Influence of Dichloroacetate (DCA) on Lactate Production and Oxygen Consumption in Neuroblastoma Cells: Is DCA a Suitable Drug for Neuroblastoma Therapy?

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Abstract
Many cancer cells metabolize glucose preferentially via pyruvate to lactate instead to CO₂ and H₂O (oxidative phosphorylation) even in the presence of oxygen (Warburg effect). Dichloroacetate (DCA) is a drug which is able to shift pyruvate metabolism from lactate to acetyl-CoA (tricarboxylic acid cycle) by indirect activation of pyruvate dehydrogenase (PDH). This can subsequently lead to an increased flow of oxygen in the respiratory chain, associated with enhanced generation of reactive oxygen species (ROS) which may cause apoptosis. In order to investigate if DCA may be suitable for neuroblastoma therapy, it was investigated on three human neuroblastoma cell lines whether DCA can reduce lactate production and enhance oxygen consumption. The data show, that DCA (in the low millimolar range) is able to reduce lactate production, but there was only a slight shift to increased oxygen consumption and almost no effect on cell vitality, proliferation and apoptosis of the three cell lines investigated. Therefore, DCA at low millimolar concentrations seems to be only of minor efficacy for neuroblastoma treatment.

Introduction
Many malignant cells metabolize glucose via pyruvate preferentially to lactate even in the presence of oxygen (aerobic glycolysis, Warburg effect), [1]. Since glycolysis is far less efficient with respect to ATP production than oxidation of glucose to CO₂ and H₂O, tumor cells compensate this drawback by accelerated glucose metabolism and high glucose uptake. This process is influenced by hypoxia inducible factor 1 (HIF-1) [2] and several different oncogenic pathways (myc, PI3K/AKT, ras-pathways) which accelerate glucose metabolism by enhancing glucose-transporter expression and increasing activity of glycolytic enzymes, among them lactate dehydrogenase A (LDHA) [3, 4, 5]. The preferred glucose metabolism to lactate is obviously advantageous.
for cancer cells since a reduced consumption of oxygen is associated with a reduced generation of reactive oxygen substrates (ROS) and may be protective against apoptosis [6]. Additionally, due to the increased turnover of glucose, more metabolites for autonomous cancer cell proliferation such as ribose-5-phosphate (nucleic acid synthesis) and NADPH (lipid synthesis) can be provided [7]. Furthermore, lactic acidosis in the microenvironment of cancer cells facilitates the breakdown of extra-cellular matrix allowing tumor expansion, increasing cell mobility/metastatic potential and activation of angiogenesis [8]. Therefore, disturbing the glycolytic pathway of tumor cells offers new therapeutical possibilities for the development of anticancer drugs. In 2007 Bonnet et al. published a study examining cancer cell metabolism, showing that the investigated cancer cells have hyperpolarized mitochondria and a low expression of the voltage gated potassium channel Kv1.5, both contributing to apoptosis resistance [6]. They presented a possible drug which would specifically target the described metabolism: Dichloroacetate (DCA), a known substance in the treatment of children with lactic acidosis [9], which shifts the glucose metabolism from pyruvate $\rightarrow$ lactate in direction pyruvate $\rightarrow$ acetyl-CoA by inhibiting pyruvate dehydrogenase kinase (PDK, especially PDK2) [10] (Fig. 1). PDK is a gate keeping enzyme that - by inactivating pyruvate dehydrogenase (PDH) - regulates the flux of pyruvate into the mitochondria to the tricarboxylic acid (TCA) cycle: When PDH is inhibited (e.g. by HIF-1) less pyruvate will enter the mitochondria thus reducing mitochondria associated production of ROS. Inhibiting PDK with DCA, thereby activating PDH, would target this specific metabolism. Bonnet et al. found that DCA decreased the mitochondrial membrane potential, augmented the mitochondrial complex I-based ROS production and activated K$^+$ channel Kv1.5 in cancer but not in normal cells. They postulated that in this way DCA induces mitochondria-dependent apoptosis, decreases proliferation and inhibits tumor growth while neither affecting noncancerous cells nor causing severe systemic toxicity.

In following studies on cancer cells in vitro [11-15] some showed cytotoxic effects at clinically relevant concentrations (0.5-1 mM), others required suprapharmacologic levels (10-100 mM). Investigations of preclinical models delivered promising results supporting the hypothesis that DCA has the ability to change tumor metabolism, even though the antitumor effect relies on the model tested with some tumor cell lines that do not respond to DCA treatment at all [16].

Recently a study of DCA treatment in human cancer was published [17], in which DCA was used in combination with surgery, temozolomide and radiation for the treatment of five patients with glioblastoma multiforme with promising clinical results in four of them. Moreover a case of a patient with Non-Hodgkin’s Lymphoma who showed a complete remission after administering DCA was reported [18].

Taken together the recent developments could make DCA one of the new possible drugs in anticancer therapy which target specifically cancer cells without harming normal cells [19]. The aim of our cell culture study was to analyse whether DCA could be a suitable substance for treatment of neuroblastoma by shifting glucose metabolism from lactate to oxidative respiration (Fig. 1).

Materials and Methods

Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), (S)-(+) Camptothecin, and dichloroacetic acid (DCA) were purchased from Sigma (Steinheim, Germany). Fetal Niewisch/Kuçi/Wolburg/Sautter/Krampen/Deubzer/Handgretinger/Bruchelt
calf serum (FCS), L-glutamine, penicillin/streptomycin, RPMI-1640 medium and trypsin/EDTA, were obtained from Biochrom (Berlin, Germany). Phosphate buffered saline without (PBS) and with Ca²⁺ and Mg²⁺ (PBS⁻⁻) were purchased from Gibco® 14040 invitrogen (Auckland, USA). The test kits for the determination of D-glucose and L-lactate, respectively, were obtained from R-Biopharm AG (Darmstadt, Germany). The QproteomeTM Mitochondria Isolation Kit was purchased from Qiagen® GmbH (Hilden, Germany). 2% sodium sulphite in 0.01 M sodium borate was provided by the Hospital pharmacy Tuebingen (Germany). Cacodylate buffer 0.1 M, glutaraldehyde (2.5%), araldite and agar-agar were provided by the Institute of Pathology and Neuropathology, University of Tuebingen (Germany).

Cell lines and cell culture

Three human neuroblastoma cell lines were used: Kelly (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), SK-N-SH (American Type Culture Collection, Maryland, USA), and LS cells, established by R. Handgretinger (University of Tuebingen, Germany) [20]. Cells were grown as monolayers in RPMI-1640 supplemented with heat inactivated fetal calf serum (10%), L-glutamine (2 mM) and penicillin/streptomycin (100 U/ml/100 µg/ml). Cell cultures for cultivation and cell suspensions for experiments were maintained at 37°C in a 95% air/5% CO₂ atmosphere at 100% humidity. The medium was changed depending on the cell line in intervals of 24 to 72 hours.

Mitochondria: Isolation and Electron Microscopy

Isolation of mitochondria from Kelly, SK-N-SH and LS cells was performed using the Qproteome Mitochondria isolation kit according to the instruction of the provider. Isolated mitochondria were resuspended in Storage buffer (provided by Qiagen® GmbH (Hilden, Germany)). Protein content of the suspensions was measured with the Lowry assay [21]. For electron microscopy, fractions of the isolated mitochondria-suspensions (Protein content SK-N-SH: 265 µg/100 µl, Kelly: 57 µg/100µl, LS: 190 µg/100µl) were centrifuged. For 2-4 hours, the pellets were fixed with glutaraldehyde (2.5%), buffered in 0.1 M cacodylate-buffer (pH 7.4). Thereafter, the pellets were transferred to agar-agar and afterwards washed with cacodylate buffer, dehydrated in (50-100%) ethanol and embedded in araldite. Ultrathin sections (ca. 50 nm) were prepared with a Leica Ultracut R-Ultramikrotom [Leitz Wetzlar, Germany] and contrasted with lead citrate. The electron microscopic pictures were taken with the EM-10 Electron microscope (Carl-Zeiss, Oberkochen, Germany).

MTT Test (proliferation and vitality assay)

Neuroblastoma cells were seeded in 96-well plates (1.5 x 10⁴ cells/200 µl cell culture medium). After 24 hours, cell cultures were incubated with DCA (adjusted at pH 7.0 with NaOH; final concentration: 250-2000 µM) for 48 hours. After incubation, cell viability was assessed by MTT assay according to Mosmann [22]: The supernatants were discarded, 100 µl RPMI-1640 containing 0.5 mg MTT/ml were added and incubation was carried out for 3 hours at 37°C. After addition of 100 µl solvent (82% isopropanol, 9% Triton X-100, 9% 1 mM HCl) plates were placed on a horizontal shaker over night in the dark. Absorbencies were measured in a microplate reader at 570 nm wavelength and a reference wavelength of 630 nm.

Apoptosis assay

For measuring apoptosis by DCA the homogeneous caspase assay (Roche, Mannheim, Germany) was used and carried out according to the manufactures instructions. Kelly, SK-N-SH and LS cells were seeded on 96 well plates. DCA (final concentration: 1mM) and camptothecin (4µg/ml) as well as PBS without DCA and DMSO (f.c.:0.1%) without camptothecin were added to about 20.000 and 40.000 cells, respectively and incubated for 4-24 hours at 37°C. Thereafter substrate working solution, containing DEVD-R110 (Asp-Glu-Val-Asp-Rhodamine110) was added and incubation was carried out for 1.5 hours at 37°C. Free rhodamine110 was measured fluorometrically (extinction: 499 nm; emission: 521 nm). A standard calibration curve for Rhodamine 110 was used in parallel.

Oxygen consumption

Oxygen consumption of whole cell- and isolated mitochondrial suspensions was measured continuously with a Clark-type electrode in a (closed 1 ml water-jacketed chamber containing a small magnetic stirrer, which was kept at 37°C through a circulating water bath), connected to a biological oxygen monitor (Strathkelvin Instruments, Glasgow, Scotland). Oxygen consumption was recorded over time and calculated from the slope of the graph representing decreasing pO₂ values in the chamber. The electrode quantifies dissolved O₂ in the medium as a mean to assess intact cell and mitochondrial respiration. The electrode was calibrated at 37°C with air saturated water [about 223 mM O₂] and a solution of 2% sodium sulphite in 0.01 M sodium borate buffer [0 mM O₂]. For experimental settings with human neuroblastoma cells, cell suspensions with 2x10⁶ cells/ml PBS⁺⁺ (containing different concentrations of glucose) or RPMI-1640 (containing about 11 mM glucose), were prepared. For experimental settings with mitochondria suspensions, 0.1 ml containing 100 µg cell protein were suspended in a total of 1 ml buffer solutions [Saccharose (0.225 M), K-phosphate-buffer (10 mM, pH 7.4), MgCl₂ (5 mM) and KCl (20 mM)]. Oxygen consumption was calculated as nanomoles O₂ per 2x10⁶ cells and minute for cells and as nanomoles O₂ per 1 mg protein and minute for isolated mitochondria.

Glucose consumption and lactate production

For measuring the dependence of oxygen consumption and lactate production from glucose concentration in the medium (Table 1), 2x10⁶ cells were resuspended in 1 ml PBS⁻⁻ with or without 1 mM or 5 mM glucose, respectively, and were incubated in the water jacket chamber of the Clark Electrode at 37°C. From each cell suspension 50 µl aliquots were taken after 0 and 60 minutes incubation time. After centrifugation (3 minutes at 400 g), supernatants were frozen at -20°C until glucose and lactate determination. For measuring the effects of DCA on cell proliferation, glucose consumption, lactate
**Table 1.** Glucose and oxygen consumption and lactate production in neuroblastoma cells incubated in PBS++ containing different glucose concentrations. 1 [nmol/(min×2×10^6 cells)], 2 Percentage of the theoretically possible lactate production when assuming that through glycolysis 1nmol glucose delivers 2 nmol lactate [%].

| Cell line | Glucose consumption | Lactate production | % Glucose consumption | O_2 consumption |
|-----------|---------------------|--------------------|-----------------------|-----------------|
| Kelly     | 0 mM Glucose        | 0                  | -                     | 4.7             |
|           | 1 mM Glucose        | 4                  | 1.5                   | 19              | 4.2             |
|           | 5 mM Glucose        | 1.8                | 2.3                   | 64              | 2               |
| SK-N-SH   | 0 mM Glucose        | 0                  | -                     | 1.8             |
|           | 1 mM Glucose        | 2.5                | 0.8                   | 16              | 1.6             |
|           | 5 mM Glucose        | 2.3                | 3                     | 65              | 1.5             |
| LS        | 0 mM Glucose        | 0                  | -                     | 2.1             |
|           | 1 mM Glucose        | 4.6                | 5                     | 54              | 4.1             |
|           | 5 mM Glucose        | 8.1                | 11.2                  | 69              | 4.6             |

**Table 2.** Influence of DCA on vitality/proliferation of Kelly, LS and SK-N-SH cells (MTT-test). Cells were incubated with DCA (250µM-2mM) for 48 hours. % of untreated control (100%), n=4 (Kelly: n=2), independent experiments, each 4 fold. Mean ± SD.

| Cell line | Control 250 µM DCA | 500 µM DCA | 750 µM DCA | 1 mM DCA | 2 mM DCA |
|-----------|---------------------|------------|------------|----------|----------|
| Kelly     | 100±11              | 97.5±14    | 83.1±20    | 80.3±17  | 85.5±13  | 85.6±19  |
| SK-N-SH   | 100±6.3             | 109±2.6    | 103±4.1    | 96.8±12.2| 102±5.9  | 98.4±7.0 |
| LS        | 100±2.8             | 102±2.3    | 97±1.2     | 96±5.7   | 97±6.1   | 89.3±6.1 |

**Table 3.** Changes of proliferation, oxygen- und glucose consumption and lactate production in neuroblastoma cells after 72 hours incubation with 1mM DCA. 1 [nmol/(min×2×10^6 cells)].

| Cell line | Cell number [×10^6] | O_2 consumption | Glucose consumption | Lactate production |
|-----------|---------------------|-----------------|---------------------|--------------------|
| Kelly     | Control 35.4        | 2.05            | 1.94                | 2.4                |
|           | + DCA 31.4↓         | 2.78↑           | 1.66↓               | 1.7↓               |
| SK-N-SH   | Control 12.5        | 2.76            | 0.6                 | 1.31               |
|           | + DCA 9.84↓         | 2.56↓           | 0.49↓               | 1.05↓              |
| LS        | Control 14.2        | 4.23            | 2.48                | 2.44               |
|           | + DCA 12.0↓         | 4.21            | 1.82↓               | 1.21↓              |

**Statistical analysis**

Two- sided unpaired t-tests were used. P value of < 0.05 was considered statistically significant.

**Results**

The aim of this work was to analyze whether DCA is able to shift pyruvate from lactate to acetyl-CoA for enhanced oxygen consumption. Therefore, lactate production vs. oxygen consumption in the presence of different glucose concentrations was analyzed in the first sets of experiments [Fig. 2A, Fig. 2B].
Oxygen consumption of neuroblastoma cells and their mitochondria

Fig. 2A shows the dependence of oxygen consumption of the three neuroblastoma cells from the glucose concentration: Compared to the physiological concentration (~5 mM glucose) was set as 100% (mean ± SD): Kelly cells: 2.96 ± 1.86 nmol oxygen/(min x 2x10^6 cells), n=5; SK-N-SH cells: 1.11 ± 0.49 nmol oxygen/(min x 2x10^6 cells), n=5; LS cells: 5.49 ± 1.3 nmol oxygen/(min x 2x10^6 cells), n=2. Isolated mitochondria from these neuroblastoma cells showed different morphologies (Fig. 2B). Electron microscopic pictures showed in all cases normal mitochondria with a moderately condensed and heavily condensed matrix. Kelly and SK-N-SH preparations had similar ratios of normal and moderately condensed mitochondria, whereas the LS mitochondria had the highest amount of heavily condensed mitochondria. The basal respiration rates increased in all three cell lines in the presence of succinate showing significant changes in Kelly and LS, but not SK-N-SH cells [Kelly: P = 0.015; LS: P = >0.0001; SK-N-SH: P = 0.088] (Fig. 2B).

Lactate production vs oxygen consumption of neuroblastoma cells in dependence of glucose concentration

Table 1 shows that under physiological conditions (5 mM glucose) all three neuroblastoma cell lines converted > 60% of glucose to lactate despite the presence of
Fig. 4. Activation of caspases (splitting of Rhodamine 110 from DEVD-R110) in SH-N-SH, LS and Kelly cells after incubation with DCA (1mM) and camptothecin (4 µg/ml, positive control). About 20,000 and 40,000 cells, respectively, were treated in 96 well plates with both substances for 6 and 24 hours. In parallel, cells were incubated with solvents (DMSO for camptothericin and PBS for DCA) Activity of caspases was measured fluorimetrically (RFU: relative fluorescence units). Standard calibration: $y = 542802x + 11312$, $R^2 = 0.999$; 1µM Rhodamine110 $\sim 563.300$ RFU.

Effects of DCA on neuroblastoma cells

DCA inhibits pyruvate dehydrogenase kinase and therefore indirectly stimulates pyruvate dehydrogenase. This should cause a shift from pyruvate $\rightarrow$ lactate to pyruvate $\rightarrow$ acetyl-CoA, and, subsequently, to an increased oxygen consumption. First, the effect of DCA on cell proliferation/vitality in clinically relevant concentrations (0.25–2 mM) was investigated using the MTT-test (Table 2). Under these conditions, no significant reduction could be observed after a 48 h incubation period with 1mM DCA [Kelly: $P = 0.894$; SK-N-SH: $P = 0.233$; LS: $P = 0.917$] Some effects were observed after a prolonged incubation period in cell culture flasks (Table 3), which was associated with the expected continuous decrease of lactate production (Fig. 3): After a 72 h incubation period, there was a decrease in lactate production in the presence of 1 mM DCA of 29% (Kelly), 20% (SK-N-SH) and 50% (LS), respectively, compared to untreated controls (Table 3). In parallel, glucose consumption decreased, but except for Kelly cells, oxygen consumption only slightly increased. Taken together, although a reduction in lactate production occurred in all three cell lines, there was no significant increase in oxygen consumption (and, therefore, probably no proportional increase in ROS-production necessary for inhibition of cell growth and vitality).

In accordance with this are the results obtained in the apoptosis assays. Whereas the positive control camphothecin caused clear effects on all three
neuroblastoma cell lines, DCA (1 mM) was completely ineffective on SK-N-SH and Kelly cells and showed only small effects on LS cells. The test was carried out several times using different cell concentrations and incubation periods. Fig. 4 shows exemplary the effects using 20,000 and 40,000 cells after 6 and 24 hours incubation time periods.

Discussion

It was reported that DCA can kill malignant cells by ROS production, which is associated with the enhanced consumption of oxygen in the OXPHOS reaction [6]. One presupposition for DCA efficacy is that mitochondrial pathways are not severely disturbed. However, in agreement with Warburg’s original assumption (which, nevertheless, proved to be not obligatory correct [23]), in many tumor cells different critical steps in mitochondrial pathways are defect. This is, therefore, in addition to direct enhancing effects on glycolysis by myc and other oncogenes, another, indirect reason for the preferred glucose consumption by glycolysis: Mutations of the mitochondrial TCA enzymes SDH (succinate dehydrogenase) and of fumarate hydratase (FH) lead to accumulation of succinate or fumarate, respectively. These substrates can stabilize HIF-1α (and, therefore, support glycolysis) even in the presence of oxygen by inhibition of prolyl hydroxylases, which are necessary for the α-ketoglutarate dependent degradation of HIF-1α [24].

Other TCA cycle associated (iso) enzymes, isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2), frequently found in gliomas and AML, also stabilise HIF-1α by inhibition of prolyl hydroxylases activity probably by lowering α-ketoglutarate or because of competitive inhibition by 2-hydroxyglutarate. Due to specific mutations, IDH1 and IDH2 are able to further reduce α-ketoglutarate to 2-hydroxyglutarate (Isocitrate → α-ketoglutarate → 2-hydroxyglutarate) [24, 25]. Furthermore, p53 mutations, frequently observed in many malignant cells, can also hamper mitochondrial pathways and, therefore, also support glycolysis in an indirect way: Under normal conditions, p53 supports induction of SCO2 (synthesis of cytochrome c oxidase) leading to proper formation of oxidative phosphorylation complexes [26]. Interestingly, p53 mutations were not found in neuroblastoma, but p53 can be inhibited in these cells through microRNA (miR-380-p) [27, 28]. Taken together, many different causes (already detected and still probably to be detected in the future) can be responsible for the reduced respiratory activity in cancer cells.

In agreement with these observations, Feichtinger et al. found that neuroblastoma cells have only a reduced capacity to consume oxygen in the OXPHOS reaction [29]. This was not due to mutations in one of the SDH subunits (as observed in the biochemically related phaeochromocytoma cells), but due to a general reduction in complex I, II, IV activities in the respiratory chain which may be associated with a low copy number of mitochondrial DNA detected by the authors in neuroblastoma cells.

In our own experiments shown in this paper, we observed only small effects of 1-2 mM DCA on neuroblastoma cells: Although lactate production could be reduced, oxygen consumption was only marginally enhanced, apoptosis was almost completely absent and cell growth was only slightly inhibited. Furthermore, a young boy with neuroblastoma brain metastases was treated with DCA after conventional therapy in our hospital. Although the drug was well tolerated the patient died in the meantime. Taken together we conclude that DCA is probably not very effective against neuroblastoma. In contrast, Vella et al published recently that undifferentiated, but not differentiated neuroblastoma cells were sensitive to DCA at very high concentrations (5 mM and 50 mM, respectively) [30]. Using 1-2 mM DCA (our study) no significant shift from glycolysis to OXPHOS could be observed. On the other hand, the low consumption rate of oxygen should be proportionally accompanied by low endogenous generation of ROS. This will probably lead to a down regulation of ROS-protecting systems in mitochondria with the consequence that these cells should be more vulnerable to ROS generating drugs [31].

In summary, treatment of a neuroblastoma patient with DCA was not successful and, in vitro, all three neuroblastoma cells investigated were almost not influenced by 1-2 mM DCA. Although this may differ in other, undifferentiated neuroblastoma cells, especially at much higher DCA concentrations [30], we conclude that DCA is probably not a favourite new drug for neuroblastoma therapy.

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