Salinomycin and dichloroacetate synergistically inhibit Lewis lung carcinoma cell proliferation, tumor growth and metastasis

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A R T I C L E   I N F O

A B S T R A C T

Drug combination is considered to be the cornerstone of cancer treatment. Simultaneous administration of two or more drugs but at lower doses not only increases cytotoxic effects on tumor cells, but also reduces side effects and possibly overcomes drug resistance. Salinomycin is a well-known cancer stem cell killer, and dichloroacetate is a pyruvate dehydrogenase kinase inhibitor that exclusively targets cells with altered mitochondrial activity, a characteristic being common to most of the cancer cells. In our recent study, we have demonstrated that salinomycin exerted a cytotoxic effect on colorectal carcinoma cells in the 2D and 3D cultures and provided evidence that the mechanism of their synergy was mediated by dichloroacetate-dependent inhibition of the activity of multidrug resistance proteins. In the current work, we confirmed the synergistic cytotoxic properties of salinomycin and dichloroacetate in the 2D and 3D cultures of Lewis lung carcinoma (LLC1) cells. To verify if a synergistic effect of these compounds persisted *in vivo*, we performed series of experiments using a syngeneic LLC1-C57BL/6 mouse model and demonstrated that combination therapy with salinomycin and DCA increased the survival rate of allografted mice, inhibited metastatic site formation and reduced the populations of cancer stem cells as well as cells that underwent the epithelial-to-mesenchymal transition.

Our results demonstrate that a synergistic effect of salinomycin and dichloroacetate exists not only *in vitro* but also *in vivo* and suggest their benefits in the treatment of metastatic cancers.

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1. Introduction

Combination therapy is one of the most accredited strategies for cancer treatment, which is able to improve efficacy, reduce toxicity, diminish drug dose, and possibly overcome drug resistance. In our previous *in vitro* study, we have shown that the combination of salinomycin (SAL) and dichloroacetate (DCA) caused a synergistic cytotoxic effect in colorectal cancer cell lines DLD-1 and HCT116 and provided evidence that a synergistic action of these two drugs was linked to inhibition of multidrug resistance protein (MRPs) activity by DCA [1]. SAL and DCA have never been tested in combination before; yet, due to their potent cytotoxic action that targets two different cell subpopulations within a tumor, it could be a novel attractive therapy in oncology.

Cancer cells acquire a metabolic phenotype caused by Warburg effect, manifesting by enhanced glycolysis, lactic acid fermentation, and reduced mitochondrial oxidative phosphorylation, reactions being potential therapeutic targets for cancer treatment. DCA is one of the few glycolytic inhibitors that targets pyruvate dehydrogenase kinase (PDK) triggering a switch from glycolysis to oxidative phosphorylation in mitochondria [2]. DCA possesses several characteristics that make it a desirable candidate for cancer therapy: it does not affect healthy cells [3], it has an excellent bioavailability [2], and it is cost-effective. DCA mechanism of action has been studied extensively, and it has been used to treat lactic acidosis in

clinical settings for many years; therefore, side effects, including neuropathy and gastrointestinal disturbances, have been annotated as well [4].

Failure of treatment with traditional chemotherapy is often associated with heterogeneity of a neoplastic tissue. A small sub-population of tumor cells known as cancer stem cells (CSCs) is thought to be ultimately responsible for tumor progression and recurrence, treatment resistance, and metastatic site formation [5–8]. 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 and other [9,10]. A number of studies [11–14] attempted to reveal how SAL exerts its cytotoxic effects; however, no conclusive mechanism of its action has been determined so far.

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 [15,16], whereas favorable anti-metastatic results of SAL have been demonstrated only with high doses of SAL (e.g. 8 mg/kg) in mouse models [9,10].

In the present study, we investigated the effects of DCA, SAL, and the combination of both agents (SAL + DCA) on Lewis lung carcinoma (LLC1) cells in vitro in 2D and 3D cell culture models and in vivo in an allograft LLC1-C57BL/6 mouse model. We analyzed effects of this therapy on tumor growth, metastatic site formation, and properties of EMT and CSC.

2. Materials and methods

2.1. Cell lines, reagents, and mice

The murine metastatic Lewis lung carcinoma (LLC1) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultivated in an RPMI-1640 cell culture medium and supplemented according to manufacturer's protocol. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Stock with SAL (Sigma Aldrich, USA) was prepared at a concentration of 5 mM. DCA (Sigma Aldrich, USA) was prepared in concentration of 300 mM. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA) prepared in 5 mg/mL stock. AO (ThermoFisher Scientific, USA) and PI (Invitrogen, CA, USA) at 5 mg/mL and 1 mg/mL, respectively.

C57BL/6 mice (8–12-week-old; male) 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. They were housed under constant environmental conditions with a 12-h light-dark cycle. 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 [17] alongside the approval of the State Food and Veterinary Service, Lithuania (G2-85).

2.2. Cell viability assay

Viability analysis of LLC1 cells after treatment was assessed by MTT analysis according to manufacturer's instructions and synergistic effects were evaluated as described earlier [1].

2.3. 3D cell culture experimental design

Multicellular tumor spheroids were formed and analyzed as described earlier [1]. AO and PI staining was applied to determine cell apoptosis. Spheroids were then examined under a fluorescence microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, NY, USA).

2.4. In vivo experiments

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 4) combination of 3 mg/kg SAL and 200 mg/kg DCA. Solution for injections was prepared in 0.9% saline. Injections were given into the peritoneum on the daily basis. All mice were labeled, and tumors were measured every two days with a caliper. The tumor volume was calculated by the formula: $V = (W2 \times L)/2$ (1), where W is the tumor dimension at the widest point and L is the tumor dimension at the longest point. Each animal was weighed at the time of administration, so that the dosage could be adjusted to achieve the required dose (mg/kg) reported. Only mice weighing 18–22 g were included in the study. 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.

2.5. Immunohistochemistry

Tumors and organ tissues from the control, DCA-, SAL-, and SAL + DCA-treated groups were analyzed by immunohistochemistry. Slices for analysis were taken from various areas of the tumor and organs. Tissues were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections (2 μm in thickness) of paraffinized samples were deparaffinized, rehydrated, and stained with eosin and hematoxylin (H&E, Sigma-Aldrich). Slides for marker characterization were incubated for 45 min with 5% BSA and incubated overnight at 4 °C with the primary antibodies against CD133 (Abcam), CD44 (Ventana), vimentin, E-cadherin (Ventana), and Ki-67 (Abcam). Then specimens were incubated for 45 min at 37 °C with the appropriate peroxidase-conjugated secondary antibody and visualized using the ultraView Universal DAB Detection Kit (Ventana Medical Systems, USA, Roche Diagnostics GmbH, Germany) following the manufacturer's instructions. Quantification of markers was performed by counting the number of mitotic figures per 10 high power fields (40 ×). Samples were examined using OLYMPUS BX43 microscope (OLYMPUS U-TBI-3 1H64849, Japan), pictures were taken with OLYMPUS cellSens Entry 2.1 (Build 17342, Japan). For each tumor or organ sample at least 5 sections were stained and carefully screened by an independent pathologist, in a blinded manner (n = 4 mice for each group).

2.6. Statistical analysis

All data were expressed as mean ± standard error mean (SEM). Statistical analysis was performed using SPSS v.20 (IBM, Armonk, NY, USA) and Sigma Plot 10 software packages (Systat Software, San Jose, CA). Synergism of SAL + DCA 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). The log-rank test was applied for survival analysis, and the Student's t-test was used for all other analyses. P-values of <0.05 were considered significant.

3. Results

3.1. SAL and DCA have a synergistic cytotoxic effect on Lewis lung carcinoma cells in 2D and 3D cultures

We have previously shown that DCA in combination with SAL exerted a strong synergistic cytotoxic effect on human colorectal cancer cell lines DLD1 and HCT116 in 2D and 3D cultures (6). In the present study, first we attempted to reproduce this effect in the mouse LLC1 cell line. We performed a series of dose response experiments and estimated IC_{50} values for monotherapies of DCA and SAL that were 28.26 mM and 0.66 µM, respectively (Fig. 1A and B). We chose several doses below IC_{50} from both monotherapies (10 and 20 mM of DCA and 0.05 and 0.5 µM of SAL) and tested their combination in the experiments of a non-constant ratio drug design. Such combination exerted a significant reduction of LLC1 cell viability and was substantially more effective than the sum of both monotherapies (Fig. 1C). As illustrated in Fig. 1D, the combination index (CI) was below 1 for all tested combinations, indicating synergy.

Next, by employing the multicellular spheroid technique, we examined if SAL + DCA therapy retained its synergistic effect in the 3D cell culture. Imaging of the spheroids was performed after exposure to different treatments after 48, 96, and 144 h (Fig. 2A). Drop in the spheroid size was observed in all groups, and the strongest effect was observed in the combination treatment group at all time points (Fig. 2A). After 48 h of monotherapy with SAL and DCA, the spheroid size decreased only by 7% and 15%, respectively, while combination treatment reduced the spheroid size by 29% as compared with the control group (Fig. 2A and B). Moreover, after 96 h in the SAL + DCA group and after 144 h in all 3 groups (SAL, DCA, and SAL + DCA), the spheroid structure began to disintegrate. Acridine orange (AO) and propidium iodide (PI) staining revealed that only cells in the spheroid core were viable (green color, AO positive) and the cells located around the core were dead (red color, PI positive) (Fig. 2C).

3.2. DCA in combination with SAL significantly suppresses tumor growth in LLC1-C57BL/6 mice

To explore the antitumor effects of SAL, DCA, and their combination *in vivo*, we established a mouse allograft model using the LLC1 cell line. LLC1-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. We compared tumor growth dynamics across 4 groups during the period of 6–16 days (Fig. 3A). At day 6, tumors were palpable and equal in size, and at this time point, mice started to receive the corresponding treatment. At day 16 in the control group, tumors of the first mice started to reach the maximal volume (1500 mm^3) and these mice were euthanized. 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. Kaplan−Meier survival curves of mice after tumor inoculation receiving various treatment regimens are presented in Fig. 3B.

3.3. Combination therapy with SAL and DCA inhibits metastasis formation in the lungs of LLC1-C57BL/6 mice

After tumor inoculation, 48 animals were divided into 4 groups (12 each) and at day 6 started to receive the corresponding treatment. At day 16 (10 days after the treatment), 4 mice from each group were randomly selected for histological evaluation for metastases as well as cell proliferation, EMT and CSC markers (these animals were not included in the survival and tumor growth analysis). All mice that received control solution or monotherapy (SAL, DCA) treatments were positive for lung metastasis, while in
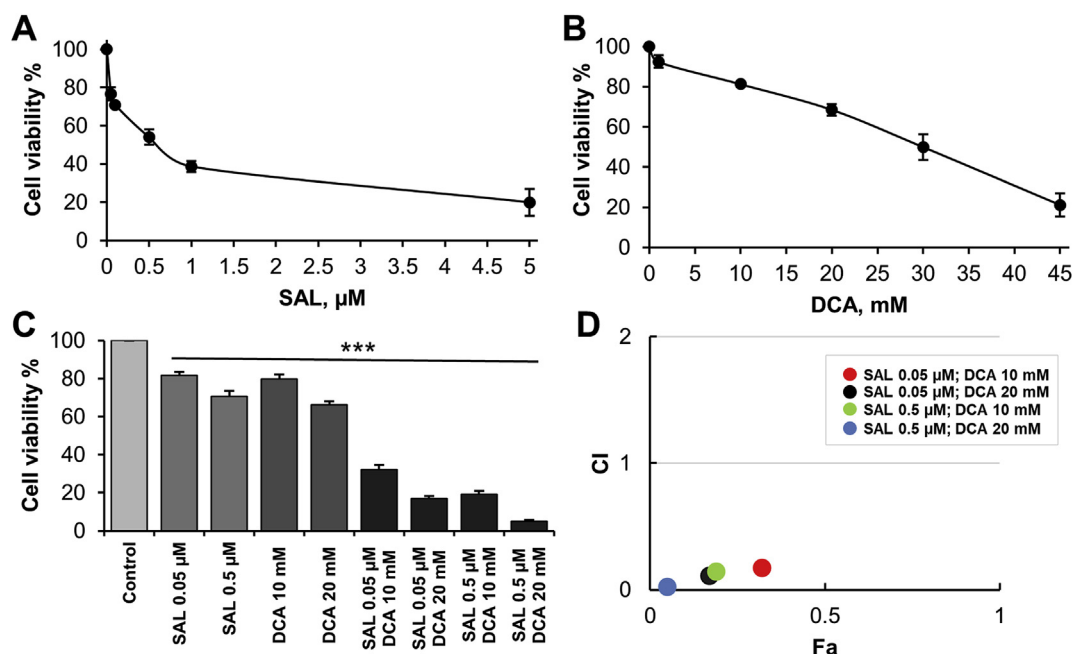

Fig. 1. Cytotoxic effects of DCA and SAL in monotherapy and combination on LLC1 cells in the 2D culture determined by MTT analysis. (A, B) dose response curves of SAL (A) and DCA (B) in monotherapies after 48 h of treatment. (C) Summary of cytotoxic effects of SAL and DCA on LLC1 cell viability in monotherapy and combination. (D) Fa-CI plot for the combination treatment of SAL and DCA using different drug ratios. Data are expressed as mean ± SEM calculated from 3 independent experiments. ***p < 0.001.
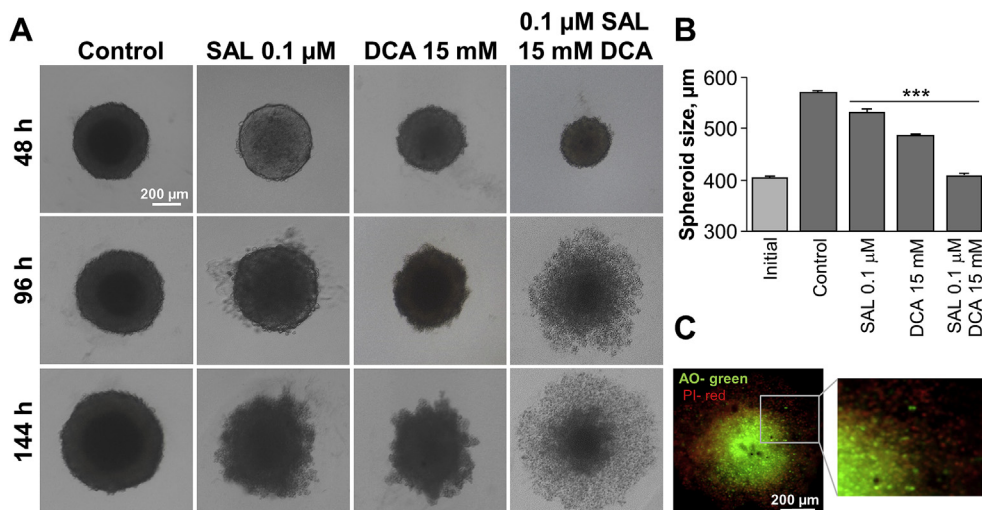
Fig. 2. Cytotoxic effects of SAL, DCA and SAL + DCA on LLC1 cells in the 3D culture. (A) Representative images of LLC1 cells after 48, 96, and 144 h of treatment with SAL, DCA, and SAL + DCA. (B) Comparison of cytotoxic effects of 0.1 μM SAL, 15 mM DCA, and their combination 48 h after the treatment. Data are expressed as mean ± SEM calculated from 3 independent experiments. ***$p < 0.001$. (C) Cell viability assessment after 96 h of SAL + DCA treatment by acridine orange (AO) and propidium iodide (PI) double staining. Viable cells are green; late apoptotic and necrotic cells are orange and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
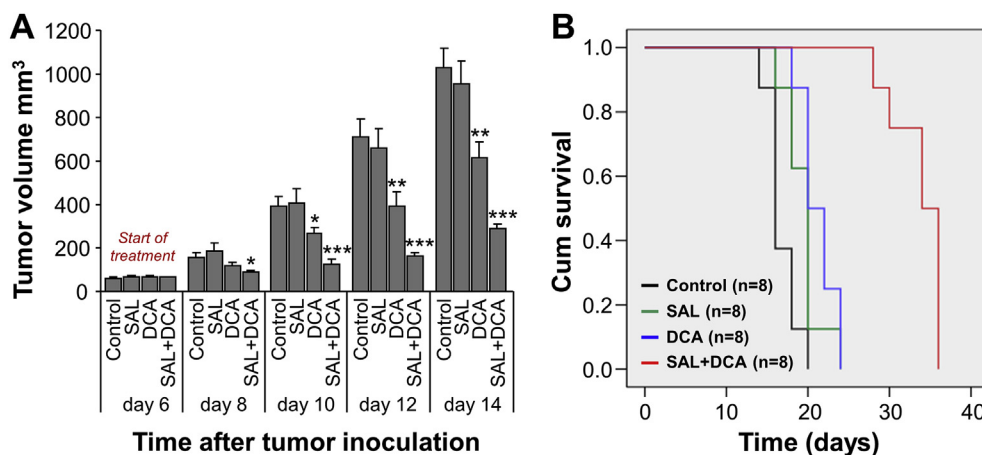

Fig. 3. Combination treatment of SAL and DCA delays tumor development in LLC1-C57BL/6 mice. (A) Tumor growth in mice after treatment with SAL, DCA, and SAL + DCA. Bar graphs represent the mean tumor volume ±SEM of 8 mice at each time point. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (B) Kaplan-Meier 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.

the combination therapy group (SAL + DCA), none of the mice had detectable metastasis (Fig. 4). We also tested animals for metastatic lesions in the liver; however, at this point, no mice in any of the 4 animal groups had any hepatic lesions developed.

3.4. Combination therapy of SAL and DCA suppresses expression of EMT, CSC, and proliferation markers in tumors of LLC1-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 from 4 mice of each group which were previously selected for metastatic evaluation were stained with the following markers: vimentin and E-cadherin for EMT; CD133 and CD44 for CSCs; and Ki-67 for proliferation (Fig. 5). 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) (Fig. 5). 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 (Fig. 5). 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.
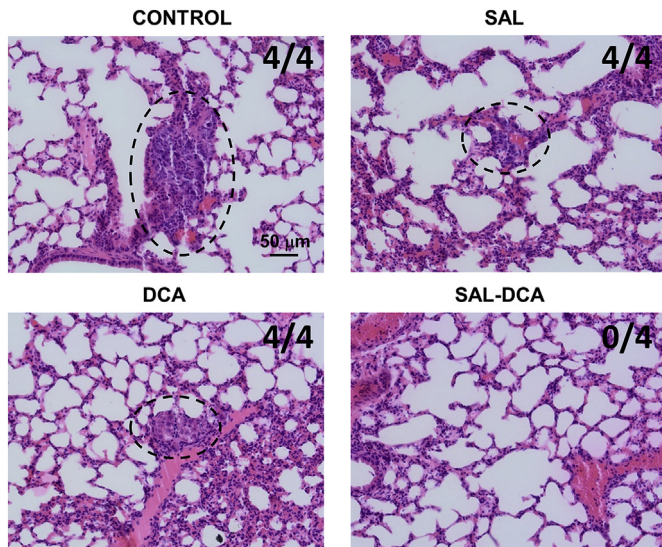
Fig. 4. Effect of SAL, DCA and SAL + DCA on metastasis formation in LLC1-C57BL/6 mice. H&E staining of paraffin-embedded sections of the lungs. Metastases are encircled with black dashed lines. Numbers on the top right corner of each image indicate a ratio of metastatic mice number versus tested mice number. Images were captured at 200 × magnification.

The results of Ki-67 staining were in alignment with the tumor growth rate and mice survival (Fig. 3); 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 (Fig. 5).

4. Discussion

The majority of solid tumors can be characterized by the unique abnormality called the Warburg effect (40). Targeting this pathological phenomenon has laid the basis for the development of innovative chemotherapeutic strategies in addition to conventional cytotoxic drugs. 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. In the present study, we demonstrated that co-administration of DCA, a known PDK inhibitor, and SAL, a potent CSC killer, could more effectively repress the growth of LLC1 cells compared to monotherapies alone *in vitro* and reduce tumor growth as well as metastasis *in vivo*.

This study is the continuum of our initial research [1] in which for the first time we have presented the synergistic cytotoxic SAL-DCA effect on colorectal cancer cell lines and proposed a possible mechanism of its action. Moving forward, in the current study, we tested our hypothesis in a mouse model to see if combination maintains its anticancer effects.

We have chosen a LLC1-C57BL/6 mice 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 [18]. The chosen mouse model and LLC1 cells due to their aggressive nature conveniently served for evaluation of disease progression and metastatic processes. In addition to these advantages, we believe that the synergistic cytotoxic properties of
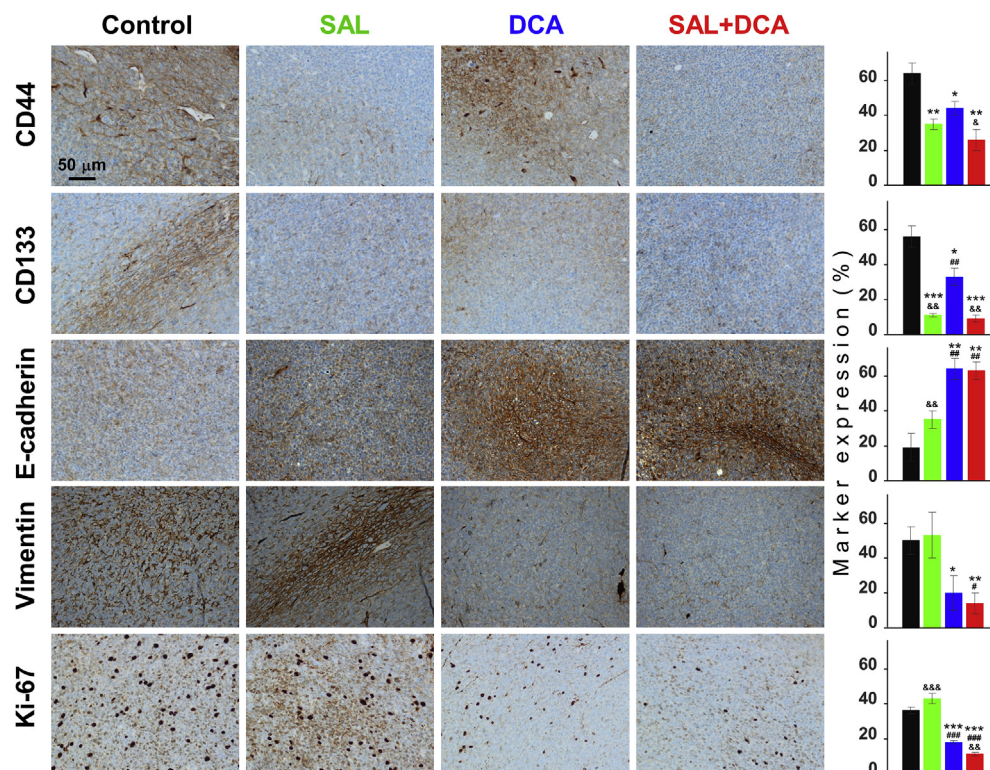

Fig. 5. Effect of SAL, DCA, and SAL + DCA on the expression of CSC, EMT and cell proliferation markers in the primary tumors of LLC1-C57BL/6 mice. Representative images of immunohistochemical staining of sections prepared from tumors after 10 days of treatment with SAL, DCA, and SAL + DCA, and bar graphs of quantitative evaluation of marker expression. Data are presented as mean ± SEM (n = 4 for each mice group). CSC markers are CD133 and CD44; EMT markers - E-cadherin and vimentin; proliferation marker - Ki-67. Images were captured at 200 × magnification. *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.

SAL-DCA are not cancer type specific since we previously reported similar synergistic effects in colorectal cancer cell lines [1].

Metastasis is the primary cause of cancer morbidity and mortality [19–21]. 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 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 LLC1-C57BL/6 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).

CSCs not only are an important player in metastasis formation, but also significantly contribute to the development of chemotherapeutical resistance and tumor relapse. Long-term effects of SAL-DCA therapy was not in scope of this study due to short longevity of mice with a tumor of such aggressive type; however, further investigations may serve to disclose possible benefits of this therapy in overcoming drug resistance and preventing tumor reoccurrence.

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.

Funding

Availability of data and materials

The datasets used and analyzed for the present study are available from the corresponding author upon reasonable request.

Authors' contribution

AS and IS designed the study and drafted the manuscript, AS, IS and RP performed experiments in 2D and 3D cell cultures, AS, JAK, KZ and VB performed animal experiments, AB was an independent pathologist who blindly performed interpretations of histology results, AS, IS, KS and SJ analyzed and interpreted the experiments. All authors reviewed the manuscript.

Ethics approval and consent to participate

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 [17] alongside the approval of the State Food and Veterinary Service, Lithuania (No. G2-85).

Declaration of competing interest

The authors declare no conflicts of interests.

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Transparency document

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