for studying the effects of DCA because of their versatility and rapid proliferation. Additionally, as Desagher and Martinou (2000) mention in their review "Mitochondria as the central control point of apoptosis", HeLa cells have been used to demonstrate the link between changes in mitochondrial processes and suppression of apoptosis in cancer cells.

Hence, the aim of this project is to determine whether DCA will be effective in inducing apoptosis in HeLa cells in different concentrations of DCA.

Methods

The cells used were from the HeLa cell line, obtained from the University of Belgrade, Serbia. The medium in which the cells were plated was a 10% FBS (fetal bovine serum), 0.1% penicillin/streptomycin RPMI-1640 medium, and were washed with a standard PBS (phosphate buffered saline). The cell passage was performed by washing with PBS (2×10 mL), then addition of trypsin (0.2 mg/mL), incubation for 3-4 minutes followed by physical stress to the plate, after which the trypsin was neutralized with RPMI medium and the cell solution was transferred to new plates.

DCA Treatment. The HeLa cells were exposed to 5 different concentrations of DCA: 0.5, 1, 5, 10, 20 mM. The concentrations were produced by first making a "stock" 20 mM DCA in RPMI medium solution, and then diluting it to the other 4 concentrations. The control was pure RPMI medium, i.e. a 0 mM DCA solution. Measurements were performed after 24 hours of addition of the medium, and after 48 hours. For the 48-h cells, the medium was changed after 24 h had passed, maintaining the according values of DCA in the medium.

MTT and Crystal Violet Assays. The methods of the Laboratory Practicum, "Basis of Cell Biology" (Osnove ćelijske biologije), published by the University of Belgrade were followed for the MTT and Crystal Violet apoptosis assays.

The necessary solutions were made: 0.5 mg MTT/mL RPMI medium, 1 : 10 Crystal Violet in PBS, 33% acetate acid solution in water. The cells were washed with PBS, treated with MTT, incubated, and washed with DMSO. Then, their absorbance at 570 nm was measured with a UV/Vis spectrophotometer and results were recorded.

After the MTT assay was performed, the same plates were washed with PBS, fixed with methanol, colored with Crystal Violet solution, washed with water, and then with acetic acid. After the addition of the acid, the absorbance at 570 nm was measured again using a UV/Vis spectrophotometer.

The same method was used for the 24-h and the 48-h HeLa cell treatment.

Propidium Iodide/DAPI Staining. Cells detached from the dishes were detached with trypsin and resuspended with a pipette. The remaining cells were harvested and transferred to a sterile glass in a micro-titer plate with 6 wells with 2 mL RPMI medium and incubated. The cells were re-washed with PBS, fixed with 2 mL 4% formaldehyde and incubated at –20°C. After rehydration with PBS, the cells were stained with 1.5 mL 125 nM propidium iodide (PI), after which they were washed and colored with 1.5 mL 0.01% DAPI. After washing with PBS, the samples were kept in PBS until they were screened with a confocal microscope.

Results

The results from the MTT assay from the HeLa cells treated with DCA solution for 24 hours are presented in the Figure 1.

The results from the MTT assay from the HeLa cells treated with different concentrations

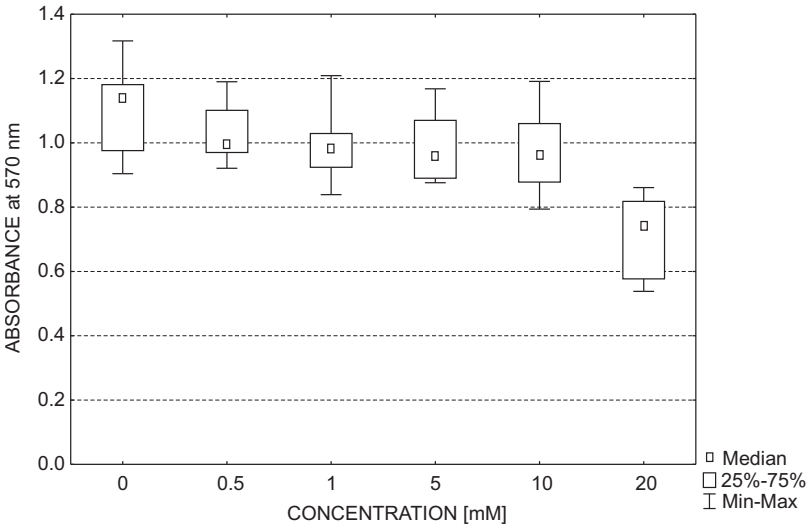

Figure 1.
Values of MTT Assay for 24-h DCA-treated HeLa cells

Slika 1.
Vrednosti MTT eseja HeLa ćelija za period inkubacije od 24 h

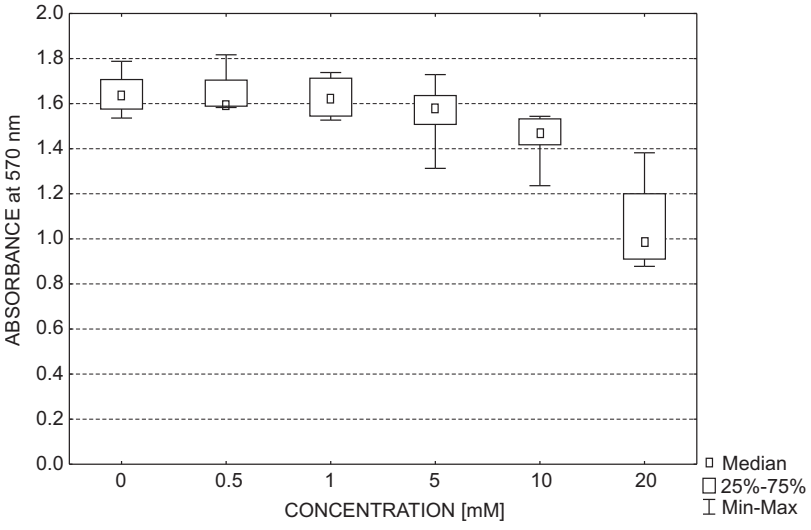

Figure 2.
Values of MTT Assay for 48-h DCA-treated HeLa cells

Slika 2.
Vrednosti MTT eseja HeLa ćelija za period inkubacije od 48 h

Table 1. Treatment of HeLa cells with DCA for 24 and 48 hours

Incubation period	Comparisons with Control				
	0.5 mM	1 mM	5 mM	10 mM	20 mM
24 hours	p = 0.19	p = 0.06	p = 0.01*	p = 0.04*	p = 0.00*
48 hours	p = 0.91	p = 0.63	p = 0.19	p = 0.00*	p = 0.00*

of DCA solution for 48 hours are presented in the Figure 2. The results of the Crystal Violet assay were consistent with the results of the MTT assay and confirmed, qualitatively and quantitatively, the decreasing trend of absorbance in relation to increasing DCA concentrations.

Mann Whitney U test was performed on the values obtained from the MTT assay and the following results were obtained (Table 1). Statistically significant difference ($p < 0.05$) are marked by asterix.

Discussion and conclusion

In this study, the results show that as DCA concentrations increase the cell-induced apoptosis in HeLa tissue increases, too. In the MTT assay, DCA concentrations above 5 mM (including 5, 10 and 20 mM) proved to induce apoptosis in both cases – after treatment for 24 and 48 hours – while 1 mM DCA concentrations had a significant difference only in the cells treated for 24 hours. Cells treated with 0.5 mM DCA weren't statistically different from the control in either case. The Crystal Violet assay supported the MTT assay, which shows that the cell death which was significant was caused by apoptosis and not necrosis, thus supporting the hypothesis that DCA stops the apoptosis suppression in cancer cells.

The role of DCA is in reversing mitochondrial remodeling (hyperpolarization), preventing the cancer cells from suppressing apoptisis. Because DCA is a small molecule (150 Da) explains its high bioavailability, which can reach 100% if administered orally, and the fact that it can enter commonly unattainable sites, including the brain.

First, to explain the "remodeling" of the mitochondria: cancer cells (non-small cell lung cancer, breast cancer and glioblastoma) have hyperpolarized mitochondria (Bonnet *et al*. 2007; Chen 1988). The hyperpolarization was linked to suppressed levels of mitochondria-derived reactive oxygen species (ROS) and decreased activity of plasma membrane K^+ channels. All these characteristics are compatible with apoptosis prevention, and could be products of a lessened mitochondrial activity, since decreased pyruvate levels (in the mitochondria) would cause decreased ROS production and closing of the K^+ channels. Additionally, less pyruvate in the mitochondria (and therefore the decreased glucose oxidation) would result in compensatory glycolysis – which was tied to apoptosis suppression. Michelakis and associates (2008) point to how several enzymes involved in glycolysis are also regulators of apoptosis, for instance activation of the glycolysis-enzyme hexokinase leads to suppression of apoptosis. Hexokinase has been noticed to be upregulated in many cancers (Kim and Dang 2006). Increased hexokinase levels would, thus, add to the hyperpolarization of the mitochondria.

In considering potential treatments that would induce apoptosis in cancer cells, and therefore in evaluating DCA as one of these potential treatments, the goal is to revert the cell from glycolysis back to glucose oxidation. Just inhibiting glycolysis would be not only useless, but also deterrent, because numerous bodily tissues depend on glycolysis, too, for energy; thus, the treatment would affect these tissues also, causing necrosis. Indeed, the wanted effect is apoptosis – which is common in normal tissue, but suppressed in cancer tumors – as opposed to necrosis, which can affect normal cells just as it affects cancer cells. One of the ways to enhance glycolysis to glucose oxidation is to activate the PDH (Pyruvate Dehydrogenase complex), which would bring pyruvates in the mitochondria and,

thus, increase the level of glucose oxidation. The utility of DCA is shown here: it inhibits PDK (Pyruvate Dehydrogenase Kinase), thus activating PDH and causing an increase in pyruvate uptake, and a decrease in lactate levels.

By increasing the glucose oxidation of the cell, DCA depolarizes the mitochondria – returning its membrane potential to normal-cells levels. Because it only returns the membrane potential values to normal, it doesn't have an effect on the non-cancerous tissue. All the above features, which were linked to mitochondrial depolarization and also linked to apoptosis suppression, were also returned to normal: ROS values increased and expression of K^+ channels increased. Thus, apoptosis was induced in the cells, and Bonnet *et al*. have shown that DCA decreases tumor growth both *in vivo* and *in vitro*, in non-small cell lung cancer, breast cancer, glioblastoma cell lines (2007) and endometrial (Wong *et al*. 2008) and prostate cancer cells (Cao *et al*. 2008).

Wong *et al*. demonstrated DCA reduced endometrial cancer cell viability in a dose-dependent manner without having an effect on normal cells, by inducing apoptosis with both cytochrome c and apoptosis-inducing factor efflux from the mitochondria. Their proposed mechanism was mitochondrial-regulated pathways, which include several molecular mechanisms: regulation of mitochondrial membrane potential, intracellular Ca^{2+} levels, and lowering survivin expression. Regulation of membrane potential and intracellular Ca^{2+} levels were related to decreased ROS values and decreased expression of K^+ channels, which links DCA to the apoptosis--induction mentioned above. Survivin is a new human gene which codes for a complex apoptosis inhibitor, which becomes expressed in transformed cancer cells – discharged from the mitochondria to the cytoplasm, where it prevents capase (cysteine-aspartic protease) activation and, thus, inhibits apoptosis (Ambrosini *et al*., 1997; Li *et al*. 1998).

The results from our study supported the hypothesis that DCA may reverse the suppressed mitochondrial apoptosis and could lead, at least in some extend, in less tumor growth.

References

Ambrosini G., Adida C., Altieri D. C. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nature medicine*, **3** (8): 917.

Austin E. W., Okita J. R., Okita R. T., Larson J. L., Bull R. J. 1995. Modification of lipoperoxidative effects of dichloroacetate and trichloroacetate is associated with peroxisome proliferation. *Toxicology*, **97** (1-3): 59.

Bonnet S., Archer S. L., Allalunis-Turner J., Haromy A., Beaulieu C., Thompson R. *et al*. 2007. A Mitochondria-K^+ Channel Axis is Suppressed in Cancer and its Normalization Promotes Apoptosis and Inhibits Cancer Growth. *Cancer cell*, **11** (1): 37.

Cao W., Yacoub S., Shiverick K. T., Namiki K., Sakai Y., Porvasnik S., Urbanek C., Rosser C. J. 2008. Dichloroacetate (DCA) sensitizes both wild-type and over expressing Bcl-2 prostate cancer cells *in vitro* to radiation. *The Prostate*, **68**: 1223.

Chen L. B. 1988. Mitochondrial membrane potential in living cells. *Annual review of cell biology*, **4** (1): 155.

Crabb D. A., Yount E. A., Harris R. A. 1981. The metabolic effects of dichloroacetate. *Metabolism*, **30** (10): 1024.

Desagher S., Martinou J-C. 2000. Mitochondria as the central control point of apoptosis. *Trends in cell biology*, **10** (9): 369.

Dypbukt J. M., Ankarcrona M., Burkitt M., Sjoholm A., Strom k., Orrenius S., Nicotera P. 1994. Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *Journal of Biological Chemistry*, **269**: 30553.

Gatenby R. A., Gillies R. J. 2004. Why do cancers have high aerobic glycolysis? *Nature Reviews Cancer*, **4** (11): 891.

Kim J-W., Dang C. V. 2006. Cancer's molecular sweet tooth and the Warburg effect. *Cancer research*, **66** (18): 8927.

Kim J-W., Dang C. V. 2005. Multifaceted roles of glycolytic enzymes. *Trends in biochemical sciences*, **30** (3): 142.

Li F., Ambrosini G., Chu E. Y., Plescia J., Tognin S., Marchisio P. C., Altieri D. C. 1998. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*, **396**: 580.

Madhok B. M., Yeluri S., Perry S. L., Hughes T. A., Jayne D. G. 2010. Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells. *British journal of cancer*, **102**: 1746.

Masters J. R. HeLa cells 50 years on: the good, the bad and the ugly. *Nature Reviews Cancer*, **2** (4): 315.

Michelakis E. D., Webster L., Mackey J. R. 2008. Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. *British journal of cancer*, **99** (7): 989.

Pan J. G., Tak W. M. 2007. Metabolic targeting as an anticancer strategy: dawn of a new era? *Science Signaling*, **381**: 14.

Sun R. C., Fadia M., Dahistrom J. E., Parish C. R. Board P. G., Blackburn A. C. 2010. Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth *in vitro* and *in vivo*. *Breast cancer research and treatment*, **120** (1): 253.

Wong J. Y. Y., Hugins G. S., Debidda M. 2008. Dichloroacetate induces apoptosis in endometrial cancer cells. *Gynecologic oncology*, **109** (3): 394.

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Uticaj dihloroacetata na indukciju apoptoze u HeLa ćelijama

Aerobna glikoliza ili Varburgov efekat, je jedinstvena karakteriska metabolizma ćelije raka koja može biti potencijalni target za terapiju. Prebacivanjem metabolizma ćelije od oksidacije glukoze na glikolizu, može se potisnuti apoptoza ćelije. Nedavno je pokazano (Madhok *et al*. 2010; Sun *et al*. 2010) da dihloroacetat (DCA) može izazvati promenu glikolitičkog fenotipa, aktivirati apoptozu i proizvesti citotoksične efekte u nekim tumorskim ćelijama. Medutim, direktni preklinički dokazi antitumornih efekata DCA objavljeni su samo za rak pluća, glioblastom i rak dojke, endometrijalni i rak prostate. U ovom radu, HeLa ćelijske linije humanog kancera uterusa izlagali smo dejstvu rastućih koncentracija DCA (0.5, 1, 5 i 10 i 20 mM) kako bismo proučili *in vitro* citotoksičnost DCA i njegov efekat na indukciju apoptoze. Skrining antiproliferativne aktivnosti ove supstance urađen je pomoću dva testa citotoksičnosti 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijum bromid (MTT) i kristal violet testa. Rezultati MTT testa pokazali su da je DCA povećao apoptozu u HeLa ćelijama na dozno zavistan način i posle 24- i 48-časovnog tretmana u koncentraciji iznad 1 mM. I rezultati dobijeni probom kristal violeta pokazali su da je smrt ćelija izazvana apoptozom, a ne nekrozom. Dobijeni rezultati podržali su hipotezu da DCA može preinačiti potisnutu mitohondrisku apoptozu i može, barem u izvesnoj meri, usporiti rast tumora.