Liposomal simvastatin sensitizes C26 murine colon carcinoma to the antitumor effects of liposomal 5-fluorouracil in vivo

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Abstract
5-Fluorouracil-based therapy remains the main approach in colorectal cancer, even though there are still some drawbacks, such as chemoresistance. In this study we combined 5-fluorouracil encapsulated in long-circulating liposomes with simvastatin, also encapsulated in long-circulating liposomes, that was previously proved to exert antitumor actions on the same tumor model. The production of angiogenic/inflammatory proteins was assessed by protein array and the production of markers for tumor aggressiveness (Bcl-2, Bax, and nuclear factor [NF]-κB) were determined by western blot analysis. Intratumor oxidative stress was evaluated through measurement of malondialdehyde level by HPLC, and through spectrophotometric analysis of catalytic activity of catalase and of total antioxidant capacity. Immunohistochemical analysis of tumors for CD31 expression was assessed. Intratumor activity of MMP-2 by gelatin zymography was also carried out. Our results revealed that combined therapies based on liposomal formulations exerted enhanced antitumor activities compared with combined treatment with free drugs. Sequential treatment with liposomal simvastatin and liposomal 5-fluorouracil showed the strongest antitumor activity in C26 colon carcinoma in vivo, mainly through inhibition of tumor angiogenesis. Important markers for cancer progression (Bcl-2, Bax, and nuclear factor [NF]-κB) and intratumor antioxidants showed that liposomal simvastatin might sensitize C26 cells to liposomal 5-fluorouracil treatment in both regimens tested. The outcome of simultaneous treatment with liposomal formulations was superior to sequential treatment with both liposomal types as the invasive capacity of C26 tumors was strongly increased after the latest treatment. The antitumor efficacy of combined therapy in C26 colon carcinoma might be linked to the restorative effects on proteins balance involved in tumor angiogenesis.

KEYWORDS
5-fluorouracil, combined therapy, liposomes, resistance, simvastatin
1 | INTRODUCTION

Chemotherapy of colorectal cancer is limited to the use of few licensed drugs, such as 5-fluorouracil (5-FU), oxaliplatin, irinotecan, capecitabine, cetuximab, panitumumab, and bevacizumab. However, 5-FU-based chemotherapy remains the backbone of colorectal cancer treatment. Despite its anticancer effects through disruption of DNA synthesis and inhibition of protein synthesis, 5-FU monotherapy was reported by Johnston and Kaye to have low response rates, in the range of 10%-20%, whereas the combination of 5-FU with other chemotherapeutic drugs, such as irinotecan, increased the response rate to 40%-50%. These limitations of 5-FU-based therapies are due to drug resistance, and its low bioavailability caused by rapid degradation of 5-FU in the liver by dihydropyrimidine dehydrogenase. Our previous studies showed that tumor-targeting properties of long-circulating liposomes (LCL) could enable 5-FU to accumulate into the colon carcinoma tissue, and to act more efficiently compared with conventional chemotherapy based on the free treatment with the same drug. Thus, LCL ensured the passive tumor accumulation of 5-FU, due to the enhanced permeability of tumor vasculature as compared to healthy endothelium (referred to as “the enhanced permeability and retention” effect), and increased the therapeutic index of 5-FU. Several studies revealed that the coadministration of chemosensitizers could improve the outcome of 5-FU therapy in colorectal cancer. In this regard, statins increased the chemosensitivity of tumor cells to 5-FU in colorectal and bile duct cancer. Among statins, simvastatin delivered by LCL (LCL-SIM) has strong antitumor activity in C26 colon carcinoma through cytotoxic effects and suppressive actions on tumor angiogenesis and inflammation, as shown by our previous studies. Based on these recent findings, we investigated whether the antitumor activity of 5-FU encapsulated in LCL (LCL-5-FU) could be enhanced after its administration in combination with LCL-SIM in C26 murine colon carcinoma-bearing mice. To this aim, we assessed the effects of this novel combined tumor-targeted treatment on tumor growth, and on the main protumor processes involved in colon carcinoma development, ie, angiogenesis, inflammation, oxidative stress, resistance to apoptosis, and invasiveness.

2 | MATERIALS AND METHODS

2.1 | Preparation of LCL-FU and LCL-SIM

Both liposomal formulations were prepared by lipid film hydration method followed by extrusion, using a lipid molar ratio of 9.5:0.5:1.22 (1,2-dipalmityl-sn-glycerol-3-phosphocholine [DPPC; Lipoid], N-[(carboxylmethoxy)polyethyleneglycol-2000]-1,2-distearoyl-sn-glycerol-3-phosphoethanolamine (Na-salt) [PEG-2000-DSPE; Lipoid], cholesterol [CHO; Sigma-Aldrich], and SIM [Biocon]) for LCL-SIM. For LCL-5-FU, a lipid molar ratio of 9.5:0.5:1 (DPPC:PEG-2000-DSPE:CHO) was used, as previously described. Both formulations were previously optimized in terms of PEG content and drug encapsulation as reported. The mean size (115 nm for LCL-SIM and 180 nm for LCL-5-FU) and narrow size distribution (polydispersity index approximately 0.1 for both formulations) favor passive tumor accumulation.

2.2 | Cell line and in vivo murine tumor model

C26 murine colon carcinoma cells (Cell Lines Service) were cultured in RPMI-1640 medium as described previously. In vivo colon carcinoma tumor model was induced by s.c. injection of 10⁶ C26 cells in the right flank of Balb/c mice (Cantacuzuno Institute, Bucharest, Romania); tumors became palpable at day 5 after cell inoculation, as described previously. The tumor volume was determined according to the formula: \( V = 0.52a^2b \), where \( a \) is the smallest and \( b \) is the largest superficial diameter in millimeters. Each experimental group consisted of 5-6 mice. Experiments were carried out according to the national regulations and were approved by the university animal experiments ethical committee (registration no. 31375/06.04.2015).

2.3 | Assessment of antitumor activity

To assess the antitumor activity of the combined tumor-targeted therapy, mice received 2 i.v. injections of 5 mg/kg SIM and 1.2 mg/kg 5-FU, either liposomal formulation or free form. Two dosing schedules were compared, namely, simultaneous treatment (at days 8 and 11 after tumor cell inoculation), and sequential treatment (pretreatment with SIM at days 7 and 10 after tumor cell inoculation, followed by 5-FU after 24 hours). Each dose was selected on the basis of our previous studies regarding the antitumor activity of LCL-SIM and LCL-5-FU given as single liposomal therapy on C26 colon carcinoma in vivo.

2.4 | Assessment of the production of key proteins for tumor development by western blot analysis

After mice were killed at day 12, tumors were collected, weighed, and snap frozen in liquid nitrogen. Tumor tissues from each experimental group were lysed as previously described, and the protein concentration was measured using the biuret method. The production of the active form of nuclear factor [NF]-κB (2 µg total protein) (polyclonal rabbit IgG anti-mouse pNF-κB-p65; Santa Cruz Biotechnology), Bcl-2 (40 µg total protein) (monoclonal anti-rabbit Bcl-2; Cell Signaling Technology), and Bax (25 µg total protein) (rabbit polyclonal IgG anti-Bcl-2-associated X protein; Cell Signaling Technology) were determined by western blot analysis, along with β-actin (rabbit polyclonal IgG anti-mouse β-actin; Sigma-Aldrich) as the loading control, as described previously. The secondary Ab used was goat anti-rabbit IgG-HRP-conjugated (Santa Cruz Biotechnology). The expression levels of these proteins were determined and represented as a percentage from their control expression.

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levels. The Bcl-2/Bax production ratio was represented as the ratio between these percentages. The final results represent mean ± SD of 3 independent experiments.

2.5 | Determination of angiogenic/inflammatory protein production in tumors

To determine the effects of the combined therapy on the production levels of angiogenic/inflammatory proteins in tumor tissue, we undertook screening for 24 proteins involved in angiogenesis using the RayBio Mouse Angiogenic Cytokine Antibody Array kit (RayBiotech) as described previously. The production of each angiogenic/inflammatory protein in tumor tissue lysates was determined in duplicate, and represented as mean ± SD of 2 independent experiments.

2.6 | Quantification of malondialdehyde levels

To assess the levels of oxidative stress in tumors treated with different regimens of the combined therapy, we measured malondialdehyde (MDA) by HPLC, as we previously described. The results were expressed as nanomoles of MDA, normalized per milligram of protein from tumor lysates. Each sample was determined in duplicate.

2.7 | Measurement of intratumor catalase activity

The catalytic activity of catalase was assessed using the method described by Aebi. We measured catalase activity of different treated tumor lysates as we previously described. Catalase activity is expressed as units of catalytic activity normalized per milligram of protein.

2.8 | Determination of total antioxidant capacity in tumors

To determine the nonenzymatic antioxidant capacity of tumors treated with combined therapy with 2 regimens of administration, we used the method first described by Erel, and we applied the same protocol described previously. The results were expressed as micromoles of trolox equivalents normalized to milligrams of protein.

2.9 | Immunohistochemical evaluation of neovascularization of tumor tissue after liposomal treatment

To assess the treatment effects on the formation of new blood vessels into the tumor tissue, immunohistochemical analysis for anti-CD31 Ab (rabbit polyclonal IgG anti-mouse CD31; ab124432, Abcam) was diluted 1000-fold. We used the following scoring system to evaluate the area of positive immunoreaction: 0.5, 5%-20%; 1, 20%-40%; 2, 40%-60%; 3, 60%-80%; and 4, 80%-100%.

2.10 | Determination of intratumor expression and activity of MMP-2 by gelatin zymography

To evaluate the activity of MMP-2 activity from tumor lysates prepared in nonreducing conditions, 20 µg proteins were separated on 0.1% gelatin and 7.5% acrylamide electrophoretic gels under denaturing conditions, as previously described. After gels were stained with Coomassie blue, 2 major areas of gelatinolytic activity were revealed on the zymographic gels, at 62 kDa (corresponding to the active form of MMP-2) and 72 kDa (corresponding to zymogen form of MMP-2, pro-MMP-2). The area of the enzyme activity was determined by densitometry analysis of the white bands using ImageJ software.

2.11 | Statistical analysis

Data from different experiments were reported as mean ± SD. Statistical comparisons of the effects of different treatments on tumors were evaluated by 1-way ANOVA followed by Bonferroni’s multiple comparisons test. The differences in angiogenic/inflammatory protein production after different treatments were determined by 2-way ANOVA with Bonferroni’s test for multiple comparisons. The scores for immunoreaction intensities of tumor sections after different treatments were analyzed by using the rank-based non-parametric Kruskal-Wallis test with Dunn’s test for multiple comparisons. All statistical analyses were undertaken using GraphPad Prism Software version 6 for Windows. A value of $P < .05$ was considered significant. Multivariate data analysis of protein array data, ie principal component analysis (PCA), was carried out using SIMCA15 Software (Sartorius Stedim Biotech). All variables were scaled to unit variance.

3 | RESULTS

3.1 | Antitumor activity of liposomal and free 5-FU and SIM after simultaneous and sequential treatment

To compare the antitumor activity of the 2 treatment regimens, mice received 2 i.v. injections containing 5 mg/kg LCL-SIM and 1.2 mg/kg LCL-5-FU on day 8 and day 11 after tumor induction, in the case of the simultaneous schedule treatment; in the sequential regimen, mice received 5 mg/kg LCL-SIM on days 7 and 10, and 1.2 mg/kg LCL-5-FU on days 8 and 11. The same schedule was used for the treatment with free drugs. The antitumor activity was monitored daily from day 7 after tumor induction (when the
average tumor volume was approximately 80 mm$^3$), until day 12, and the results were assessed by measuring the area under the tumor growth curve (AUTC) of different treated groups (Figure 1A) and tumor volumes at day 12 (when mice were killed) (Figure 1B). Our data indicated that LCL encapsulation of 5-FU and SIM enhanced the antitumor efficacy of both combined liposomal treatments tested on C26 colon carcinoma-bearing mice (Figure 1). Nevertheless, the strongest antitumor activity was noted after sequential treatment with LCL formulations, as AUTC as well as tumor volumes at day 12 were almost totally decelerated (approximately 82%-85% inhibition of tumor growth compared with control tumors, $P < .0001$; Figure 1) after this treatment.

### 3.2 | Effects of different treatments on intratumor apoptosis

To investigate whether different treatments affected the antiapoptotic capacity of C26 colon carcinoma, we evaluated the expression level of Bcl-2, an essential protein for the expression of the metastatic phenotype of colon cancer cells, being associated with the failure of chemotherapy. Our results revealed strong and similar suppressive effects of both sequential therapies (based on liposomal as well as free drugs) on the intratumor production of Bcl-2 (70%-80% reduction of protein levels compared with its control production ($P < .0001$)) and only moderate inhibitory effects of each simultaneous therapy (25%-40% reduction of protein levels compared with its control production) on the same protein levels in C26 colon carcinoma in vivo (Figure 2A).

To evaluate the apoptotic effects of different treatments on C26 colon carcinoma tumors, we assessed the expression levels of Bax protein, also involved in the effectiveness of 5-FU therapy on colon cancer cells. Our results showed that none of the treatments affected the levels of Bax protein statistically significantly compared to its control production (Figure 2B). Nevertheless, except for simultaneous treatment with free drugs, all treatments applied did not induce the settlement of cancer cell chemoresistance as these treated tumors showed 2-5-fold reduction of the Bcl-2/Bax production ratio (Figure 2C, $P = .02$), which could predict the sensitivity of colon cancer cells to 5-FU therapy.

### 3.3 | Effects of different treatments on tumor inflammation

To assess the molecular mechanisms of action of different treatments on C26 colon carcinoma inflammation, we evaluated the changes in the production of a key transcription factor, NF-κB, which plays an important role in inflammation associated with cancer cell proliferation as well as in the supportive processes for tumor progression, such as angiogenesis. Moreover, this transcription factor was reported to be constitutively activated in colorectal cancer. Therefore we evaluated the production levels of phosphorylated p65 subunit of NF-κB, which is associated with the activation of NF-κB. In Figure 3, our results showed that the coadministration of free drugs in each regimen enhanced the production of the phosphorylated p65 subunit of NF-κB, by
5 times in the simultaneous treatment regimen, and by 3 times in the sequential treatment regimen, whereas combined liposomal therapies did not statistically significantly affect the activation of NF-κB.

3.4 Effects of different treatments on C26 colon carcinoma-associated angiogenesis

To compare the different treatments effects on tumor angiogenesis, the production of 24 proteins involved in angiogenesis and inflammation were quantified by undertaking a protein array, and the results were subjected to a multivariate data analysis, using PCA. Differences in protein profiles highlighted through OPLS-DA model performance parameters (Table S1), also supported that sequential treatment with liposomal formulations has the strongest suppressive effects on the production of angiogenic proteins compared to control.

Furthermore, we show in detail in Table 1 the percentage of inhibition (−) or stimulation (+) of expression for each proangiogenic/proinflammatory protein (Table 1A), and for each antiangiogenic/antiinflammatory protein (Table 1B) in tumor tissue, induced after each combined liposomal treatment compared with control production of the same proteins. More specifically, LCL-SIM + LCL-5-FU strongly affected the production of granulocyte colony-stimulating factor, granulocyte/macrophage-colony stimulating factor, monocyte colony-stimulating factor, insulin growth factor-II, interleukin (IL)-1α, IL-9, IL-13, monocyte chemoattractant protein-1, Fas ligand, and thrombopoietin (by 50%-80% inhibition, $P < .001$ and $P < .0001$), whereas the production of IL-1β, IL-6, IL-12p40, tumor necrosis factor-α, eotaxin, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and leptin was almost totally depleted (by 80%-100% inhibition, $P < .0001$) by the same treatment (Table 1B). Nevertheless, except for the production of platelet factor-4 (PF-4) and IL-12p70, which were affected moderately, the levels of all other antiangiogenic and antiinflammatory proteins were strongly to very strongly affected by LCL-SIM + LCL-5-FU (Table 1B).
Effects of combined therapy on intratumor oxidative stress

As our previous study revealed, the oxidative stress level has a great impact on tumor development, promoting C26 murine colon carcinoma cell proliferation in an oxidative stress-dependent manner. Therefore, we evaluated the effects of different treatments on the oxidative stress levels by measuring the intratumor levels of MDA, a general oxidative stress marker, as well as measuring the levels of enzymatic and nonenzymatic antioxidants (catalytic activity of catalase and total antioxidant capacity [TAC]).

As Figure 5A shows, all treatment regimens, except simultaneous treatment with liposomal formulations, exerted antioxidant actions on C26 colon carcinoma by decreasing intratumoral MDA levels. The highest reduction of MDA levels (approximately 40%, \( P < .01 \)) was induced by the liposomal formulations given in a sequential schedule.

The assessment of the enzymatic activity of catalase in different treated groups has shown dissimilar actions of free drugs to liposomal formulations. Thus, treatment with free drugs in both regimens increased the enzymatic activity of catalase (enzymatic activity was twice that of the control, \( P < .001 \) for simultaneous treatment and \( P < .05 \) for sequential treatment), but liposomal formulations did not statistically significantly affect (\( P > .05 \)) the enzymatic activity of catalase compared with control (Figure 5B). Moreover, except for the liposomal formulations given sequentially, which did not affect the TAC compared with control (\( P > .05 \)), all other treatments produced increased TAC, approximately doubling the levels of trolox equivalents compared with the control group (\( P < .001 \)), as can be observed in Figure 5C. Together, these data suggest that sequential treatment with liposomal drug formulations exerted moderate antioxidant actions of C26 colon carcinoma in vivo.

Impact evaluation of antiangiogenic activity of combined liposomal drug treatments on their antitumor efficacy

As the antitumor activity of each combined liposomal drug treatment was mainly based on the antiangiogenic action, we investigated the efficacy of these therapies on C26 colon carcinoma progression with regard to their impact on tumor neovascularization and tumor invasive capacity.

Effects of liposomal combined therapies on tumor neovascularization

To evaluate the effects of liposomal combined treatments on vascularization in C26 colon carcinoma, tumors were immunohistochemically analyzed with regard to the expression of CD31 as a marker for proliferating endothelial cells. Our results showed a significant reduction of the expression of CD31 in tumors treated with the sequential regimen LCL-SIManteLCL-5-FU compared with its control levels (Figure 6). This finding might suggest a tight connection between inhibition of neovascularization and strong reduction of the proangiogenic protein production (Table 1A) after sequential treatment with liposomal drugs. In contrast, after simultaneous treatment with LCL-SIM and LCL-5-FU, the expression of endothelial marker CD31 in tumors was slightly increased compared with its expression in control tumors (Figure 6, \( P < .05 \)). This action might be related to the ineffectiveness of this treatment to annihilate neovascularization.
pathways regulated by bFGF (Table 1A) as the expression of this angiogenic protein was only slightly affected by this treatment.

3.6.2 | Effects of liposomal combined therapies on invasive and metastatic capacity of C26 colon carcinoma tumors

As several studies associated the therapeutic inhibition of angiogenesis with an increase of invasiveness and metastatic capacity of tumor cells,38-40 we investigated whether liposomal combined therapies given in both regimens affected the activity of MMP-2, an important player in colorectal cancer metastasis.41 Our data showed that sequential regimen of liposomal therapy induced an increase of both the zymogen form of MMP-2 (3 times higher than control, \(P < .01\)) (Figure 7A,B) and the active form of MMP-2 (2 times higher than control, \(P < .0001\)) (Figure 7C). Notably, the simultaneous regimen of liposomal therapy did not affect the expression of MMP-2 (\(P > .05\)) (Figure 7C). In conclusion, these data suggested that simultaneous treatment with liposomal formulations in C26 colon carcinoma-bearing mice had better prognosis than sequential treatment with the same liposomal forms in terms of antitumor efficacy in this tumor model.

4 | DISCUSSION

Despite the advances in the improvement of colorectal cancer therapies based on 5-FU, there are still major limitations that impede an effective treatment outcome such as the resistance developed by tumor cells to 5-FU. Enhancement of sensitivity of cancer cells to 5-FU by chemosensitizers represent the starting point of many strategies designed to overcome this major drawback of the antitumor therapies based on this cytotoxic drug.5 In a recent study, Kodach and colleagues reported that statins increased the sensitivity of colorectal cancer cells to 5-FU by inducing differentiation of colorectal cancer cells.11 Moreover, our previous findings have shown that the antitumor efficacy of LCL-SIM on C26 colon carcinoma in vivo was mainly based on suppressive actions on tumor angiogenesis.12 In the light of these aspects, this study aimed to investigate the antitumor activity of a novel targeted combined therapy based on treatment with liposomal 5-FU together with a nonconventional anticancer drug, SIM, also encapsulated in LCL. Furthermore, we assessed the combined therapy in 2 treatment regimens, namely, simultaneous treatment with liposomal formulations (LCL-SIM + LCL-5-FU) and sequential treatment with liposomal formulations (LCL-SIM + LCL-5-FU) and sequential treatment with liposomal formulations (LCL-SIM + LCL-5-FU). Our results showed that both combined liposomal drug treatments exerted strong antitumor activities, although sequential treatment with the liposomal formulation induced the most effective inhibition of tumor growth (Figure 1A,B). Moreover, the antitumor actions of both combined treatments might suggest that SIM acted as a sensitizer for tumor cells to 5-FU, as previously reported,11,12,42,43 this effect being enhanced by its intratumor accumulation by virtue of passive tumor-targeting properties of LCL. As our previous study described the antiangiogenic proteins effects of LCL-SIM, we evaluated the impact of combined therapy on the production of angiogenic proteins. Thus, the results indicated that LCL-SIM + LCL-5-FU had the greatest impact on these proteins, followed by simultaneous treatment with liposomal formulations (Figure 4 and Table 1A). Moreover, previous studies showed the essential role of angiogenesis in colorectal cancer progression and, therefore, the susceptibility of this type of cancer to antiangiogenic therapies has been established.44 Thus, anti-VEGF therapy based on bevacizumab was proved to be first- and
second-line treatment combined with chemotherapy for colorectal cancer.\textsuperscript{44} It is known that overexpression of VEGF was associated with the progression of colorectal cancer.\textsuperscript{45} In addition to this finding, our recent study associated the failure of the therapy based on i.v. treatment with LCL-5-FU alone in C26 colon carcinoma in vivo with its stimulatory effect on the intratumor production of VEGF.\textsuperscript{8} Nevertheless, previous data have shown that several anti-VEGF therapies have limited effects on cancer progression as a result of compensatory upregulation of other proangiogenic proteins in the tumor microenvironment.\textsuperscript{46,47} In line with these findings, our data indicated

### Table 1

Effects of combined liposomal treatments on the production level of (A) proangiogenic proteins and (B) antiangiogenic proteins in C26 colon carcinoma

| Proangiogenic proteins | Percentage of inhibition (−) and stimulation (+) of proangiogenic proteins levels in C26 colon carcinoma treated with LCL-SIM + LCL-5-FU compared to control group | Percentage of inhibition (−) and stimulation (+) of proangiogenic proteins levels in C26 colon carcinoma treated with LCL-SIM + LCL-5-FU compared to control group |
|------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| G-CSF                  | $-71.31075 \pm 2.110$ (****)                                                                 | $-78.342 \pm 0.196$ (****)                                                                 |
| GM-CSF                 | $-39.1151 \pm 5.128$ (**)                                                                     | $-66.987 \pm 1.155$ (****)                                                                     |
| M-CSF                  | $-37.807 \pm 0.0934$ (**)                                                                     | $-66.076 \pm 0.311$ (****)                                                                     |
| IGF-II                 | $-27.893 \pm 7.367$ (ns)                                                                      | $-51.615 \pm 0.762$ (****)                                                                     |
| IL-1α                  | $-34.726 \pm 0.514$ (**)                                                                     | $-66.325 \pm 0.747$ (****)                                                                     |
| IL-1β                  | $-89.042 \pm 0.871$ (****)                                                                   | $-80.914 \pm 0.907$ (****)                                                                   |
| IL-6                   | $-51.675 \pm 1.822$ (****)                                                                   | $-81.723 \pm 2.951$ (****)                                                                   |
| IL-9                   | $-55.253 \pm 1.604$ (****)                                                                   | $-77.087 \pm 1.555$ (****)                                                                   |
| IL-12p40               | $-58.635 \pm 1.128$ (****)                                                                   | $-85.232 \pm 0.772$ (****)                                                                   |
| IL-13                  | $-69.761 \pm 3.277$ (****)                                                                   | $-69.523 \pm 0.699$ (****)                                                                   |
| TNF-α                  | $-67.470 \pm 1.464$ (****)                                                                   | $-81.717 \pm 2.838$ (****)                                                                   |
| MCP-1                  | $-7.635 \pm 6.645$ (ns)                                                                      | $-61.060 \pm 1.243$ (****)                                                                   |
| Eotaxin                | $-84.536 \pm 1.737$ (****)                                                                   | $-98.142 \pm 0.897$ (****)                                                                   |
| FasL                   | $-48.674 \pm 0.502$ (****)                                                                   | $-78.364 \pm 2.686$ (****)                                                                   |
| bFGF                   | $-36.4395 \pm 2.053$ (**)                                                                   | $-84.525 \pm 0.530$ (****)                                                                   |
| VEGF                   | $-96.4125 \pm 0.875$ (****)                                                                  | $-96.581 \pm 1.157$ (****)                                                                  |
| Leptin                 | $-83.6707 \pm 4.446$ (****)                                                                  | $-89.229 \pm 1.068$ (****)                                                                  |
| TPO                    | $-3.782 \pm 1.990$ (ns)                                                                      | $-52.517 \pm 7.253$ (****)                                                                   |

| Antiangiogenic proteins | Percentage of inhibition (−) and stimulation (+) of anti-angiogenic proteins levels in C26 colon carcinoma treated with LCL-SIM + LCL-5-FU compared to control | Percentage of inhibition (−) and stimulation (+) of anti-angiogenic proteins levels in C26 colon carcinoma treated with LCL-SIM + LCL-5-FU compared to control |
|------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| TIMP-1                 | $-31.528 \pm 6.598$ (‘)                                                                       | $-82.233 \pm 2.105$ (****)                                                                  |
| TIMP-2                 | $-82.814 \pm 1.322$ (****)                                                                   | $-94.071 \pm 2.138$ (****)                                                                  |
| PF-4                   | $+29.104 \pm 22.306$ (ns)                                                                    | $-40.718 \pm 4.857$ (‘)                                                                     |
| IL-12p70               | $+1.448 \pm 1.876$ (ns)                                                                      | $-37.415 \pm 1.477$ (‘)                                                                     |
| IFN-γ                  | $-57.830 \pm 6.087$ (****)                                                                   | $-69.670 \pm 0.526$ (****)                                                                   |
| MIG                    | $-76.142 \pm 5.272$ (****)                                                                   | $-93.643 \pm 0.821$ (****)                                                                   |

Protein levels after combined treatments are compared to control levels of the same proteins. Results are expressed as a percentage of the average inhibition (−) or stimulation (+) ± SD of 2 independent measurements. Statistical differences were evaluated by using 2-way ANOVA with Bonferroni's correction for multiple comparisons.

$P > .05$; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

Abbreviations: 5-FU, 5-fluorouracil; bFGF, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; FasL, Fas ligand; IFN-γ, γ-interferon; IGF-II, insulin growth factor-II; IL, interleukin; LCL, long-circulating liposome; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; MIG, monokine induced by IFN-γ; ns, not significant, PF-4, platelet factor-4; SIM, simvastatin; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor-α; TPO, thrombopoietin; VEGF, vascular endothelial growth factor.
that both combined liposomal treatments almost totally reduced not only the intratumor levels of VEGF but also the production of other important proangiogenic molecules, such as eotaxin and leptin (Table 1A). Moreover, our present data showed that LCL-SIM + LCL-5-FU also induced strong inhibition of bFGF production (by 85%, \( P < .0001 \)) while the combined therapy based on LCL-SIM + LCL-5-FU only slightly inhibited the expression of bFGF (30%, \( P < .05 \)) in these tumors. This finding might be correlated with the impact of these treatments on the neovascularization of C26 colon carcinoma tumors (Figure 6). Thus, our data have shown that LCL-SIM + LCL-5-FU slightly increased the expression levels of CD31, a marker for proliferating endothelial cells, as a result of inefficient suppression of proangiogenic protein bFGF in the tumor microenvironment.\(^46,47\) Additionally, LCL-SIM + LCL-5-FU reduced intratumor formation of new blood vessels as a consequence of its decelerating effects on the production of all proangiogenic molecules (Figure 6 and Table 1A).

In addition to these important antitumor effects, the moderate antioxidant actions of LCL-SIM + LCL-5-FU (Figure 5A-C) might contribute to the amplitude of the anticancer activity of this treatment in C26 colon carcinoma-bearing mice.

To link the antiangiogenic actions of both combined liposomal treatments with their impact on tumor aggressiveness, the intratumor production of essential markers for C26 colon carcinoma progression, such as the inflammatory transcription factor NF-κB and important regulators of apoptosis in response to 5-FU therapy (Bcl-2 and Bax),\(^29,30,48\) were assessed. Thus, it seems that neither of the combined liposomal treatments induced NF-κB activation (Figure 3) associated with colorectal cancer progression,\(^69\) but strongly and similarly lowered the intratumor production ratio of
Bcl-2/Bax (Figure 2A-C). In line with our studies, Violette et al.\textsuperscript{28} previously reported the involvement of high levels of Bcl-2 and low production of Bax in the resistance of colon cancer cells to 5-FU therapy.

Nevertheless, our data regarding the assessment of invasive and metastatic capacity of tumors after combined liposomal treatments suggested that LCL-SIM + LCL-5-FU did not significantly affect the expression of MMP-2 (Figure 7A-C), whereas LCL-SIMantelCL-5-FU stimulated the production and activity of this key player in colorectal cancer metastasis.\textsuperscript{41} This major drawback of LCL-SIMantelCL-5-FU might be directly connected with almost total depletion of tissue inhibitor of metalloproteinase (TIMP)-1 and -2 levels\textsuperscript{50} in C26 carcinoma, while simultaneous treatment with LCL-SIM + LCL-5-FU still preserved the production of TIMP-1 in these tumors (Table 1B). Moreover, LCL-SIM + LCL-5-FU did not affect the production of other antiangiogenic/anti-inflammatory proteins, PF-4 and IL-12p70, in the C26 colon carcinoma microenvironment (Table 1B), which could also contribute the beneficial outcome of this treatment. To support these data, the new concept of targeting angiogenesis, so-called vascular normalization, is based on the suppression of proangiogenic factors and the preservation of antiangiogenic molecules in the tumor microenvironment.\textsuperscript{51,52} Thus, the restoration of intratumor vessel structure and function could increase blood perfusion and finally benefit other cancer therapies (including chemotherapy).

In addition to the effects of LCL-SIM + LCL-5-FU on antitumor protein production, the slight increase in tumor neovascularization (Figure 6) might prove the antitumor efficacy of this therapy over sequential treatment with LCL-SIM and LCL-5-FU in C26 colon carcinoma-bearing mice.

Together, our data suggested that both combined therapies based on the treatment with LCL-SIM and LCL-5-FU strongly inhibited the growth of C26 murine colon carcinoma in vivo through strong antiangiogenic actions on the tumor microenvironment. Although sequential combined liposomal treatment exerted the strongest antitumor activity as a result of almost total depletion of angiogenic protein production in the C26 carcinoma microenvironment, the prognosis of therapy based on simultaneous treatment with liposomal forms is superior to the sequential regimen. Thus, C26 tumors treated with LCL-SIMantelCL-5-FU had higher invasive capacity compared with tumors treated with LCL-SIM + LCL-5-FU. The beneficial outcome of this therapy on C26 colon carcinoma evolution might be connected to its suppressive actions on proangiogenic protein production balanced by preservation of the expression of some antiangiogenic molecules in the tumor microenvironment that could contribute to the restoration of normal vasculature and finally ensuring the success of chemotherapy based on liposomal delivery of 5-FU in these tumors.
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FIGURE 7 Effects of liposomal combined therapies on the activity of MMP-2 in tumor tissue. A, Gelatin zymography gel. B, Percentage of expression levels of pro-MMP-2 in tumor lysates after liposomal treatments compared to control. C, Percentage of expression levels of the active form of MMP-2 in tumor lysates after liposomal treatments compared to control. Results represent the mean of percentage MMP-2 activity of duplicate measurements ± SD. *P < .05; **P < .01; ****P < .0001. ns, not significant (P > .05). Treatment groups: Control, untreated (tumors in mice treated with PBS); LCL-SIM + LCL-5-FU, 5 mg/kg simvastatin delivered by long-circulating liposomes (LCL-SIM) and 1.2 mg/kg 5-fluorouracil delivered by LCL (LCL-5-FU) at days 8 and 11 after tumor cell inoculation; LCL-SIMateLCL-5-FU, 5 mg/kg LCL-SIM at days 7 and 10, and 1.2 mg/kg LCL-5-FU at days 8 and 11 after tumor cell inoculation, respectively.

DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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