Dichloroacetate and PX-478 Exhibit Strong Synergistic Effects in a Various Number of Cancer Cell Lines.

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Abstract

**Background:** One key approach for anticancer therapy is drug combination. Drug combinations can help reduce doses and thereby decrease side effects. Further, the likelihood of drug resistance is reduced. Distinct alterations in tumour metabolism have been described in past decades, but metabolism has yet to be targeted in clinical cancer therapy. Recently, we found evidence for a synergism between dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor and HIF1-α inhibitor PX-478. In this study, we aimed to analyse this synergism in cell lines of different cancer types and to identify the underlying biochemical mechanisms.

**Methods:** The dose-dependent antiproliferative effects of the single drugs and their combination were assessed using MTT or SRB assays. FACS, Western blot and HPLC analyses were performed to investigate changes in reactive oxygen species levels, apoptosis and the cell cycle. Additionally, real-time metabolic analyses (Seahorse) were performed with DCA-treated MCF-7 cells.

**Results:** The combination of DCA and PX-478 produced synergistic effects in all eight cancer cell lines tested, including colorectal, lung, breast, cervical, liver and brain cancer. Reactive oxygen species generation and apoptosis played important roles in this synergism. Furthermore, cell proliferation was inhibited by the combination treatment.

**Conclusions:** Here, we found that these cancer metabolism-targeting compounds exhibited potent synergism across all tested cancer cell lines. Thus, we highly recommend the combination of these two compounds for progression to in vivo translational and clinical trials.

1. Background

1.1 Introduction

In the last decade, combinatorial approaches for cancer therapy have become increasingly popular (1). Drugs designed to act against individual molecular targets can hardly combat a multigenic disease such as cancer (2). However, synergistic drug combinations can lead to reduced drug doses with less pronounced side effects, increased response rates and attenuated likelihoods of drug resistance (1–3).

In a previous work (ZITAT), we screened 14 selected compounds, including dichloroacetate (DCA) and PX-478, for synergistic interactions on cancer cell lines. In detail, we conducted a screen in which we analysed all possible pairwise combinations at concentrations below the 25% maximal effective concentration (EC\textsubscript{25}) in HT-29 and MCF-7 cells. The combination of DCA and PX-478 displayed significantly stronger effects on cell viability than either single compound. In HT-29 cells, the combination proved to be particularly effective, decreasing the cell viability by an additional 62% beyond the summed effect of each single agent. The viability of MCF-7 cells was also decreased by the combination treatment but to a lower extent than in HT-29 cells (11%). This pattern does not demonstrate or define that this combination is synergistic, only that synergism is highly likely. Therefore, we aimed to further investigate this combination in the present study.

A widely accepted method of analysing synergism introduced by Chou and Talalay (4) quantifies synergism over the whole dose-response curve based on the mass action law.

1.2 Compounds
DCA inhibits pyruvate dehydrogenase kinase (PDK), leading to increased pyruvate dehydrogenase activity and partially reversing the Warburg effect (5). The Warburg effect switches metabolism towards anabolism while inhibiting apoptosis (6, 7). In cancer cells, suppression of the mitochondrial-K⁺ channel axis can underlie apoptosis resistance. DCA was found to normalize this axis and thereby induce the apoptosis of cancer cells (8, 9). In addition to its effects on the mitochondrial membrane potential, DCA is believed to lead to a significant increase in reactive oxygen species (ROS) generation, which plays an important role in the induction of apoptosis (10–14). By contrast, other authors reported that DCA may function as a sensitizer for ROS induced alterations, but did not significantly increase ROS production per se (13, 15). The DCA dose as well as the metabolic state of the cell may impact DCA-mediated ROS production. In a study by Wong et al., DCA induced apoptosis via mitochondrial regulated mechanisms in different endometrial cancer cells but did not affect non-cancerous cells (9). In addition, DCA has been shown to downregulate autophagy, thereby leading to enhanced tumour cell apoptosis and attenuated cell proliferation (16). Additionally, DCA positively regulates p53 activity (17) and is approved for therapy of hereditary lactic acidosis (18). In the other hand, different groups have identified off-target effects of DCA, such as activation of the AMPK signalling pathway, antagonization of acetate and disruption of tyrosine catabolism (19–23).

Since DCA is approved for therapy of hereditary lactic acidosis, much is known about its pharmacodynamic and pharmacokinetic profile (18, 24). In a phase 1 trial of DCA in adults with recurrent malignant brain tumours, oral administration of DCA was feasible and well tolerated when integrated with genetic-based dosing (25).

PX-478 interferes with the transcription and translation of hypoxia-inducible factor-1α (HIF1α) and leads to diminished deubiquitination of HIF-1α (26), thereby inducing apoptosis and cell cycle arrest (27, 28). In addition, PX-478 induces elevated levels of ROS (28). In oesophageal squamous cell cancer, PX-478 induces apoptosis and reduces cell proliferation and inhibits epithelial-mesenchymal transition (29). Welsh et al. identified a correlation between antitumor effects in human xenografts and HIF1α levels (30). Further, Aebersold et al. identified HIF1-α expression as a negative prognostic factor in oropharyngeal cancer (31).

HIF-1α inhibits the production of mitochondrial ROS as a reaction to ROS accumulation, hypoxia and cytokine stimulation. Interestingly, these effects are achieved partially through a decrease in the production of acetyl-CoA via upregulation of PDK-1 and −3, the direct targets of DCA (32, 33). Furthermore, HIF-1α also abolishes intracellular ROS accumulation independent of mitochondrial metabolism by directly increasing FOXO1 expression and thereby inducing expression of the antioxidant enzyme SESN3 in pancreatic carcinoma (28).

DCA-mediated inhibition of PDK leads to HIF1α inhibition and, thereby, angiogenesis (34). Furthermore, studies have shown that cancer cells exhibiting the Warburg effect gaining resistance to anoikis, a form of programmed cell death triggered by loss of cell-cell contacts, which is relevant to metastasis. This process can be reversed via ROS generation by enhanced oxidative glycolysis (35, 36). Additionally, mitochondrial biogenesis is regulated by HIF-1α (37). On the one hand, ROS play an important role in the induction of cancer cell apoptosis or necrosis (10). On the other hand, ROS can also induce tumour formation and growth, e.g., transformation of ovarian epithelial cells expressing H-RasV12 (38). Low levels of ROS can stimulate cell proliferation, whereas high levels of ROS lead to apoptosis and irreversible damage to cancer cells (36) Thus, ROS play a critical role in various types of human cancers.
Both compounds studied here were well tolerated in clinical studies (39, 40) and were not toxic to non-cancerous tissues. Agnoletto et al. demonstrated that DCA exerts cytotoxic activity towards primary B-cell chronic lymphocytic leukaemia (B-CLL) patient samples but not towards normal peripheral blood mononuclear cells obtained from healthy individuals when used at the same range of concentrations. HIF1α expression has been detected in solid tumours, including breast, colon and lung adenocarcinomas, but not in normal breast, colon or lung tissues (41).

In this study, we examined the effects of the combination of DCA and PX-478 on eight cancer cell lines and the non-cancerous cell line HEK-293. In addition, we studied the impact of the combination on ROS generation, apoptosis induction and cell cycle arrest.

2. Methods

2.1 Cell culture

The breast cancer cell lines MCF-7 and MDA MB-231 were a kind gift from Göran Landberg (Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg, Sweden). The colon cancer cell line HT-29, the hepatocellular cancer cell line HEPG2, the cervical cancer cell line HeLa and the adenocarcinoma lung cancer cell lines A549 and H441, as well as the non-cancerous cell line HEK-293, were purchased from the American Type Culture Collection (ATCC). The glioblastoma cell line U251 was a kind gift from Kai Murk (Charité Berlin, Germany). A549, HEK-293, HeLa, HEPG2, HT-29, MCF-7 and U251 cells were cultured in DMEM and H441 and MDA-MB-231 cells in DMEM/F12. All media contained penicillin/streptomycin (100 U ml⁻¹), L-glutamine (DMEM: 584 mg l⁻¹, DMEM/F12: 365.1 mg l⁻¹) and 10% heat-inactivated foetal calf serum (PAN Biotech, Germany). The humidified incubator was set at 37 °C with 5% CO₂. Cells were harvested using 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in PBS.

2.2 Compounds

PX-478 (Hölzel Diagnostika Handels GmbH, Cologne, Germany) and DCA (Sigma-Aldrich, Munich, Germany) were dissolved in distilled water.

2.3 Cell viability and cell proliferation assays

A total of 0.75 × 10⁴ A549, 1 × 10⁴ HEK-293, 0.3 × 10⁴ HeLa, 0.6 × 10⁴ HEPG2, 1.5 × 10⁴ HT-29, 0.5 × 10⁴ MCF-7, 1.5 × 10⁴ MDA-MB-231, 1 × 10⁴ H441 and 0.3 × 10⁴ U251 cells per well were seeded in flat bottom 96-well plates. After 24 hours, when cells were approximately 50% confluent, DCA, PX-478 or the combination was added. After 48 hours of further incubation, either a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Bio-Techne GmbH, Germany) or a sulforhodamine B (SRB) assay was performed. The MTT assay was performed according to the manufacturer's instructions. For the SRB assay, cells were fixed with 10% trichloroacetic acid (w/v) and stained with 0.06% SRB in 1% acetic acid for 30 minutes. Cells were then repeatedly washed with 1% acetic acid (v/v) and dissolved in 10 mM Tris (pH 10.5). The protein mass was measured by determining the optical density at a wavelength of 492 nm in a microplate reader. All experiments were performed independently three times with at least 2 technical triplicates (mostly with 3).
Dose-response curves were generated using GraphPad Prism 7.05 statistical analysis software. The half-maximal effective concentration (EC\textsubscript{50}) of each compound was determined via nonlinear regression.

### 2.4 Confirmation of synergism

Synergism was evaluated for four to seven concentrations (mostly with 6), as suggested by Chou and Talalay (4).

Cells were treated with the combination of DCA and PX-478 at a constant EC\textsubscript{50}:EC\textsubscript{50} ratio as well as with the single compounds alone. Significant differences between each single compound and the combination were assessed by an unpaired t-test. Only concentrations with p-values of ≤ 0.05 for both single compounds compared to the combination were considered to exhibit significant differences and are marked with an asterisk (*) in the figures.

Combination indices (CIs) were calculated using CompuSyn software (42). The CI is a quantitative value indicating the synergism of a drug combination at specific concentrations. A value of less than 0.9 indicates synergism (the lower the CI, the stronger is the synergism). Values from 0.9 to 1 indicate a nearly additive effect, and a CI value of greater than 1.1 indicates antagonism (43, 44). CI values were calculated as follows:

\[
CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}
\]

In the numerators, (D\textsubscript{1}) and (D\textsubscript{2}) are the concentrations of drug 1 and drug 2, respectively, in the drug combination that have a certain effect on cell viability (x %). In the denominators, (D\textsubscript{x1}) and (D\textsubscript{x2}) are the concentrations of each drug alone (drug 1 or drug 2, respectively) that are necessary to obtain the same effect (x %) as the drug combination (both drug 1 and drug 2). The concentrations (D\textsubscript{x1}) and (D\textsubscript{x2}) were calculated by CompuSyn with reference to the cell viability data for the respective compounds. To enhance analytical robustness, most concentrations of the compounds were doubled. Therefore, potential calculation errors were minimized, as suggested by Zhao et al. (45). To generate the median-effect plots, the following equation was used:

\[
D_x = D_m \left[ \frac{fa}{1 - fa} \right]^{1/m}
\]

where D\textsubscript{m} is the median effective dose, m is the slope of the median-effect curve, and fa is the fraction affected. Since calculation of a CI value is appropriate only when neither single compound has an effect close to 100%, the respective CI values are not shown in the Results Sect. (45). All data collected in this study can be found in the additional file 1 (additional file 1).

### 2.5 Membrane lipid oxidation rate

HT-29 cells were seeded in 10 cm diameter Petri dishes and treated with the EC\textsubscript{50} dose of DCA, the EC\textsubscript{50} dose of PX-478 or the combination after 24 hours when the cells were approximately 80% confluent. After incubation for an additional 48 hours, cells were harvested with trypsin, pelleted and resuspended in 500 µl of PBS. For lipid
extraction, cells were homogenized in a mixture of methanol:chloroform:water (2:1:1 by volume) using a modified Bligh/Dyer method. The extracted lipid suspension was bubbled with argon to prevent artificial oxidation. Then, alkaline hydrolysis was carried out, and the resulting free fatty acids were analysed by reversed-phase HPLC (RP-HPLC). Arachidonic acid and its oxygenated derivatives 10-/15-hydroxyeicosatetraenoic acid (HETE) were identified by their specific retention times and UV spectra and were quantified via integration (46).

2.6 Flow cytometric analysis:

Samples were analysed with BD FACS Calibur and Cell Quest.

2.6.1 Detection of intracellular ROS

Intracellular ROS were detected via an oxidation-sensitive fluorescent probe (2’,7’-dichlorodihydrofluorescein diacetate [H2DCFDA], Bio-Techne GmbH, Germany). HeLa and MCF-7 cells were seeded in 6 cm diameter Petri dishes and treated after 24 hours at a confluence of 50%. Cells were treated with the EC50 dose of DCA, the EC50 dose of PX-478 or the combination for 48 hours. Then, cells were harvested and washed twice with PBS. Next, cells were incubated with 50 µM H2DCFDA at 37 °C for 20 minutes in the dark and were then put on ice. Cells were washed 2 more times before being analysed by flow cytometry.

2.6.2 Evaluation of apoptosis by Annexin-V-FITC and propidium iodide staining

HeLa and MCF-7 cells were seeded in 6 cm diameter Petri dishes and incubated for 24 hours to a confluence of approximately 60%. After 24 hours, cells were treated with PX-478, DCA or the combination and harvested 48 hours later. The following concentrations were used: HeLa cells—EC50 DCA and 0.5 x EC50 PX-478; MCF-7 cells—EC50 DCA and EC50 PX-478. Cells were washed twice with PBS, put on ice immediately, transferred to binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl2; pH 7.4) and stained with Annexin-V-FITC (Hölzel Diagnostika Handels GmbH, Germany) in the dark according to the manufacturer’s instructions. After 15 minutes, propidium iodide (50 µg/ml) was added, and cells were analysed by flow cytometry.

2.7 Western blot analysis

For Western blotting, cells were seeded in 6 cm diameter Petri dishes, grown to approximately 80% confluence and treated with the noted compounds. Twenty-four hours later, cells were washed with PBS and lysed with lysis buffer (50 mM β-glycerophosphate pH 7.6, 1.5 mM EGTA, 1.0 mM EDTA, 1% (v/v) Triton X-100, 0.2% (v/v) protease inhibitor cocktail, 0.4% (v/v) PMSF, 100 mM natrium vanadate, 500 mM NaF) on ice for 60 minutes. The lysed cells were centrifuged for 15 minutes at 4 °C and 11,000 x g, and the supernatants were denatured by heat. The samples were separated under reducing conditions by 10% SDS-PAGE and transferred to nitrocellulose membranes (Thermo Fisher, Rockford, USA). The membranes were subsequently blocked overnight and were then incubated with the corresponding primary antibodies in blocking buffer (5% non-fat dry milk/TBS/0.1% Tween 20 for the anti-PARP antibody and 5% BSA/TBS/0.1% Tween 20 for all other antibodies) overnight at 4 °C. The primary antibodies and the corresponding working concentrations are listed in Table 1. After incubation with appropriate peroxidase-conjugated secondary antibodies, proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Fisher Scientific, Bonn, Germany). Signals were visualized using a VersaDoc™ 4000 MP and QuantityOne® 4.6.5 software (BioRad Laboratories, Munich, Germany) and quantified using ImageJ 1.52a software (National Institute of Health, USA; version 1.8.0_112).
2.8 Metabolic assays

MCF-7 cells were seeded in an XF 96-well culture microplate (Agilent, Santa Clara, USA) at 3 x 10^4 cells per well in 180 µl of prewarmed assay medium. After 24 hours, a mitochondrial respiration assay or glycolytic rate assay was performed with a Seahorse XFe96 Analyzer (Agilent Technologies). For the mitochondrial respiration assay, the oxygen consumption rate (OCR) was measured using the mitochondrial stress test procedure in XF media (non-buffered DMEM containing 10 mm glucose, 2 mm L-glutamine and 1 mm sodium pyruvate). The glycolytic rate was measured in accordance with the Agilent Seahorse XFp hGlycolytic Stress Test Kit instructions. After four measurements of either the baseline OCR or baseline extracellular acidification rate (ECAR), DCA solution was injected into the appropriate wells to the desired working concentration. Before each measurement, the assay medium was gently mixed to restore normal oxygen tension and pH in the microenvironment surrounding the cells. Two hours after treatment with DCA (6 measurements), the actual mitochondrial respiration assay or glycolytic stress test was performed. When metabolic analysis was complete, the cells were immediately fixed, and an SRB assay was performed as described above for data normalization. Graphs were produced using GraphPad Prism 7.05 statistical analysis software. Glycolytic capacity and maximal respiration (Fig. 5) were calculated as follows:

- maximal respiration (OCR) = (maximum rate measured after injection of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone [FCCP]) – (non-mitochondrial respiration rate)
- non-mitochondrial respiration (OCR) = minimum rate measured after injection of rotenone & antimycin A
- Glycolytic capacity (ECAR) = (maximum rate measured after injection of oligomycin) – (non-glycolytic acidification rate)
- non-glycolytic acidification (ECAR) = minimum rate measured after injection of 2-deoxy-D-glucose (2DG).

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.05 statistical analysis software. Differences with a p-value of ≤ 0.05 were considered significant: Significant differences compared to the control are marked with an asterisk (*), while significant differences between the combination and both, the control and each single compound, are marked with two asterisks (**). All experiments were performed with at least 2 technical and 3 biological replicates.

3. Results
3.1 The combination of DCA and PX-478 produces synergistic effects in eight cancer cell lines

First, we evaluated the effects of DCA and PX-478 on eight cancer cell lines: A549 and H441 (lung cancer), MCF-7 and MDA-MB-231 (breast cancer), HeLa (cervical cancer), HepG2 (hepatocellular cancer), HT-29 (colon cancer) and U251 (glioblastoma) (Fig. 1). For all cell lines, SRB or MTT assay was performed. Compounds were combined at a fixed EC$_{50}$:EC$_{50}$ ratio. To evaluate synergism, we calculated CI values for different concentrations over the whole dose-response curve using the program CompuSyn, in accordance with the method of Chou and Talalay (4). A CI of less than 0.9 indicates synergism. The lower the CI value, the stronger is the synergistic interaction for the indicated concentration. The EC$_{50}$ values used for treatment in the combinatorial experiments were determined for all cell lines in preceding experiments and are henceforth referred to as the approximated half-maximal effective concentration (EC$_{50}$a) values (47) (see additional file 1). The actual EC$_{50}$ values for the experiments conducted herein were calculated afterwards (see Table 2).

While the combination showed synergistic effects in all cell lines at lower concentrations, the combination showed synergistic effects in the adenocarcinoma cell line A549 and the hepatocellular cancer cell line HEPG2 over the complete dose-response curve, with CI values ranging from 0.61 to 0.87 and 0.56 to 0.79, respectively.

As shown in Table 2, which lists the EC$_{50}$ data and best CI values from the dose-response curves, synergism was observed in all analysed cell lines, with the lowest CI values in MCF-7, HT-29 and HEPG2 cells (Table 2).

The lowest CI value (CI = 0.4), indicating the strongest synergy for the experimental data points across the eight cell lines, was obtained in MCF-7 cells at 0.125 x EC$_{50}$a. The second lowest CI value (CI = 0.54) was obtained in HT-29 cells at 0.8 x EC$_{50}$a, while the third lowest (CI = 0.56) was obtained in HEPG2 cells at 0.5 x EC$_{50}$a. We included CI values primarily relying only on experimental data and eliminated CI values for concentrations where the effect of either single compound was too close to 100%, as suggested by Zhao et al. (45). Interestingly, the combination of DCA and PX-478 strongly affected cell viability or the protein mass in all cell lines, leading to a left shift in the dose-response curves. The combination treatment allowed the concentration of each single drug to be noticeably reduced (Table 2). For example, in MCF-7 cells, the EC$_{50}$ values of DCA and PX-478 were reduced by 68% and 64%, respectively. Collectively considering all cell lines, the EC$_{50}$ values of the compounds were profoundly reduced by an average of 57% when used in combination relative to when used as single agents.

Comparison of the EC$_{50}$ values of PX-478 in HT-29 and MDA-MB-231 cells indicates that noticeably higher doses were needed in these cell lines than in the other cell lines, indicating resistance to PX-478. For MDA-MB-231 cells, the resistance to PX-478 resulted in relatively weak synergism compared to that in the other cell lines, as reflected in the CI values as well as the dose-response curve, where the curve for DCA is close to that for the combination (Table 2 and Fig. 1). However, clear synergism was shown for HT-29 cells even though a higher dose of PX-478 was required. In these cells, the resistance did not inhibit the synergism.
Table 2 lists the EC$_{50}$ values for DCA, PX-478 and the combination of both in all tested cell lines. The EC$_{50}$ values were calculated via curve fitting with the program GraphPad Prism. In the last column, the lowest CI value indicating synergism (CI < 0.9) is listed.

### 3.2 The combination of DCA and PX-478 shows limited effects on the non-cancerous cell line HEK-293

This work aimed to present a potential therapeutic strategy for cancer by targeting cancer metabolism, which should not affect normal tissue. Therefore, we analysed the effects of DCA, PX-478 and their combination on the non-cancerous cell line HEK-293, which was derived from human embryonic kidney cells (Fig. 2). HEK-293 cells were incubated to a confluence of 50% and were then treated with different concentrations of DCA, PX-478 and the combination (Fig. 2A). Only minimal effects were observed for PX-478 and the combination, and only for DCA a slight reduction in the protein mass was seen, at concentrations of 10 and 15 mM (10 and 19% of that in the respective untreated cells – control cells).

Further, we compared the effect of combination treatment with 15 mM DCA and 15 µM PX-478 in HEK-293 cells to the effect of similar doses in the cancer cell lines used to generate the dose-response curves in Fig. 1. If no similar dose had been used in a specific cell line, lower concentrations were selected for comparison. For example, in MCF-7 cells, 10 mM DCA and 4 µM PX-478 led to a reduction of 52% in the protein mass compared to that in the corresponding control cells. This effect was significantly stronger than that of 15 mM DCA and 15 µM PX-478 on HEK-293 cells (97% protein mass, p = 0.000007). The six tested cell lines that were sensitive to PX-478 were significantly more sensitive to the combination at a comparable concentration (Fig. 2B). However, no significant difference was found in HT-29 cells. MDA-MB-231 cells were not compared, since due to the described resistance, no doses of PX-478 close to 15 µM were used. In conclusion, 6 of the 8 cancer cell lines were significantly more sensitive than the immortalized non-cancerous cell line HEK-293 to the combination of DCA and PX-478.
3.3. The combination of DCA and PX-478 increases ROS levels as well as it leads to apoptosis and cell cycle arrest.

The existing data for PX-478 and DCA suggest some predominant theories concerning the mechanisms underlying their synergism. In the following experiments, the effects of this combination on increasing reactive oxygen species generation, arresting the cell cycle and inducing apoptosis were investigated.

3.3.1 The combination of DCA and PX-478 increases ROS levels in HT-29, MCF-7 and HeLa cell lines

To investigate the relevance of the combination to ROS production, we performed HPLC measurements with HT-29 cells to analyse the oxidation of arachidonic acid derivatives (Fig. 3A). DCA-treated cells showed a non-significant (21%, \( p = 0.21 \)) increase in the 5- and 10-HETE levels compared to those in control cells. In cells treated with PX-478, the oxidation ratio was significantly increased by 58% compared to that in control cells (\( p = 0.04 \)). The combination treatment led to a 109% increase in the oxidation ratio, which was significantly higher than that observed for the control treatment (\( p = 0.02 \)) but did not differ significantly from that observed for PX-478 alone (\( p = 0.22 \)).

Furthermore, we evaluated the relevance of this combination to ROS via FACS analysis with H2DCFDA in HeLa, MCF-7 and HT-29 cells (Fig. 4). H2DCFDA reacts with ROS, and fluorescent dichlorofluorescein (DCF) can be measured in the FL1 channel. The results shown in Fig. 4B confirmed our HPLC results in HT-29 cells. FACS analysis showed that compared to control treatment, DCA did not affect ROS activity in any cell line. ROS production was significantly increased in HeLa cells (2–12%, \( p = 0.008 \)) but not in MCF-7 cells (3–4%, \( p = 0.37 \)) or HT-29 cells (7–10%, \( p = 0.089 \)) treated with PX-478 alone compared to control cells. Compared to the single compounds, the combination led to significant increases of 28% (\( p = 0.021 \)), 16% (\( p = 0.0002 \)) and 37% (\( p = 0.014 \)) in HeLa, MCF-7 and HT-29 cells, respectively. Thus, as our results in HeLa, MCF-7 and HT-29 cells suggest, increased ROS is likely to play an important role in the synergism of DCA + PX-478 combination treatment.

3.3.2 The combination of DCA and PX-478 leads to apoptosis and a reduction in proliferation

Western blot analyses of PARP/cleaved PARP, Ser795-phosphorylated Retinoblastoma protein (pRB1) and Cyclin D1 were performed in HT-29 and MCF-7 cells (Fig. 5). In MCF-7 cells, two concentrations of DCA and PX-478 (\( EC_{50} \) and 0.5 \( x \) \( EC_{50} \)) and the respective combinations were analysed. In HT-29 cells, the level of cleaved PARP was significantly higher in cells treated with the combination than in cells treated with the single compounds (\( p = 0.002 \)). In MCF-7 cells, the combination led to the highest levels of cleaved PARP at both doses, with significant differences compared to control and DCA-treated cells, but nonsignificant differences compared to PX-478-treated cells (\( p = 0.086 \) and \( p = 0.087 \)). However, via FACS analysis with Annexin-V-FITC staining, we identified significantly increased levels of programmed cell death for the combination of DCA and PX-478 in MCF-7 cells compared to PX-478 cells (Fig. 6). While 12% of PX-478-treated cells were Annexin-V-FITC-positive, the percentage increased to 20% after combination treatment (\( p = 0.004 \)). Thus, we concluded that apoptosis is a relevant factor for this synergism in HT-29 and MCF-7 cells.
For both cell lines, pRB1 levels were significantly lower in combination-treated cells than in single compound-treated cells and control cells (Fig. 5). Furthermore, we observed an interesting effect of the combination in MCF-7 cells: while PX-478 alone did not affect the level of pRB1 at 0.5 x EC\textsubscript{50} and EC\textsubscript{50}, DCA led to decreased levels of pRB1 (52% and 54%, respectively). For the combination, pRB1 levels were 33% of those in control cells at the lower concentration and 25% of those in control cells at the higher concentration (\(p = 0.027\) and 0.046 compared to the single compounds, respectively). These data suggest that DCA has a limited effect on pRB1 levels in MCF-7 cells, while the combination can affect pRB1 phosphorylation more strongly.

Furthermore, we used Western blotting to evaluate the impact of the compounds on Cyclin D1 levels. In HT-29 and MCF-7 cells, the level of Cyclin D1 exhibited the greatest reduction for the combination treatment (\(p = 0.009\) and \(p = 0.005\), respectively, compared to control treatment). However, the differences with respect to each single compound were nonsignificant (Fig. 5). Collectively, these data suggest that the combination of DCA and PX-478 synergistically reduces cell proliferation.

3.4 The effect of DCA was verified via real-time measurement of metabolism (Seahorse XFe96)

To verify the effects of DCA on anaerobic and aerobic glycolysis, studies with the Seahorse XFe96 Analyzer were performed (Fig. 7). We measured real-time changes in the OCR and the ECAR. Two hours after treatment with DCA, the protocols for the mitochondrial respiration assay and the glycolytic rate assay were performed. The results supported the hypothesis that DCA increases the influx of pyruvate into mitochondria, which led to a 42% increase in maximal respiration (\(p = 0.004\)). In addition, we observed a 73% reduction in the glycolytic capacity when DCA was added (\(p = 0.0001\)).

4. Discussion

In this study, we demonstrate that the combination of DCA and PX-478 acts synergistically in all 8 cancer cell lines tested (Fig. 1) but affects non-cancerous HEK-293 cells only slightly (Fig. 2). Furthermore, induction of ROS and apoptosis, as well as inhibition of cell cycle progression, play important roles in this synergism.

A primary question is whether the combination of DCA and PX-478 should be used only in cancer cells with a specific currently active metabolic state. The effect of DCA depends on the metabolic status of a cancer cell (48–52). Interestingly, DCA induces enhanced mitochondrial activity, ROS production and apoptosis in ovarian cancer cells, which generate energy mainly via oxidative phosphorylation (OXPHOS). However, in cancer cells where the Warburg effect is active, significantly higher concentrations were needed, possibly because of the higher expression of mitochondrial uncoupling protein 2 (49). Consistent with these findings, Chaundary et al. found that prostate cancer cells with mitochondrial dysfunction were less sensitive to DCA than control cells (50). In contrast, other studies have suggested that cells relying primarily on the Warburg effect are the most sensitive to DCA (51, 52). In conclusion, DCA dosing is affected by the metabolic state of cancer cells, and the precise kind of mitochondrial dysfunction seems to be relevant. This specificity is reflected by the different EC\textsubscript{50} concentrations of DCA in our 8 different cancer cell lines (Table 2). Additionally, the strength of the synergy between DCA and PX-478 varied across the cell lines (Fig. 1). The metabolic state of cancer cells seems to be relevant but may not be critical for the synergism described here.
The heterogeneity of the DCA mediated effects in different cancer cell lines can also be seen when our real-time metabolic assay results are compared with those of Tataranni et al. and Lucido et al. (14, 53). DCA strongly increased maximal respiration and decreased glycolytic capacity in MCF-7 cells (Fig. 7), while in pancreatic carcinoma and head and neck squamous cell cancer, both glycolytic capacity and maximal respiration were decreased. Consistent with our findings, Ma et al. found increased maximal respiration in non-small cell lung cancer cells treated with DCA (54).

Notably, as Tataranni et al. stated, the efficacy of DCA in inhibiting cancer cell growth is not always causally related to its effects on PDH activity (14). In the same work, these researchers found evidence that cancer stem cells in pancreatic cancer cell lines might also be affected by DCA treatment.

Via its effect on HIF-1α, PX-478 has already shown synergistic potential with different compounds. In combination with arsenic trioxide (ATO), PX-478 increases ROS and, likely, ROS-induced apoptosis (28). This mechanism, as our data suggest, is very likely to apply also to the combination of DCA + PX-478. Interestingly, both DCA and PX-478 mediate antitumoural effects through inhibition of PDKs, which can partially explain the synergism observed here. While DCA suppresses PDK-1, HIF-1α increases PDK-1 expression (32, 33). Thus, PX-478 reinforces the primary effect of DCA indirectly, thereby synergistically increasing ROS production when combined with DCA, as our data suggest (Figs. 3 and 4). These results and those of Lang et al. support the hypothesis that PX-478, as a HIF-1α inhibitor, may be beneficial for many therapeutic approaches. This explanation might apply to other known HIF-1α inhibitors as well.

Anoikis, a specific type of apoptosis induced by matrix detachment, activates both the intrinsic and extrinsic apoptotic pathways (55). Matrix detachment stimulates mitochondrial ROS generation, which acts as a signalling mechanism for anoikis. Cells treated with a certain amount of an antioxidant acquire resistance to anoikis (56). As Kamarajugadda et al. showed, forced activation of PDH and the consequent increase in mitochondrial respiration leads to enhanced ROS production triggered by matrix detachment, leading to anoikis. Additionally, these researchers showed that the expression of PDK leads to resistance to anoikis (35). Resistance to anoikis is an important factor supporting metastasis (35, 36). Considering our data regarding apoptosis and ROS production, this mechanism may also play an important role in the described synergism (Figs. 3, 4 and 5).

As radiation therapy and many chemotherapeutics also act through elevated levels of ROS, their combination with metabolism-targeting agents such as DCA and PX-478 is likely to be beneficial (36).

As DCA has attracted considerable attention in recent years, many examples of synergism have been detected. 5-fluorouracil, platinum-based chemotherapy, a SIRT2 inhibitor, Metformin, omeprazole + tamoxifen, sorafenib, erlotinib and gefitinib, have been shown to have synergistic effects in combination with DCA in vivo and in vitro (12, 54, 65, 57–64).

As Ward et al. proposed, the detected synergism between DCA and metformin can be best exploited with another pro-oxidant compound (12). As our study suggests, this compound could be PX-478, which would lead to an interesting triple combination. Another interesting example of synergy, found by Sun et al., is that DCA is more effective in a liposomal formulation (66). DCA and metal-organic framework Fe^{2+} synergistically increase ROS levels and are more selective in vivo in liposomal formulations. This synergism, which relies mainly on ROS production, could be combined with the synergism described here. Incorporating PX-478 into the liposomal formulation may be even more beneficial for ROS production and tumour cytotoxicity overall, as our data and
those of Lang et al. suggest (28) (Fig. 2). PX-478 acts as a radiosensitizer in vitro and in vivo (27, 67), can induce immunogenic cell death in vivo when combined with gemcitabine (68), and synergizes with ATO in vivo (28).

Clinical trials with DCA in cancer therapy, congenital lactic acidosis and pulmonary arterial hypertension have been performed during recent decades and are ongoing (25, 69, 70). Although DCA has not yet been implemented in clinical cancer treatment regimens, interest in DCA has not decreased. In one clinical trial in patients with previously treated metastatic breast or non-small cell lung cancer (ClinicalTrials.gov Identifier: NCT01029925), Garon et al. concluded that DCA should be used for patients with a longer life expectancy and should potentially be used in combination with cisplatin (62). Chu et al. drew similar conclusions from another clinical trial in patients with recurrent and/or metastatic solid tumours (ClinicalTrials.gov Identifier: NCT00566410), concluding that DCA is not feasible and will not show efficacy when used as a single agent but could act as an apoptosis sensitizer in combination with a more cytotoxic treatment and in patients with a less advanced disease stage (71). A phase 1 clinical trial of PX-478 (2010) in patients with advanced solid tumours showed that PX-478 was well tolerated at low doses, with consistent HIF1α inhibition, and prolonged the duration of stable disease (40). However, PX-478 seems to have been abandoned as an anticancer drug, as no further clinical trials with PX-478 have been registered. If PX-478 is used in combination with DCA, obstacles such as its dose-limiting toxicity could be eliminated. We believe that synergism is an important strategy for successfully including promising compounds such as DCA and/or PX-478 in cancer therapy. Our dose-response curves indicated that the concentrations of DCA and PX-478 could be reduced by an average of approximately 57%. Considering the concentrations of DCA achieved in clinical studies and our EC₅₀ values in the different cell lines tested, we concluded that a combination of DCA and PX-478 can help attain the concentrations needed for a therapeutic effect.

4.1 Limitations

In this study, we focused on the effect of the specific compounds and their combination rather than identifying whether a certain effect can be directly linked to a specific mode of action of a single compound. Those conclusions have to be drawn considering the existing data for the single compounds. While DCA has a rapid onset of action (see the results of the real-time metabolic assays), inhibition of the HIF-1α transcription factor via PX-478 takes much longer. Therefore, we performed measurements after 24 or 48 hours of incubation, which means that we did not consistently quantify the individual effects of DCA and PX-478.

4.2 Conclusion

Considering the evidence generated by various research groups about the effects of DCA and PX-478 and the promising synergism between the two compounds presented here, commencement of in vivo trials is recommended. If the combination is further supported, phase 1 clinical trials are recommended. In addition, the use of this combination with conventional chemotherapy may be an interesting translational approach.

5. List Of Abbreviations

CI: combination index
DCA: Dichloroacetate
6. Declarations

6.1 Ethics approval and consent to participate

Not applicable.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary files (additional file 1 and additional file 2).

6.4 Competing interests

The authors declare that they have no competing interests.

6.5 Funding

Jonas Parczyk received a stipend by the Berlin Institute of Health.

6.6 Author Contributions

Conceptualization of the project was done by JP, JR and AK. Experiments were performed by JP, JR, CP, MS, HW, NN, HK and AK. AK and JP were responsible for project administration and supervision. Writing and editing was done by JP, JR, AK, BE and KD.

All authors read and approved the manuscript.

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**Figures**

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**Figure 1**

Synergistic interactions between DCA and PX-478 in all 8 cell lines. Figure 1 shows the dose-response curves for DCA, PX-478 and their combination in eight different cell lines as well as the respective CIs (shown to the right of each dose-response curve). Cells were seeded in 96-well plates and treated at a confluence of approximately 50%. Forty-eight hours later, an SRB (protein mass) assay was performed. For HT-29 cells, an MTT assay was performed. If applicable, a CI was calculated with CompuSyn for each concentration. A CI of less than 0.9 indicates synergism, a CI between 0.9 and 1.1 indicates a nearly additive effect, and a CI of greater than 1.1
indicates antagonism. Concentrations were established as fractions of the previously approximated EC50 values (EC50a) at an EC50a:EC50a ratio. Concentrations for which the effect of combination was significantly different from that of both single compounds and the control (p ≤ 0.05) are marked with an asterisk (*). Synergistic interactions were confirmed for all cancer cell lines, as indicated by the CI values and predominant left shifts of the curves. Without exception, the effects of the drug combination surpassed the effects of each single compound.

**Figure 2**

The combination of DCA and PX-478 shows significantly lower effects on the non-cancerous cell line HEK-293 than on the six PX-478-sensitive cancer cell lines (A549, HeLa, HEPG2, MCF-7, U251, and H441). A: HEK-293 cells were seeded in 96-well plates and treated with 4 different concentrations of DCA, PX-478, or the combination at a confluence of approximately 50%. Forty-eight hours later, an SRB assay was performed. At the highest tested concentration (15 mM DCA and 15 µM PX-478), the combination had no significant effect (97% protein mass) compared to control (p = 0.3). Interestingly, DCA alone exerted a significant effect (81% protein mass, p = 0.026). Protein masses significantly lower than in control cells are marked with an asterisk. B: The effect of the combination of 15 mM DCA and 15 µM PX-478 on HEK-293 cells was compared to the effect of similar or lower concentrations of the combination on the tested cancer cell lines. Data points used to generate the dose-response curves in figure 1 were used for comparison. The bars are marked with an asterisk when the effect on a cancer cell line was significantly stronger than that on HEK-293 cells. All six PX-478-sensitive cancer cell lines were significantly more sensitive than HEK-293 cells to the combination of DCA and PX-478. For the exact concentrations used, please see additional file 1. The effect of comparable doses of the combination on HT-29 cells did not differ from that on HEK-293 cells. MDA-MB-231 cells were not compared, since due to the described resistance, no doses of PX-478 close to 15 µM were used. Figure 2B is not applicable for comparing effects between the different cancer cell lines, since different concentrations of the compounds were used. For comparisons of synergism, please see figure 1 and table 2.
Figure 3

The combination of DCA and PX-478 leads to increased ROS activity in HT-29 cells. Figure 3 shows HPLC analysis results and cell counts for HT-29 cells. A: HPLC results for the proportion of arachidonic acid to its oxygenated derivatives 10-/15- HETE for drug treatment compared to control treatment is presented. Cells were treated with either the EC50 dose of DCA, the EC50 dose of PX-478 or the combination. Cells treated with PX-478 alone and with the combination of DCA and PX-478 showed a significant increase in the oxidation ratio compared to that in control cells, although the difference between the combination and PX-478 was noticeable but not significant. B: The cell count as a percentage of the control cell count is presented. Treatment with the single compounds DCA and PX-478 led to significant reductions of 65 and 61%, respectively. Only 15% of the
number of control cells remained after the combination treatment. Significant differences from the control are marked with an asterisk (*), while significant differences from both the control and each single compound are marked with two asterisks (**). (C) The profound effects of DCA, PX-478 and DCA + PX-478 on cell confluence are shown. All cells were imaged at 40× magnification with a Nikon D90.

Figure 4

The combination of DCA and PX-478 leads to increased ROS activity. Figure 3 shows the ROS activity as assessed by flow cytometric analysis with H2DCFDA in HeLa, MCF-7 and HT-29 cells. Cells were incubated to a confluence of 50% and were then treated with either DCA, PX-478 or the combination for 48 hours. The following concentrations of the compounds were used: HeLa—DCA and PX-478, EC50; MCF-7 and HT-29—DCA, EC50 and PX-478, 0.5 x EC50. Panel A shows representative histograms for analysis of DCF-FITC in HeLa cells. Panel B shows the results in HeLa, MCF-7 and HT-29 cells as bar graphs. Three independent experiments were performed. Significant results compared to the control are marked with an asterisk (*). The combination of DCA and PX-478 led to the highest ROS activity in all cell lines, which was significantly increased compared to that in the corresponding control, DCA-treated and PX-478-treated cells (marked with two asterisks [**]).
The combination of DCA and PX-478 leads to increased levels of cleaved PARP and reduced levels of Cyclin D1 and pRB1 in HT-29 and MCF-7 cells. Cells were incubated to a confluence of approximately 80% and treated with DCA and PX-478 at the following concentrations: HT-29—DCA, EC50 and PX-478, 0.5 x EC50; MCF-7—either DCA and PX-478, 0.5 x EC50 or EC50. Twenty-four hours later, the cells were harvested, and Western blot analyses were performed. A: Three independent Western blots are shown for each antibody except for β-actin (only one representative blot is shown here). The here presented blots are cropped, please see additional file 2 for full length blots. B: Results are presented as fold change relative to control. Significant differences are marked with an asterisk (*). The level of cleaved PARP was significantly increased in HT-29 cells treated with the combination (p = 0.00002) compared to HT-29 cells treated with the single compounds. For MCF-7 cells, a clear trend was visible for both concentrations (p = 0.086 or p = 0.087, respectively). Significant differences compared to control
are marked with an asterisk (*); significant differences between the combination of DCA and PX-478 and each single compound are marked with two asterisks (**). The combination of DCA and PX-478 significantly reduced cyclin D1 and pRB1 levels in both cell lines and at both concentrations in MCF-7 cells.

Figure 6

DCA + PX-478 induced significantly higher levels of apoptosis than DCA or PX-478 alone in MCF-7 and HeLa cells. Figure 6 shows the results of flow cytometric analysis with Annexin-V-FITC and propidium iodide in MCF-7 and HeLa cells. At 60% confluence, MCF-7 cells were treated with either the EC50 dose of DCA or the EC50 dose of PX-478, while HeLa cells were treated with the EC50 dose of DCA and 0.5 times the EC50 dose of PX-478. Three independent experiments were performed. Panel B shows bar graphs indicating the percentages of Annexin-V-FITC-positive MCF-7 and HeLa cells. Significant differences compared to the control are marked with an asterisk (*). Representative dot plots are shown for MCF-7 cells in panel A. The combination of DCA and PX-478 led to the greatest percentage of Annexin-V-FITC-positive cells, and the percentage was significantly different from that of control, DCA-treated and PX-478-treated cells for both cell lines (**) (p = 0.004 and 0.042 for MCF-7 cells and HeLa cells, respectively).
Figure 7

Mitochondrial respiration assay and glycolytic stress test with DCA. Figure 7 shows the results of the mitochondrial respiration assay (A) and glycolytic stress test (B) of DCA-treated MCF-7 cells performed with a Seahorse XFe96 analyser. Three independent experiments were performed with at least four technical replicates. After measurement of the baseline OCR and ECAR, the EC50 dose of DCA was injected. After six measurement cycles, oligomycin (inhibition of ATP-synthase), FCCP (uncoupling agent) and rotenone & antimycin A (inhibition of respiratory chain) were added for the mitochondrial respiration assay (A), and glucose, Oligomycin and 2-DG (inhibition of glycolysis) were added for the glycolytic rate assay (B). Three measurement cycles were performed after each chemical was added. A: As shown, DCA increased the maximal OCR and thereby the maximal respiration of MCF-7 cells. B: Further, DCA reduced the maximal ECAR and thereby the glycolytic capacity. See the Methods section for the exact calculation procedures and definitions of glycolytic capacity, maximal respiration, non-mitochondrial OCR and non-glycolytic acidification rate. In MCF-7 cells, DCA increased the maximal respiration by 42% and decreased the glycolytic capacity by 73% ($p = 0.004$ and 0.0001, respectively).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• Additionalfile2.pptx
• Additionalfile1.xlsx