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Salinomycin and Dichloroacetate Synergistically Inhibit Cancer Cells *in vitro* and *in vivo*

SUMMARY OF DOCTORAL DISSERTATION

Natural Sciences,
Biology (N 010)

VILNIUS 2020

This dissertation was written between 2014 and 2019 at the Laboratory of Human Genome Research, Institute of Biosciences, and the Department of Biological Models, Institute of Biochemistry, Life Sciences Center, Vilnius University; the National Cancer Institute; and the Institute of Cardiology, Lithuanian University of Health Sciences. The research was supported by the National Cancer Institute.

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Sinergistinis citotoksinis salinomicino ir dichloroacetato poveikis vėžinėms ląstelėms *in vitro* ir *in vivo*

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ABBREVIATIONS

BCECF-AM	2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein
AO	Acridine orange
CBNX	Carbenoxolone
CI	Combination index
CSC	Cancer stem cells
DCA	Dichloroacetate
EMT	Epithelial–mesenchymal transition
Fa	Fraction affected
FC	Fold change
H&E	Eosin and hematoxylin
LLC1	Lewis lung carcinoma
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MRP	Multidrug resistance protein
PDK	Pyruvate dehydrogenase kinase
P-gp	P-glycoprotein
pH_i	Intracellular pH
PI	Propidium iodide
ROI	Region of interest
RT-qPCR	Real-time quantitative polymerase chain reaction
SAL	Salinomycin
SAL + DCA	Combination of SAL and dichloroacetate
SEM	Standard error of the mean

INTRODUCTION

Combination therapy is the cornerstone of cancer treatment. The simultaneous application of cytotoxic drugs potentiates their efficacy compared with monotherapy because it targets the key pathways in a synergistic or an additive manner. Such therapy is likely to diminish drug resistance, while simultaneously providing cytotoxic benefits such as inhibition of tumor growth, decrease of cancer stem cell (CSC) population, reduction of metastatic potential, and induction of apoptosis (1).

Salinomycin (SAL) is a monocarboxylic polyether ionophore that has been discovered in high throughput screening as a potential anti-cancer drug selectively targeting breast cancer stem cells (2). This finding resulted in numerous experiments performed on other types of cancer cells, which confirmed an initial hypothesis (3-9). Side effects of SAL reported in clinical studies include tachycardia and mild tremor; however, none of the severe side effects such as alopecia, nausea, myelodepression, or gastrointestinal distress characteristic of traditional chemotherapeutic drugs has been documented (10).

Dichloroacetate (DCA) is a small synthetic molecule that is known as a pyruvate dehydrogenase kinase inhibitor. Its anticancer properties involve reversing the Warburg effect by switching ATP production back to oxidative phosphorylation (11-15); reduction of mitochondrial membrane potential, and activation of mitochondrial potassium channels, which subsequently contribute to the induction of apoptosis in various cancers through the release of proapoptotic molecules such as cytochrome c and apoptosis-inducing factor (16, 17). Several features of DCA make it an attractive candidate for cancer therapy: it has a minimal effect on healthy cells (12), good bioavailability (17), and is a low-cost drug. Additionally, DCA has been used to treat patients with congenital lactic acidosis in clinic settings for more than 40 years, hence its side effects are already well studied (18, 19). In the last decade, a number of articles have been published in favor of DCA, and it was proposed as an effective drug to treat neuroblastoma, breast, colon, lung, prostate, and other cancers (14, 20-22). A successful

1 phase clinical trial to treat patients with recurrent malignant brain tumors was completed in 2014 and it concluded DCA as safe, tolerable, and feasible for chronic administration (22). Another 1 phase clinical trial performed with DCA on various advanced solid tumors supports these data (23). Side effects caused by DCA can be categorized in two groups: neurological such as peripheral neuropathy, sedation, mood fluctuations, or disorientation and gastrointestinal such as heartburn, nausea, vomiting, or indigestion (24).

A great number of scientific reports have shown that the multidrug resistance phenotype in tumors correlates with the increased expression of particular ABC transporters, so-called multidrug resistance proteins (MRPs). P-glycoprotein (P-gp) was the first identified ABC transporter, and it is thought to be responsible for multidrug resistance in the majority types of cancer. Some papers have suggested SAL as a possible P-gp inhibitor (8), while DCA so far has never been identified to possess such characteristics. On the other hand, a recent study performed *in vivo* on mice with glioblastoma tumors has proposed that DCA could be used for prompt intracellular acidification that, in turn, may enhance the effectiveness of chemotherapeutic treatment (25, 26).

Metastatic spread of cancer cells is one of the greatest challenges in cancer treatment. Diagnosis and treatment of metastasis are complicated and are associated with poor disease outcome. It has been previously reported that DCA exerts a significantly stronger inhibition toward metastasis formation when it is used in combination with other compounds, such as bicarbonate or metformin (27, 28), whereas favorable antimetastatic results of SAL have been demonstrated only with high doses of SAL (e.g., 8 mg/kg) in mouse models (29, 30).

A biological process, known as epithelial–mesenchymal transition (EMT), is a trans-differentiation of epithelial cells into invasive mesenchymal cells and is a key factor for metastasis formation and cancer progression (31). The EMT phenomenon is a good target for cancer treatment, especially for patients who are at risk of developing metastasis or already have metastatic lesions. SAL was previously reported to inhibit EMT (32), whereas DCA, to our knowledge, has never been investigated for such properties.

In the present study, we investigated the effects of DCA, SAL, and the combination of both agents on the colorectal cancer cell lines DLD-1 and HCT116 as well as Lewis lung carcinoma (LLC1) cells *in vitro* in 2D and 3D cell culture models and *in vivo* in an allograft model subcutaneously injecting LLC1 cells in C57BL/6 mice. *In vitro* studies were designed to investigate the potential mechanism of SAL + DCA action, and *in vivo* studies were aimed at analyzing the effects of this therapy on tumor growth, metastatic site formation, and properties of EMT and CSC.

The aim of the study

To conduct the preclinical trials of SAL + DCA on human colorectal cancer cell lines DLD-1 and HCT116 in vitro and LLC1 *in vitro* as well as *in vivo* in an allograft model subcutaneously injecting LLC1 cells in C57BL/6 mice and to investigate the possible mechanism of its synergistic cytotoxic action.

Objectives of the study

1. To investigate the cytotoxic effects of SAL, DCA and their combination on the viability of cancer cell lines DLD-1, HCT116, and LLC1 in 2D and 3D cell cultures;
2. To reveal the possible mechanism of synergistic cytotoxic action of SAL + DCA combination;
3. To investigate SAL + DCA therapeutic potential in the treatment of metastasized lung cancer in C57BL/6 mice with LLC1-induced tumors;
4. To evaluate the antimetastatic potential of SAL + DCA combination in C57BL/6 mice with LLC1-induced tumors;
5. To examine SAL + DCA effects on the expression of EMT, CSC and proliferation markers in C57BL/6 mice

Relevance of the research and its scientific novelty

Drug combination represents one of the most accredited strategies of cancer therapy that is able to improve drug efficacy, diminish drug

dosage, reduce toxicity and possibly overcome drug resistance. In the present study, we not only aimed to achieve all of these benefits of combinational therapy, but also we targeted tumor microenvironment by choosing two drugs that eradicate two different cell subpopulations within a tumor.

A small subpopulation of tumor cells known as CSCs is thought to be ultimately responsible for tumor progression and recurrence, treatment resistance, and metastatic site formation. Every year, a number of publications report important findings on CSCs; however, this knowledge is rarely adopted in clinical practice, and therefore, more profound preclinical research is needed in order to create safe and effective therapies that target CSCs. SAL is one of the most promising CSC inhibitors today, which has been proven to effectively eradicate CSCs in a variety of cancer types, such as pancreatic, breast, prostate, ovarian cancer and others (29, 30). A number of studies have also attempted to combine SAL with other chemotherapeutic drugs; however, to date, no such combination has been tested in clinical trials.

The second component of combination therapy is DCA, a pyruvate dehydrogenase kinase (PDK) inhibitor. DCA possesses several characteristics that make it a desirable candidate for cancer therapy: it does not affect healthy cells (17), it has an excellent bioavailability (12), and it is cost-effective. In the present study, for the first time, SAL + DCA combination was tested on cancer cells *in vitro* and *in vivo*.

Metastatic spread of cancer cells is one of the greatest challenges in cancer treatment. Diagnosis and treatment of metastasis are complicated and are associated with poor disease outcome. In the present study, we have also tested antimestastatic properties of SAL + DCA therapy in C57BL/6 mice with LLC1-induced tumors for the first time.

A biological process, known as EMT, is a trans-differentiation of epithelial cells into invasive mesenchymal cells and is a key factor for metastasis formation and cancer progression (31). EMT is mediated by several factors that can be tracked via expression of specific markers such as epithelial cadherin and vimentin. The EMT phenomenon is a good target for cancer treatment, especially for patients who

are at risk of developing metastasis or for those who already have metastatic lesions. SAL was previously reported to inhibit EMT (32), whereas DCA, to our knowledge, has never been investigated for such properties.

In this work, we investigated the effects of DCA, SAL, and the combination of both agents on colorectal cancer cells DLD-1 and HCT116 and on LLC1 cells *in vitro* in 2D and 3D cell culture models and provided a possible explanation for its synergistic mechanism of action. We also tested this therapy *in vivo* in an allograft model subcutaneously injecting LLC1 cells in C57BL/6 mice. We analyzed the effects of this therapy on tumor growth, metastatic site formation, and properties of EMT and CSCs.

1. MATERIALS AND METHODS

1.1 *In vitro* experiments

Human colorectal carcinoma DLD-1 and HCT116 cell lines and murine metastatic LLC1 cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and were maintained as described in our publication (33). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed in order to determine cell proliferation as described earlier. Briefly, cells were seeded in a 96-well plate and after 24 h were treated with SAL (0.01, 0.05, 0.25, 0.5, 1 μM), DCA (1, 5, 15, 30, 45 mM), or both (concentration and CI values are available in Supplementary Table 1). After treatment, cells were cultured for 48 h; then the MTT reagent was added to each well, and after 1.5 h, the absorbance of each well was measured by a plate reader at a test wavelength of 490 nm. A synergistic effect was evaluated based on the Chou-Talalay method (34).

Multicellular tumor spheroids were formed as described previously (35). After 48 h, spheroids were treated by applying monotherapy and combination therapy. Therapeutic effect was evaluated by measuring spheroid dimensions.

For apoptosis detection, we used an Annexin V–FITC apoptosis detection kit (Sigma Aldrich, USA), following the manufacturer's instructions. Cells were analyzed for apoptosis by a BD LSR II flow cytometer (BD Biosciences, USA) and an FACS Diva software package (BD Biosciences, USA).

For pH_i measurements, cells grown onto glass coverslips were loaded with a cell-permeant form of 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF-AM) (ThermoFisher Scientific, USA) by incubating cells in the Krebs–Ringer solution containing 2 μM of BCECF-AM for 5 min. Then cells were washed with an RPMI-1640 medium and transferred for fluorescence recording. The dye was alternately excited with 440-nm and 495-nm light, and the emitted light was filtered at 540 nm and recorded. The emitted light from 495-nm excitation is pH sensitive, whereas that from 440-nm excitation is relatively pH insensitive. Thus, the ratio of emitted light at two excitation wavelengths (background subtracted) is a function of pH. Ratios were converted to pH_i values based on a calibration curve.

The calcein assay was used in order to examine the effects of DCA and SAL on MRPs. Cells were loaded with calcein-AM (Molecular Probes, USA) by incubating the cells in the RPMI-1640 solution containing 4 μM of calcein-AM for 30 min at 37°C in a CO_2 incubator. The dye was excited with 490-nm light, and the emitted light was filtered at 540 nm and recorded by time lapse. Cells were periodically exposed (every 10 min) to a low-intensity light for 100 ms. Calcein fluorescence was measured in the regions of interest (ROIs) placed on every cell of the cell group.

RNA was isolated from cells using Quick RNA MiniPrep (Zymo Research, USA) following the instructions provided by the manufacturer after 4 days of seeding. Real-time qPCR was performed with Kapa SYBR Fast qPCR Master Mix (2X) (Kapa Biosystems, USA) as described earlier.

All data were expressed as mean ± standard error mean (SEM) from at least three independent experiments. Statistical analysis was performed using the Sigma Plot 10.0 software. Comparisons of two

values were performed using the Student t test. Synergism of DCA and SAL was analyzed with the Fa-CI plot, and CI calculations were done according to the Chou-Talalay method using the CompuSyn 2.0 software (ComboSyn, Inc., Paramus, NJ, USA). CI values below 1 suggest synergy, whereas CI values above 1 indicate antagonism. For the comparison of gene expression profile between 2D and 3D cell cultures, a fold change (FC) value was calculated. Only gene expression with $p < 0.05$ and an absolute FC of ≥ 1.5 were considered as significant.

1.2 *In vivo* experiments

All experiments were conducted in strict accordance with the directive of the European Parliament and of the Council on the protection of animals used for scientific purposes (36) alongside the approval of the State Food and Veterinary Service, Lithuania (No. G2-85).

C57BL/6 mice (8–12-week-old, male, weight of 18–21 g) were purchased from the Department of Biological Models, Institute of Biochemistry, Vilnius University (Vilnius, Lithuania). The mice were inhabited in plastic cages (≤ 4 mice per cage) with *ad libitum* access to water and food. A total of 48 mice were used in the experiment. To generate tumors, LLC1 cells were subcutaneously injected in the flank of C57BL/6 mice (3×10^5 cells per mouse). Once the tumors were palpable, at day 6 all mice were randomly divided into 4 groups, and drug treatment was initiated. The following doses were used in the experiment: 1) control solution (0.5% DMSO); 2) 3 mg/kg SAL; 3) 200 mg/kg DCA; and 4) combination of 3 mg/kg SAL and 200 mg/kg DCA. All mice were labeled, and tumors were measured every 5 days with a caliper. On day 10, 4 mice from each group were randomly selected and were sacrificed for histological analysis. The rest 8 mice in each group continued the study in order to obtain a survival rate in every group. For ethical reasons, the end of the experiment for each mouse was considered when tumors reached 1500 mm^3 after which a mouse was euthanized. Mice were euthanized by cervical dislocation.

Tumors and organ tissues from the control, DCA-, SAL-, and SAL + DCA-treated groups were analyzed by immunohistochemistry. Slides for marker characterization were incubated overnight at 4ºC with the primary antibodies against CD133 (Abcam), CD44 (Ventana), vimentin, E-cadherin (Ventana), and Ki-67 (Abcam).

2. RESULTS

2.1 *In vitro* results

2.1.1 DCA in combination with SAL synergistically inhibits the viability of HCT116 and DLD-1 cells in 2D culture.

Our first objective was to examine the effects of DCA and SAL in HCT116 and DLD-1 2D colorectal cancer cell cultures by applying the compounds in a variety of doses in monotherapy as well as in combination. The experimental design was made in accordance with the Chou-Talalay method for synergy quantification of a drug combination (37). **Fig. 1** shows that a number of SAL and DCA doses in combination acted synergistically in both DLD-1 and HCT116 cell lines.

These results were also confirmed by flow cytometry analysis: the combination of SAL (0.25 μM) and DCA (15 mM) produced a synergistic effect by dramatically increasing the early and late apoptotic cell populations after 48-h exposure in both HCT116 and DLD-1 cell lines compared with control and compared with the single agents (**Fig. 2**)

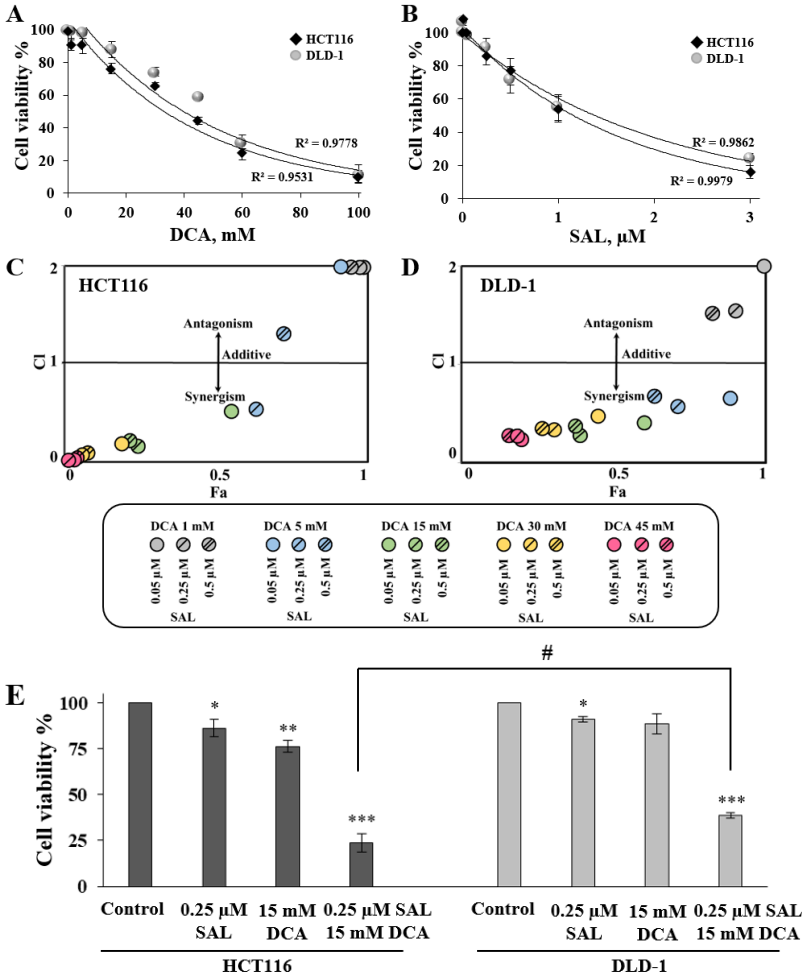

Fig. 1. Cytotoxic effects of DCA and SAL in monotherapy and in combination on colorectal cancer cells in the 2D culture determined by the MTT assay. (A, B) A dose-response curve of cytotoxic effect of DCA **(A)** and SAL **(B)** alone on DLD-1 and HCT116 cell viability after 48-h treatment. **(C, D)** Fa-CI plot analysis of combination treatment of DCA and SAL (SAL) on DLD-1 **(C)** and HCT116 **(D)** cell viability. For visual purpose, all CI values above 2 were presented as equal to 2. **(E)** Effects of 0.25-µM SAL and 15-mM DCA and their combination on HCT116 and DLD-1 cell viability after 48-h treatment, determined by the MTT assay. Data are expressed as mean ± SEM calculated from 3 independent experiments measuring cell viability in 6 wells for each condition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (compared to control); # $p < 0.05$ (compared between cell lines).

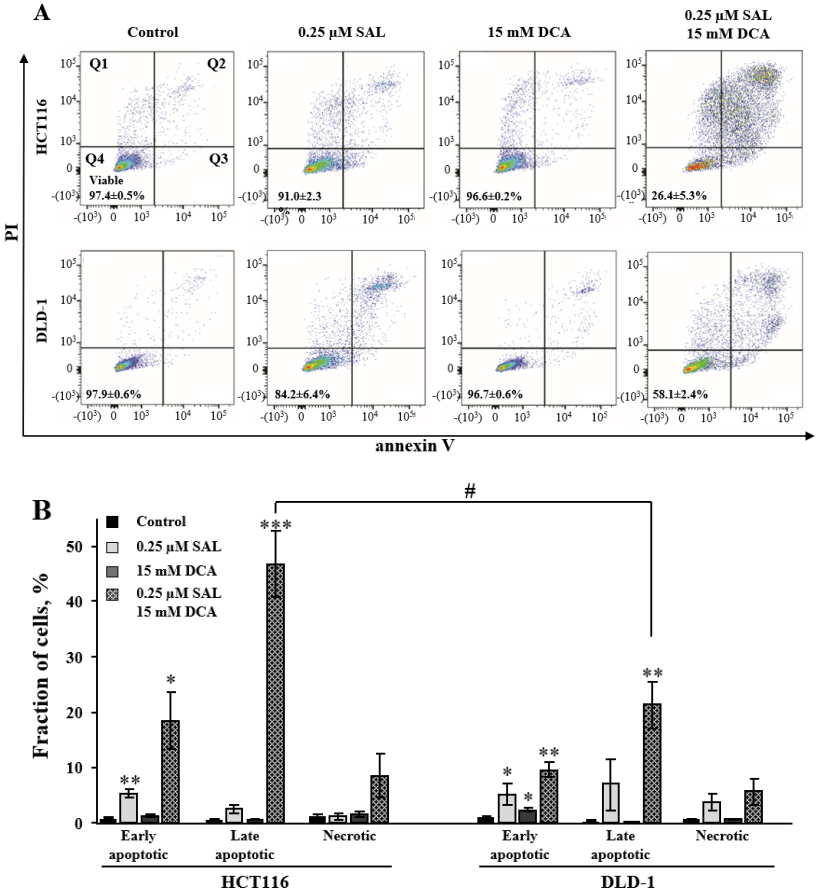

Fig. 2. Flow cytometry analysis of annexin V- and PI-stained HCT116 and DLD-1 cells, undergoing treatment with SAL, DCA and their combination. **(A)** Dot plots represent responses to therapy with indicated compound(s) for HCT116 cells and DLD-1 cells. Q1 (necrosis) shows cells negative for annexin V labeling, but positive for PI staining. Q2 (late apoptosis) shows cells positive for annexin V labeling and positive for PI staining. Q3 (early apoptosis) shows cells positive for annexin V labeling, but negative for PI staining. Q4 (viable cells) shows cells negative for both annexin V labeling and PI staining. **(B)** Effects of 0.25-µM SAL and 15-mM DCA and their combination on HCT116 and DLD-1 cell viability after 48-h treatment, determined by flow cytometry. Data are expressed as mean ± SEM, averaged from 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (compared to control); # $p < 0.05$ (compared between cell lines).

2.1.2 DCA and SAL cause a strong cytotoxic effect on colorectal cancer cell lines in the 3D cell culture.

In order to determine whether combination therapy maintains its cytotoxic effect in the 3D cell culture, we applied the multicellular spheroid technique. Spheroids were treated in monotherapy with 0.01, 0.25, 0.5, 1, and 5 μM of SAL and 1, 10, 15, 30, 45, and 60 mM of DCA or combination of these doses. Treatment efficacy was measured after 48, 96, and 144 h. As shown in **Fig. 3**, spheroids

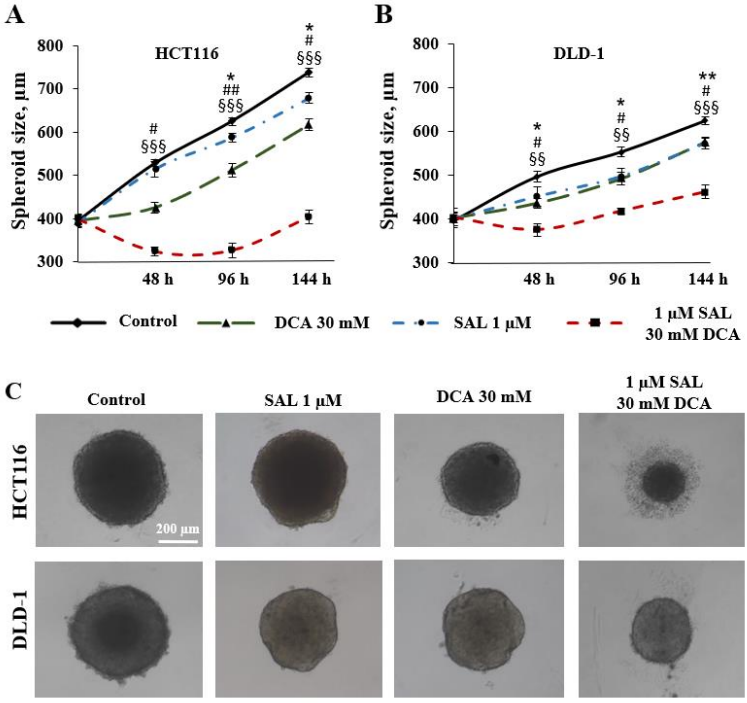

Fig. 3. Cytotoxic effects of DCA, SAL, and their combination on the 3D colorectal cancer cell culture. The effect of 1-μM SAL, 30-mM DCA and their combination on HCT116 **(A)** and DLD-1 **(B)** multicellular spheroid size after 48, 96 and 144 h of treatment. Data are expressed as mean ± SEM calculated from 3 independent experiments measuring the size of 6 spheroids for each condition. **(C)** Typical images of HCT116 and DLD-1 multicellular spheroids after 48 h of control or treatment with 1-μM SAL, 30-mM DCA, and their combination. * $p < 0.05$, ** $p < 0.01$ (SAL compared to control); # $p < 0.05$, ## $p < 0.01$ (DCA compared to control); §§ $p < 0.01$, §§§ $p < 0.001$ (SAL and DCA combination compared to control).

of both cell lines were less sensitive to drug doses that were effective in the 2D cell culture; however, a number of concentrations still acted synergistically. Interestingly, DLD-1 cell spheroids were sensitive to SAL monotherapy at all time points, whereas in HCT116 cell spheroids, a significant effect was obtained only after 96 h.

2.1.3 Expression of stem cell markers is up-regulated in the multicellular spheroid culture of DLD-1 cells

Our next objective was to investigate the rationale behind the altered chemotherapeutic sensitivity in the DLD-1 cell line, when grown in the 3D structure. We observed that in the 3D culture, DLD-1 cells were more sensitive to the monotherapy of SAL compared to HCT116 cells. Since SAL selectively targets CSCs, we tested whether switching from 2D to 3D environment could cause specific gene activation. We performed analysis of cancer cell stemness markers (*ALDH1A1*, *CEACAM5*, *ALCAM*, *LGR5*, *DPP4*, *CD133*, *CD24*, *CD29*, and *CD44*), EMT markers (*SNAIL1*, *SNAIL2*, *CDH1*, and *CDH2*), and multipotency markers (*NANOG* and *POU5F1*) in HCT116 and DLD-1 2D and 3D cell cultures in the absence of treatment. As presented in **Table 1,** no change ($FC < 1.5$ or $p > 0.05$) was observed in the regulation of multipotency genes in both cell lines, and only one marker (*SNAIL2*) was up-regulated among EMT markers in the DLD-1 cell line. In contrast, the majority of cell stemness markers (*CEACAM5*, *ALDH1A1*, *CD24*, *CD44*, and *CD133*) were significantly overexpressed in the DLD-1 cell line in a 3D environment, whereas only one stem cell marker (*DPP4*) was increased in the HCT116 cell line. These results suggest that higher potency of SAL monotherapy in the DLD-1 3D cell culture was achieved due to up-regulation of stemness genes.

Table 1. Gene expression analysis.

Gene Expression Analysis				
Genes	**Cell lines and cultures**			
	HCT116 3D/2D		**DLD-1 3D/2D**	
	FC	**P value**	**FC**	**P value**
EMT markers				
SNAIL1	–1.1	0.1821	–1.1	0.3618
SNAIL2	–1.4	0.0513	1.1	0.0429
CDH1	1.0	0.7252	1.0	0.5211
CDH2	1.1	0.3529	1.3	0.0821
Multipotency markers				
NANOG	1.0	0.9418	1.3	0.0200
POU5F1	1.2	0.1071	–1.1	0.4263
Cell stemness markers				
ALCAM	–1.2	0.0236	1.2	0.1517
CEACAM5	–1.1	0.9179	**24.3**	**0.0000**
ALDH1A1	0.0	> 0.99	**8.5**	**0.0193**
LGR5	2.2	0.2784	**–1.9**	**0.0001**
DPP4	**1.7**	**0.0004**	**–1.5**	**0.0001**
CD24	1.0	0.6359	**2.0**	**0.0001**
CD29	–1.3	0.0055	–1.0	0.4036
CD44	–1.2	0.0661	**3.4**	**0.0001**
CD133	1.1	0.1786	**1.7**	**0.0001**

Relative expression of EMT-, multipotency- and cell stemness-related genes in DLD-1 and HCT116 2D and 3D cell cultures estimated by means of RT-qPCR (all experiments were repeated in independent biological triplicates).

2.1.4 Putative mechanisms of action

2.1.4.1 The role of multidrug resistance proteins

To examine a possibility that a synergistic effect of SAL and DCA was achieved due to modulation of MRP activity, we used the calcein assay. As can be seen in **Fig. 4B–E,** none of the used concentrations of SAL had any effect on calcein removal from HCT116 and DLD-1 cells. In contrast, quite surprisingly for us, calcein fluorescence decay was significantly slowed-down by DCA. To our knowledge, this phenomenon has not been reported before. Thus, one of the possible mechanisms by which the synergistic effect of SAL is achieved could be attributed to DCA-inhibited removal of SAL from cancer cells.

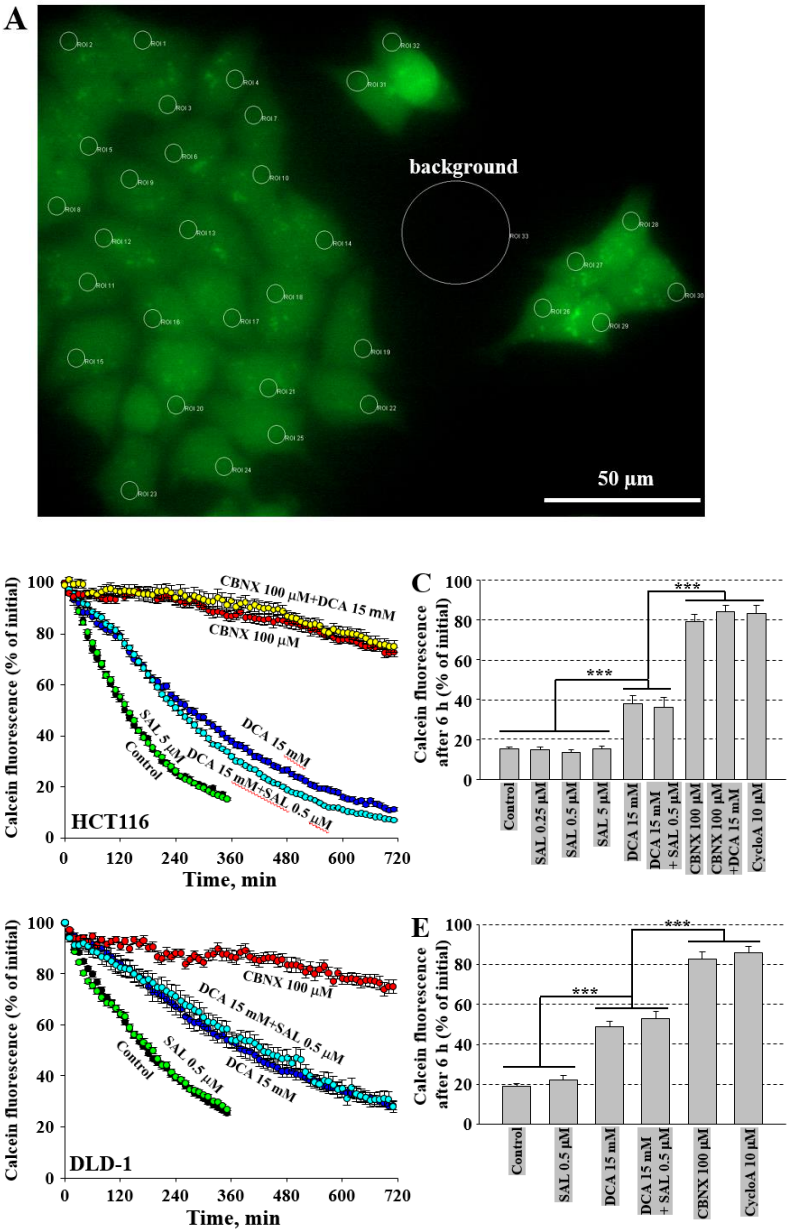

Fig. 4. DCA inhibits MRP activity in HCT116 and DLD-1 cells. (A) View of the calcein-AM-loaded HCT116 cell group with regions of interest

(ROIs) on every cell and ROI for background subtraction. **(B)** Typical calcein fluorescence decay in HCT116 cells under control (n = 38) and in the presence of SAL (5 μM; n = 32), DCA (15 mM; n = 27), DCA together with SAL (n = 32), CBNX (100 μM; n = 33), CBNX together with DCA (n = 35). **(C)** Calcein fluorescence in HCT116 cells after 6 h of recording relatively to initial fluorescence intensity under control and in the presence of indicated compounds. Data are expressed as mean ± SEM, averaged from 3 independent experiments. **(D)** Typical calcein fluorescence decay in DLD-1 cells under control (n = 37) and in the presence of SAL (0.5 μM; n = 16), DCA (15 mM; n = 17), DCA together with SAL (n = 21), CBNX (100 μM; n = 17). **(E)** Calcein fluorescence in DLD-1 cells after 6 h of recording relatively to initial fluorescence intensity under control and in the presence of indicated compounds. Data are expressed as mean ± SEM, averaged from 3 independent experiments. *** $p < 0.001$.

2.1.4.2 Intracellular pH

It is known that alkaline pH_i creates high proton gradients and oscillations that may contribute to cancer cell viability, proliferation, and invasion as well as the response of tumors to various treatments such as chemotherapy, radiotherapy, and hyperthermia (37-43). Therefore, in the next step, we tested SAL and DCA capability to modify pH_i. As shown in **Fig. 5**, SAL had no effect on pH_i. In contrast, DCA (30 mM) alone or in the presence of SAL decreased pH_i by ~ 0.2 units in HCT116 cells. Identical effects were documented in DLD-1 cells.

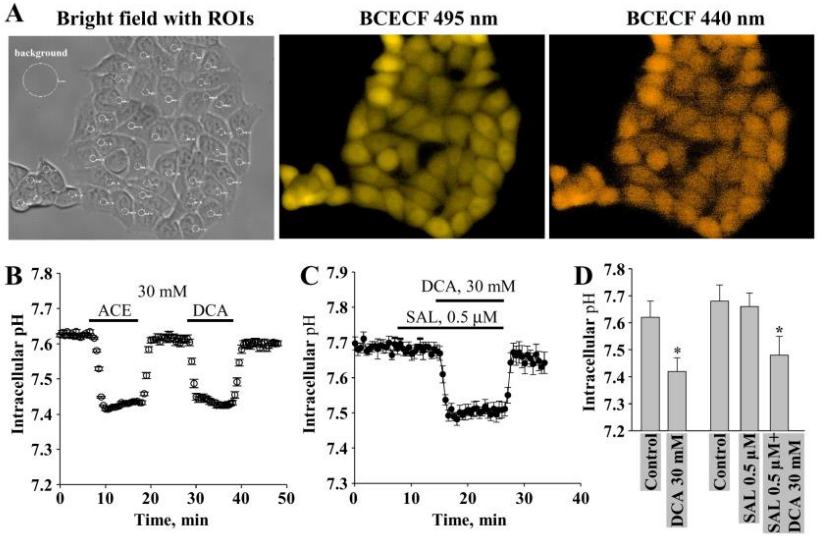

Fig. 5. The effect of SAL and DCA on pH_i in HCT116 cells. (A) Bright field and BCECF fluorescence images at 495-nm and 440-nm excitation waves. **(B)** The effect of acetate (ACE, 30 mM) and DCA (30 mM) alone on pH_i (n = 41). **(C)** The effect of SAL (0.5 μM) and DCA (30 mM) on pH_i (n = 24). **(D)** Summary of the effects of SAL and DCA on pH_i. Horizontal bars in B and C indicate exposure times to indicated compounds. Data in D are expressed as mean ± SEM, averaged from 3 independent experiments. * $p < 0.05$.

2.2 In vivo results

2.2.1 DCA in combination with SAL significantly suppresses tumor growth in LLC1- bearing mice

To explore the antitumor effects of DCA, SAL, and their combination *in vivo*, we employed a mouse allograft model using the LLC1 cell line. C57BL/6 mice were randomly divided into 4 groups and received one of the following treatments: control solution, 3 mg/kg SAL, 200 mg/kg DCA, and combination of 3 mg/kg SAL and 200 mg/kg DCA. Treatment with SAL did not affect tumor growth as compared with control. In contrast, mice treated with DCA exhibited reduced tumor growth from day 10, and at day 14, the tumor volume was 1.7-fold smaller compared with the tumor volume in the control animal group. The consistent and strongest tumor growth suppression was observed in

the combination treatment group, e.g., at day 14 of the study, the tumor volume was reduced 3.6-fold in the SAL + DCA group compared to the control group (**Fig. 6**).

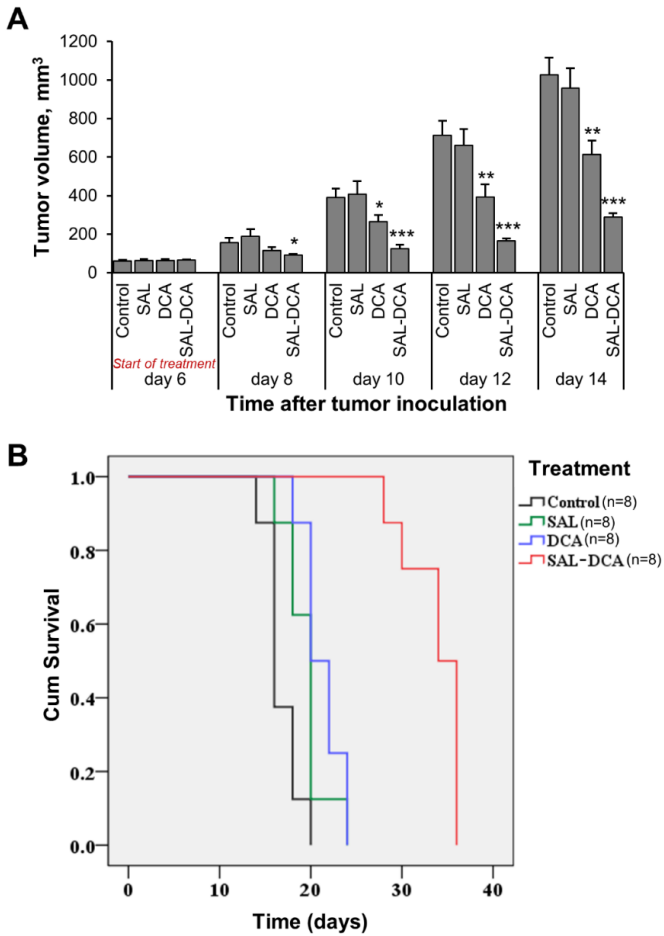

Fig. 6. Combination treatment of SAL and DCA delays tumor development in C57BL/6 mice injected with LLC1 cells. (A) Tumor growth in mice after treatment with SAL, DCA, and SAL + DCA. Bar graphs represent the mean tumor volume ± SEM of 8 mice at each time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(B)** Kaplan-Meier survival curves of mice receiving different treatments. The p values for differences in survival comparing mice treated with SAL, DCA, SAL + DCA and control mice are $p < 0.05$, $p < 0.005$ and $p < 0.0001$, respectively, as determined by log-rank analysis.

2.2.2 Combination therapy of SAL + DCA inhibits metastasis formation in the lungs of LLC1-bearing mice

At day 16 (10 days after the treatment), 4 mice from each group were randomly selected for histological evaluation of metastases as well as cell proliferation, EMT, and CSC markers. All mice that received control solution or monotherapy (SAL, DCA) treatments were positive for lung metastasis, while in the combination therapy group, none of the mice had detectable metastasis (**Table 2, Fig. 7**).

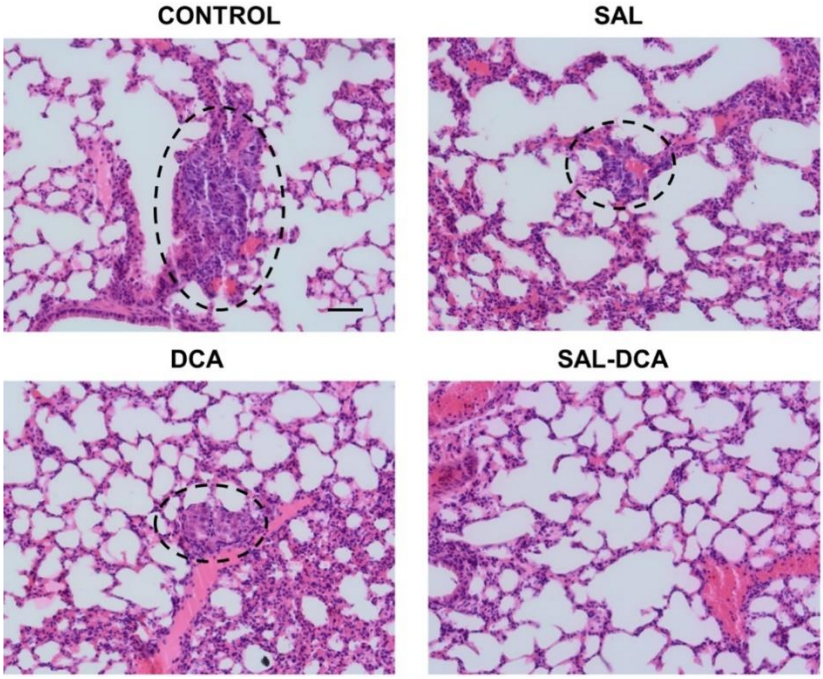

Fig. 7. SAL + DCA combination prevents metastasis formation in LLC1-bearing C57BL/6 mice. H&E staining of paraffin-embedded sections of the lungs. Metastases are encircled with black dashed lines. Images were captured at 200× magnification, scale bar = 50 μm.

2.2.3 Combination therapy of SAL and DCA suppresses expression of EMT, CSC, and proliferation markers in LLC1-induced tumors in C57BL/6 mice

In order to compare the impact of monotherapies (SAL, DCA) and combination treatment (SAL + DCA) on tumor cell stemness, EMT, and proliferation, dissected primary tumors were stained with the following markers: vimentin and E-cadherin for EMT, CD133 and CD44 for CSCs, and Ki-67 for proliferation (**Fig. 8**). Immunohistochemical analysis revealed a significant difference in the expression of EMT markers in control (E-cadherin and vimentin expression was 19 ± 8% and 50 ± 8%, respectively) compared with combination therapy (E-cadherin and vimentin expression was 63 ± 5% and 14 ± 6%, respectively) (**Table 2**). Treatment with SAL had no impact of EMT marker expression, while DCA showed similar results as combination therapy. In the DCA group, E-cadherin expression increased by 3.4-fold and vimentin expression decreased by 2.5-fold (**Table 2**).

The expression of both CSC markers was remarkably reduced by SAL alone as well as in combination with DCA, while DCA alone had a lower impact on CSC marker expression levels. In comparison with control, CD133 expression was decreased from 56 ± 6% in control to 11 ± 1% and 9 ± 2% in the SAL and SAL + DCA groups, respectively, while in the DCA group, the expression of this marker was reduced to 33 ± 5%. These results suggest that in the SAL + DCA combination, SAL is a key agent that contributes to the reduction of CSCs, whereas DCA is responsible for the inhibition of EMT. The results of Ki-67 staining were in alignment with the tumor growth rate and mice survival (**Fig. 6**): the proliferation potential was not affected by SAL, but was substantially decreased after DCA and SAL + DCA treatments. Ki-67 expression was 2-fold and 3.3-fold lower in the DCA and SAL + DCA treatment groups, respectively (**Table 2**).

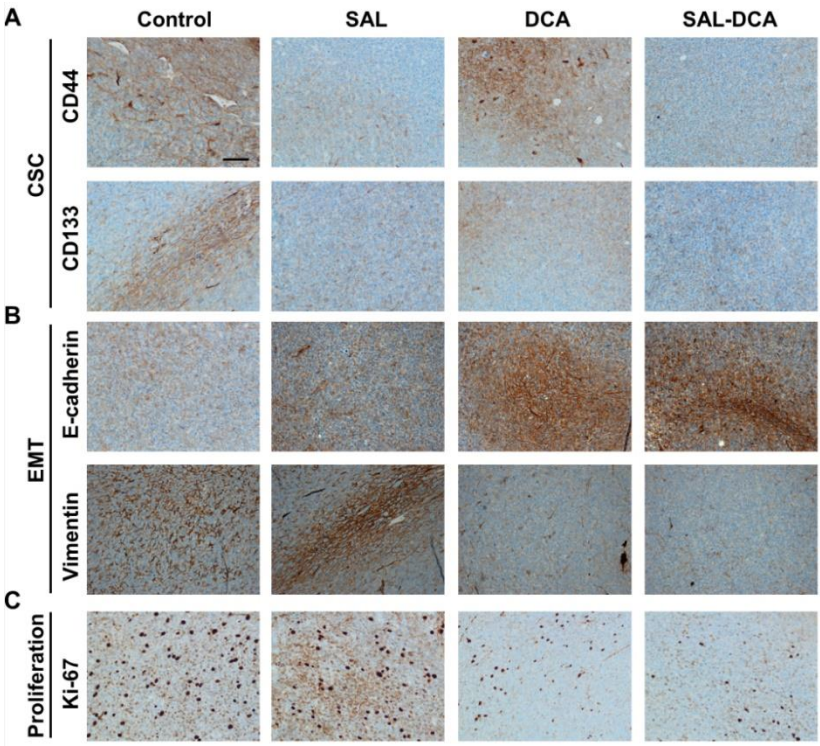

Fig. 8. Effect of SAL, DCA, and DCA combined with SAL on the expression of CSC, EMT, and cell proliferation markers in LLC1-bearing C57BL/6 mice. Immunohistochemical staining of sections prepared from LLC1-induced tumor in C57BL/6 mice after 10 days of treatment with SAL, DCA, and SAL + DCA. Protein expression levels of CSC markers CD133 and CD44 **(A)**, EMT markers E-cadherin and vimentin **(B)**, and proliferation marker Ki-67 **(C)**. Images were captured at 200× magnification, scale bar = 50 μm.

Table 2. Expression of CSC, EMT, and proliferation markers in primary tumor samples and incidence of metastatic site formation in the lungs and the liver of LLC1-bearing mice after 10 days of treatment with SAL, DCA, and their combination.

Group	Marker expression level, % (mean ± SEM)					Number of mice with metastases	
	CSC		EMT		Proliferation		
	CD44	CD133	E-cadherin	Vimentin	Ki-67	Lung	Liver
Control (n = 4)	64 ± 6	56 ± 6	19 ± 8	50 ± 8	36 ± 2	4/4	0/4
SAL (n = 4)	35 ± 3**	11 ± 1$^{***;\&\&}$	35 ± 5$^{\&\&}$	53 ± 13	43 ± 3$^{\&\&\&}$	4/4	0/4
DCA (n = 4)	44 ± 4*	33 ± 5$^{*;\#\#}$	64 ± 6$^{**;\#\#}$	20 ± 10*	18 ± 1$^{***;\ \#\#\#}$	4/4	0/4
SAL + DCA (n = 4)	26 ± 6$^{**;\&}$	9 ± 2$^{***;\&\&}$	63 ± 5$^{**;\#\#}$	14 ± 6$^{**;\#}$	11 ± 1$^{***;\ \#\#\#;\&\&}$	0/4	0/4

N = 4 mice for each group.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to SAL.
$^{\&}p < 0.05$; $^{\&\&}p < 0.01$; $^{\&\&\&}p < 0.001$ compared to DCA.

3. DISCUSSION

In the present study, we have discovered the synergistic cytotoxic effect of two drugs – SAL and DCA – in HCT116, DLD-1 and LLC1 cancer cell lines *in vitro* and proposed the explanation of its mechanism of action, which involves previously undisclosed activity of DCA. We also have confirmed synergistic properties of this combination *in vivo* in C57BL/6 mice with LLC1-induced tumors and revealed its effects on tumor growth, metastatic site formation as well as EMT and CSC progression.

The majority of solid tumors can be characterized by the unique abnormality called the Warburg effect (44). Targeting this pathological phenomenon has laid the basis for the development of innovative

chemotherapeutic strategies in addition to conventional cytotoxic drugs. DCA is one of the few glycolytic inhibitors that targets PDK, triggering a switch from glycolysis to oxidative phosphorylation in mitochondria; in other words, it reverses Warburg effect. In the last decades this small molecule has been extensively studied, and it is considered to be a promising anticancer agent.

In contrast, the CSC subpopulation, which does not proliferate malignantly yet and cannot be characterized by the Warburg effect, represents a distinct challenge for pharmacological investigations. Preclinical studies on mice and several clinical pilot trials have shown that SAL is capable to effectively eradicate CSCs and to induce partial clinical regression of heavily pretreated and therapy-resistant cancers. The ability of salinomycin to kill both CSCs and therapy-resistant cancer cells may define the compound as a novel and effective anticancer drug (10).

In this work, we have shown for the first time that the combination of SAL and DCA can produce a strong cytotoxic effect in colorectal and lung cancer cell lines. In addition to that we have proposed a possible explanation of its synergistic action. We provided evidence that DCA is a potent MDR protein inhibitor and is also capable to reduce pH_i. As a consequence to these properties, SAL has a prolonged exposure to cancer cells, which leads to cytotoxic effects.

Next, we have tested the effects of combination therapy *in vivo*. We chose the LLC1-C57BL/6 mouse model as it is the only reproducible syngeneic lung cancer model, and such models have proven to be beneficial in predicting clinical outcomes of therapy from preclinical research (45). The chosen mouse model and LLC1 cells due to their aggressive nature conveniently served for the evaluation of disease progression and metastatic processes. Our in vivo experiments showed that SAL + DCA therapy significantly reduces tumor growth in C57BL/6 mice with LLC1-induced tumors and is much more potent than both monotherapies.

Metastasis is the primary cause of cancer morbidity and mortality (46-48). Combating metastasis is complicated and often unsuccessful; therefore, on initial confirmation of the presence of malignant cells,

every effort should be made to prevent metastatic site formation. LLC1 cells are known to rapidly metastasize to the lungs, and in advanced cases, they can spread to the liver, kidney, and other organs. In the present study, we were mostly concerned about the initiation of metastatic sites and elucidation of factors that could be moderated in order to prevent this; therefore, we have tested mice for organ lesions and analyzed the dynamics of metastatic markers at the early stage of the disease. The results showed that at the time of the analysis (16 days after tumor inoculation), all mice in the control and monotherapy groups were already lung metastasis-positive, while no mice had any metastasis developed in the SAL + DCA group, suggesting that the simultaneous application of both drugs not only inhibits tumor growth, but also delays disease invasion to adjacent organs (lung, liver, kidney, and others).

Emerging evidence has shown that the induction of metastatic site formation is directly related to EMT initiation and CSC abundance (49-52). The EMT theory proposes that invasive migrating cells ascend from cancerous cells displaying epithelial properties that by building up various biochemical and genetic changes ultimately are converted into cells with mesenchymal characteristics (44, 52-56). Our results suggest that the absence of metastatic incidence in the group treated with SAL and DCA combination could be achieved due to cumulative effects on CSC and EMT processes since according to immunohistochemical analysis, SAL was responsible for CSC reduction, whereas DCA appeared to inhibit EMT. While SAL is considered as a known CSC inhibitor, to our knowledge, DCA has never been reported before to act as an EMT inhibitor. Such finding expanded our understanding about rationale behind the synergistic action of this combination therapy.

In summary, our results demonstrate that the combination of SAL and DCA acted synergistically both *in vitro* and *in vivo*. Combination treatment of SAL and DCA had favorable effects on tumor growth, metastatic site formation, EMT process, and CSC presence. We believe that versatile advantages of this therapy could be a promising approach in cancer treatment.

CONCLUSIONS

1. SAL and DCA exert a synergistic cytotoxic effect on DLD-1, HCT116, and LLC1 cancer cells *in vitro*;
2. SAL + DCA synergistic cytotoxic mechanism of action may be due to the effect of DCA on MDR and pH_i;
3. SAL + DCA therapy significantly reduces tumor growth in C57BL/6 mice with LLC1-induced tumors;
4. SAL + DCA therapy inhibits metastasis formation in the lungs of C57BL/6 mice with LLC1-induced tumors;
5. SAL + DCA therapy suppresses the expression of EMT, CSC, and proliferation markers in LLC1-induced tumors in C57BL/6 mice.

Supplementary Table 1. Concentration-dependent combined effects of salinomycin and DCA in HCT116 and DLD-1 cell lines.

Dose DCA (mM)	Dose SAL (μM)	HCT116		DLD-1	
		Fa	CI	Fa	CI
1	0.05	0.99	6.632	0.99	3.850
5	0.05	0.94	2.981	0.88	0.649
15	0.05	0.55	0.534	0.6	0.409
30	0.05	0.19	0.178	0.45	0.477
45	0.05	0.03	0.031	0.19	0.264
1	0.25	0.99	33.158	0.9	1.543
5	0.25	0.63	0.554	0.71	0.567
15	0.25	0.24	0.180	0.39	0.288
30	0.25	0.06	0.056	0.3	0.341
45	0.25	0.01	0.011	0.18	0.278
1	0.5	0.99	35.167	0.82	1.515
5	0.5	0.72	1.316	0.63	0.675
15	0.5	0.22	0.218	0.37	0.360
30	0.5	0.07	0.083	0.26	0.345
45	0.5	0.03	0.043	0.16	0.278

Fa, fraction affected; CI, combination index. CI of < 1 represents synergism, CI of 1 or close to 1 represents additive effects, and CI of > 1 represents antagonism.

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PRESENTATIONS

- "Laboratory Animals in Research", 2018, November, Vilnius, Lithuania, (oral presentation)
- "Computational Methods in Drug Design", 2018, March, Istanbul, Turkey, (oral presentation)
- COINS Conference of Life Sciences, 2018, March, Vilnius, Lithuania (poster)

- "Epigenetic Chemical Biology ECI Workshop and Core Group Meeting", 2018, February, Paris, France (poster)
- "FEBS Advanced Course in Oncometabolism", 2017, June, Coimbra, Portugal (poster)
- COINS conference of life sciences, 2017, March, Vilnius, Lithuania (poster)
- "Drug Discovery" 2016, November, Hamburg, Germany (poster)

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