BABEȘ-BOLYAI UNIVERSITY CLUJ-NAPOCA
Faculty of Biology and Geology
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

TARGETING OF THE PROTUMOR MICROENVIRONMENTAL PROCESSES BY LIPOSOMAL SIMVASTATIN-BASED TREATMENTS IN COLORECTAL CANCER

~Summary~

Scientific Supervisor
PROF. DR. NICOLAIE DRAGOȘ

PhD Candidate
LAVINIA LUCA (LUPUȚ)

CLUJ-NAPOCA
2019

BABEȘ-BOLYAI UNIVERSITY CLUJ-NAPOCA

Faculty of Biology and Geology

DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

TARGETING OF THE PROTUMOR MICROENVIRONMENTAL PROCESSES BY LIPOSOMAL SIMVASTATIN-BASED TREATMENTS IN COLORECTAL CANCER

~Summary~

Scientific Supervisor

PROF. DR. NICOLAIE DRAGOȘ

PhD Candidate

LAVINIA LUCA (LUPUȚ)

CLUJ-NAPOCA
2019

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AUTC – areas under the tumor growth curves
bFGF – basic fibroblast growth factor
CCL2 – chemokine (C-C motif) ligand 2⇔**MCP-1 –** monocyte chemotactic protein-1
CHO – cholesterol
CRC – colorectal cancer
DPPC – 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
FasL – Fas ligand
G-CSF – granulocyte-colony stimulating factor
GM-CSF – granulocyte-macrophages colony-stimulating factor
IFN-γ – interferon-γ
IGF-II – insulin-like growth factor II
IL – interleukin
IL-12p40 – IL-12 subunit p40
IL-12p70 – IL-12 subunit p70
LCL – long-circulating liposomes
LCL-SIM – long-circulating liposomal simvastatin
LCL-5-FU – 5-FU encapsulated in LCL
MDA – malondialdehyde
MIG – monokine induced by IFN-γ
MMP – matrix metalloproteinase
MPEG-2000-DSPE – N-(carbonyl-methoxypolyethylenglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Na-salt)
PF-4 – platelet factor 4
ROS – reactive oxygen species
SIM – simvastatin
TAMs – tumor-associated macrophages
TGF-β – transforming growth factor-β
TIMP –tissue inhibitor of metalloproteinases
TNF-α – tumor necrosis growth factor α
TPO – thrombopoietin
VEGF – vascular endothelial growth factor
5-FU – 5-fluorouracil

KEYWORDS: colorectal cancer, tumor-associated macrophages, long-circulating liposomes, simvastatin, 5-fluorouracil.

Chapter I. Introduction: Tumor microenvironment and colorectal cancer (CRC) therapy

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men worldwide, respectively the second commonly occurring type of cancer in women, counting approximately 1.8 million cases in 2018 (Bray et al. 2018).

Even if the anticancer therapies are mainly focussed on the genetically degenerated cells, the surrounding cells and components are active participants in the proliferation, angiogenesis and metastasis of the tumor cells. Thus, understanding the mechanisms underlying the relationship between immune infiltrated cells and tumor cells is essential in order to develop a new therapy for CRC. Besides of the involvement of the infiltrated immune cells, especially tumor-associated macrophages (TAMs), in the main processes-driven tumor development, they contribute to the therapy resistance. Therefore, the investigation of the mechanisms by which TAMs favor the proliferation of C26 colon carcinoma cells, is one of the subjects of this thesis.

Conventional CRC therapies usually target tumor cells, which are genetically unstable and the target of the drug could be modified after acquiring mutations of tumor cells. Thus, targeting the protumor processes mediated by tumor microenvironment constitute an alternative to this drawback of the conventional chemotherapy. Moreover, chemoresistance acquired by colon cancer cells and limited number of anticancer licenced drugs, highlight the necessity of developing new therapeutic agents to act as sensitizers for cancer cells to the action of the conventional chemotherapeutic agents, enhancing their antitumor effects.

In the present study, the efficacy of monotherapy in C26 colon carcinoma-bearing mice with simvastatin (SIM) incorporated in long-circulating liposomes (LCL-SIM) – as a novel therapeutic agent in CRC was assessed. Also, in the third study was evaluated the potential of LCL-SIM as sensitizer and synergistic antitumor agent in a combined therapy with 5-fluorouracil (5-FU) encapsulated in LCL (LCL-5-FU) on C26 colon carcinoma model *in vivo*.

1. Tumor microenvironment

Tumor microenvironment describes the non-malignant cells (stromal cells) present in the tumor, along with growth factors, signalling molecules, proteolytic enzymes, which interplay with tumor cells, favoring the tumor growth, invasion, metastasis, providing protection of tumor from the host immune system, and contribute to the chemoresistance (Swartz et al. 2012). These important roles of the tumor microenvironment in the development of tumors, pointed out to the

necessity of therapeutic targeting of the tumor microenvironment components, not only targeting the tumor cells.

In the tumor microenvironment are found different types of cells along with tumor cells, such as immune cells, fibroblasts, pericytes, adipocytes, endothelial cells and lymphatic cells (Balkwill F. R. et al. 2012). Stromal cells contribute to the hallmarks of cancer, referring to signalling involved in proliferation, anti-apoptotic and pro-angiogenic effects, evading growth suppressors and immune system, promoting immortality, invasion and metastasis and reprogramming energy metabolism (Hanahan and Coussens 2012).

Among infiltrating immune cells TAMs are most abundant at the tumor site and they are key-players in the inflammation related with cancer, being involved in protumor functions such as proliferation of tumor cell, tumor angiogenesis, tumor metastasis and immunosuppressive activity (Noy and Pollard 2014, Solinas et al. 2009).

Macrophages are a prominent component of inflammation and they can be either classically activated – in a phenotype known as M1 macrophages, a pro-inflammatory and anti-tumor phenotype, or alternative activated – in a M2 phenotype or close to M2 phenotype, an anti-inflammatory and protumor type. Macrophages are plastic cells and adaptable to microenvironmental stimuli, thus adopting different phenotype either as a response to cytokines and chemokines produced by cells (tumor cells) or as a response to products released by the pathogens at the site of inflammation.

TAMs are considered as a distinct M2 phenotype of polarization due to protumor functions exerted, such as matrix deposition and tissue remodelling, wound healing and tissue repair, promotion of angiogenesis, suppressive actions on adaptive immunity and production of growth and survival factors (Sica et al. 2006). Many studies associate the accumulation of TAMs in tumor tissue with a poor prognosis for patients (Noy and Pollard 2014). Therefore, TAMs represent a major interest for cancer immunotherapy.

TAMs arrived at the tumor site, undergo important phenotypic changes induced by cues from tumor microenvironment, finally adopting a protumor phenotype and release a broad spectrum of cytokines, chemokines and growth factors with a great impact on tumor angiogenesis inducing angiogenic switch (Albini et al. 2005, Lin and Pollard 2007), on tumor metastasis through matrix remodelling (Condeelis and Pollard 2006, Pollard 2004), and on immunosuppression, thus preventing the elimination of tumor cells by the immune system (Balkwill F. and Mantovani 2001, Mantovani et al. 2002, Sica et al. 2000).

2. Therapies administered in CRC

Therapy of CRC is represented by local therapies such as surgery, radiotherapy, conventional chemotherapy and targeted therapies (Grothey and Sargent 2016).

Conventional chemotherapy of CRC is based on different classes of chemotherapeutic agents such as antimetabolites (fluoropyrimidines), inhibitors of topoisomerase (irinotecan), and platinum derivates (oxaliplatin). These chemotherapeutic agents are administered alone, combined with each other or in combination with other substances that potentiate their therapeutic effects.

Fluoropyrimidines are a class of antimetabolites that act by being incorporated in DNA or ARN. **5-fluorouracil (5-FU)** is a fluoropyrimidine which is converted intracellular in three active metabolites disrupting the synthesis of ARN and inhibit the thymidylate synthase which is necessary in DNA synthesis (Longley et al. 2003).

Chemoresistance is one of the major limitation of the conventional chemotherapy that cause failure of treatments, and tumor heterogeneity is one of the causative factors (Cazin et al. 1992). In CRC chemotherapy, resistance of colon cancer cells to administration of 5-FU is one of the examples of chemoresistance (Violette et al. 2002). Combination of 5-FU with other drugs that sensitises colon cancer cells and enhance 5-FU cytotoxic effects is one of the approaches used to overcome chemoresistance in CRC therapy. Another solution to overcome this drawback is the development of new therapeutic agents that target processes that promote CRC development, not only malignant cells which could rapidly acquire resistance to cytotoxic agent. Statins have the potential to meet these requirements.

Statins are commonly used as lipid-lowering agents with a significant contribution in the prevention of cardiovascular disease, acting as competitive inhibitors of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase, a rate-limiting enzyme in the mevalonate pathway, which is involved in cholesterol biosynthesis (Alberts et al. 1980, Sirtori 2014). Apart from hypocholesterolemiant properties, statins were found to exert pleiotropic effects (Bellosta et al. 2000). Among them statins were found to have antineoplastic properties, such as inhibition of tumor growth and angiogenesis, induction of apoptosis and anti-invasive effects in tumors (Hindler et al. 2006). SIM is a FDA approved lipid-lowering agent. Moreover, several studies reported that SIM possess antitumor properties (Alupei et al. 2015, Gopalan et al. 2013, Schointuch et al. 2014, Stine et al. 2016). In a study conducted by Kodach et al., SIM was reported to increase the chemosensitivity of CRC cells to 5-FU actions (Kodach et al. 2011).

Despite their potential as anticancer agents, necessary doses of statins in order to achieve antitumor effects are substantially higher than those typically used as a treatment for

hypercholesterolemia in clinical practice. The side-effects induced by statins used in high doses, with the purpose to obtain antitumor effects, were reported in several studies (Knox et al. 2005, Thibault et al. 1996) as statin-associated myopathy, anorexia, diarrhea, fatigue and abdominal pain (Gazzerro et al. 2012). A strategy to overcome these drawbacks could be tumor-targeted delivery of statins, which could limits the side-effects on healthy tissues and could increase the doses of statins that reach at the tumor site (Licarete et al. 2015). One of the nanocarrier systems that could be used as tumor-targeted delivery of statins are liposomes (Askarizadeh et al. 2019).

Nanoparticle drug delivery take advantage of architectural particularities of tumor vasculature. Tumor blood vessels present pores with size range between 10 to 1000 nm, and this highly permeable areas allow the tailor made nanocarriers to passively extravasate in the interstitial space (Torchilin 2000). In addition to leakage tumor blood vessels, poor lymphatic drainage facilitate the accumulation of nanocarriers in tumors and this phenomenon is named as "Enhanced Permeability and Retention (EPR) effect" (Maeda et al. 2009, Matsumura and Maeda 1986). In order to reach the tumor site, nanocarriers loaded with anticancer drugs have to travel through bloodstream and this exposed them to reticuloendothelial system (RES) which rapidly cleared them. To avoid recognition and opsonisation, nanocarriers have to be coat in inert polymers to become so called "stealth" nanocarriers. Also, in order to extravasate through leaky tumor blood vessels, nanocarriers have to be size-tailored in a range of 10-200 nm (Haley and Frenkel 2008).

Among nanocarriers used for drug delivery in cancer therapy, liposomes – spherical vesicles of phospholipid bilayer, possess the capacity to encapsulate both hydrophilic (in aqueous phase) and lipophilic drugs (in phospholipid bilayer) (Malam et al. 2009).

Long-circulating liposomes are used for both preclinical studies and clinical practice (Gabizon 2001). They are liposomes grafted with polymers such as PEG (polyethylene gylcol) to avoid opsonisation with serum proteins and recognize by RES, and therefore they are named "stealth" liposomes. The prolonged time in the circulation allows LCL to accumulate at the tumor site and extravasate in the interstitial space. In the present work were used LCL, as carriers for antitumor agents SIM and 5-FU.

Few formulation of liposomes are already approved as treatment for cancer, such as doxyl (PEG-LCL with doxorubicin). For advanced and metastatic colorectal cancer two liposomal formulation are in phase II of clinical trials, Aroplatin™ (platinum analogue similar to oxaliplatin encapsulated in multilamellar liposomes) and LE-SN38 (liposomal irinotecan) (Bulbake et al. 2017). In a preclinical study LCL-oxaliplatin induced apoptosis of CRC cells compared to free form (Yang et al. 2011).

Chapter II. General objectives

The aim of this thesis was to develop a tumor-targeted therapy based on long-circulating liposomal simvastatin that is able to modulate protumor processes supported by the colon carcinoma microenvironment.

- The **first objective** of this thesis was to investigate the interaction between TAMs and C26 colon carcinoma cells that can be exploited for future anticancer therapies.
- The **second objective** of this study was to test the antitumor activity of LCL-SIM on colon carcinoma microenvironment as potential future therapy of CRC.
- The **third objective** of the thesis was to investigate whether LCL-SIM could be used to enhance the antitumor activity of LCL-5-FU in murine colon carcinoma *in vivo*.

Chapter III. Tumor-associated macrophages favor C26 murine colon carcinoma cell proliferation in an oxidative stress–dependent manner

[This chapter was published as: Luput L, Licarete E, Sesarman A, Patras L, Alupei MC, Banciu M. Tumor-associated macrophages favor C26 murine colon carcinoma cell proliferation in an oxidative stress–dependent manner. Oncol Rep. 2017; 37(4):2472-2480. doi: 10.3892/or.2017.5466. A part of this section contains results partial presented in the dissertation paper.]

1. Introduction

The role of TAMs in colon carcinoma development is still controversial. Therefore, the present study aimed to investigate the TAMs-driven processes that can affect colon cancer cell proliferation. To achieve this purpose, murine macrophages were co-cultured with C26 murine colon carcinoma cells at a cell density ratio that approximates physiological conditions of colon carcinoma development *in vivo*. In this respect, the effects of TAMs-mediated angiogenesis, inflammation, and oxidative stress on the proliferative capacity of C26 murine colon carcinoma cells were studied. To gain insights into the TAMs-driven oxidative stress, NADPH oxidase – the main pro-oxidant enzyme in macrophages, was inhibited. Our data suggested that the stimulatory effects of TAMs on C26 cell proliferation might be related mainly to their pro-oxidant actions exerted by NADPH oxidase activity, which maintains the redox status and the angiogenic capacity of tumor microenvironment. Additionally, the anti-inflammatory and pro-angiogenic effects of TAMs on tumor cells were found to create a favorable microenvironment for C26 colon carcinoma development and progression. In conclusion, our data proved the protumor role of TAMs in colon carcinoma development in an oxidative stress-dependent manner that potentiates angiogenic

capacity of tumor microenvironment. These data can offer valuable information for future tumor-targeted therapies based on TAMs "re-education" strategies.

2. Materials and methods

- **C26 murine colon carcinoma cells** (Cell Lines Service GmbH, Eppelheim, Germany) were cultured in RPMI-1640 medium (Lonza, Group AG, Basel, Switzerland), supplemented with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), as monolayer at 37^0 C in a humidified atmosphere containing 5% CO_2.
- **Co-culture of C26 tumor cells and macrophages** was prepared by seeding of C26 tumor cell suspensions on intraperitoneal murine macrophages monolayer (freshly harvested from peritoneal cavity of Balb/c mice (Cantacuzino Institute, Bucharest, Romania) and cultivated at a density ratio of 4:1 between C26 cells and macrophages, that provides an approximation of physiological conditions of colon carcinoma development *in vivo* (Herbeuval et al. 2004). Experiments were performed according to the national regulations and were approved by the university animal experiments ethical committee (registration no.31375/06.04.2015).
- The **proliferative capacity** of C26 colon carcinoma cells was evaluated on standard culture of C26 cells ($1x10^3$/well) as well as on co-culture of C26 cells with murine peritoneal macrophages (density ratio of 1:4). The cells were seeded into 96-well plates and incubated for 48h. The proliferation rate was expressed as number of absorbance units per hours of incubation (James et al. 2015) and tested by using **ELISA BrdU-colorimetric immunoassay** (Roche Applied Science, Penzberg, Germany).
- **Cells cultures were lysed** with **lysis buffer** for **whole cell lysates** containing protease inhibitor cocktail tablets (Complete, Roche Diagnostics GmbH, Mannheim, Germany) and for nuclear extraction, cell cultures were lysed with **extraction buffer** containing protease and phosphatase inhibitor tablets (Roche Diagnostics GmbH, Mannheim, Germany) and were obtained **nuclear fractions**. The protein concentration was determined by the Bradford assay (Sigma-Aldric, Germany) (Bradford 1976).
- The production levels of inflammatory/angiogenic proteins in cells cultivated under both culture conditions were investigated by performing a screening for 24 proteins involved in angiogenesis using **RayBio® Mouse Angiogenic Cytokine Antibody Array ki**t (RayBiotech Inc., Norcross, GA, USA) as described previously (Banciu et al. 2006).
- To determine the oxidative stress levels in different culture conditions was quantified the amount of malondialdehyde (MDA) through **high-performance liquid chromatography**

(HPLC) in lysates obtained from C26 cells as well as C26 cells and macrophages (Karatas et al. 2002).

- To assess the **total anti-oxidant nonenzymatic capacity** was used a **spectrophotometric** method described by Erel (Erel 2004) and **catalytic activity of catalase** was assessed via **spectrophotometric** method described by Aebi (Aebi 1984).
- To assess indirectly the role of macrophage NADPH oxidase in the generation of physiological levels of reative oxygen species (ROS) in co-culture microenvironment, cells were treated with **300 µM of apocynin**, a **NADPH oxidase inhibitor** (Santa Cruz Biotechnology, USA) for 48h. Moreover, to determine whether NADPH oxidase-generated oxidative stress can modulate C26 cell **proliferation**, **tumor angiogenesis, and inflammation**, all assays described above were performed after co-culture incubation with apocynin.
- Data from different experiments are expressed as **mean ± standard deviation (SD)**. The differences between protumor processes under standard and co-culture conditions were evaluated by using **unpaired *t* test**. The differences between the production of angiogenic proteins in cells from standard culture and co-culture were analyzed by **two-way ANOVA with Bonferroni correction for multiple comparisons**. Correlations between different parameters were evaluated by using **Pearson correlation coefficient, *r***. All statistical analyses were performed by using **GraphPad Prism version 6 for Windows**, GraphPad Software (San Diego, CA). A ***P* value** lower than 0.05 was considered significant.

3. Results and discussion

3.1. TAMs stimulate proliferation of C26 colon carcinoma cells

The effect of TAMs on C26 cell proliferation after 48h of incubation was evaluated by comparing the proliferation rate of C26 murine carcinoma cells cultivated alone with the proliferation rate of C26 cells co-cultivated with macrophages (**Fig. 1**) at a cell density ratio of 4:1 (C26 cells: macrophages) that ensures an approximation of physiological conditions of colon carcinoma development *in vivo* (Herbeuval et al. 2004) . Our data showed that C26 cells proliferated more rapidly in the presence of macrophages (by 28%, $P< 0.01$*)* than those cultivated alone. Therefore the involvement of TAMs in the coordination of main processes responsible for C26 cell proliferation was further investigated.

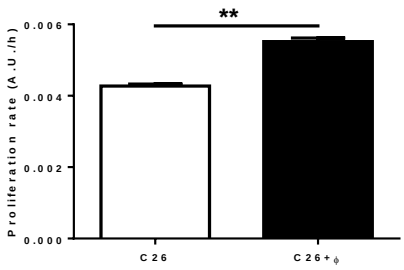

Figure 1. The effects of TAMs on C26 cell proliferation. The proliferation rate of C26 cells in standard culture as well as in the presence of TAMs was assessed after 48h of incubation with culture media. Data are shown as mean of proliferation rate, which represent the absorbance units (A.U.) per hours of incubation ± SD; n=5 measurements. C26 = C26 murine colon carcinoma cells; C26+ϕ = C26 colon carcinoma cells co-cultured with intraperitoneal murine macrophages (ϕ) at a cell density ratio of 4:1 (C26 cells: macrophages). To compare the rate of C26 cell proliferation in standard culture with C26 cell proliferation in co-culture with macrophages unpaired t-test was used and the P value are indicated as **, $P<0.01$.

3.2. The angiogenic effects of TAMs on C26 colon carcinoma cells

To assess the link between proliferative effects of TAMs on C26 cells and TAMs-driven angiogenesis, 24 proteins involved in this process were screened by protein array in C26 cell lysates and also in lysates obtained after C26 cell cultivation with TAMs for 48h.

The results presented in **Figure 2A** have shown an overall enhancement of the production of the angiogenic proteins with 112% in the cell lysates obtained under co-culture conditions compared to their production in C26 cells cultivated alone.

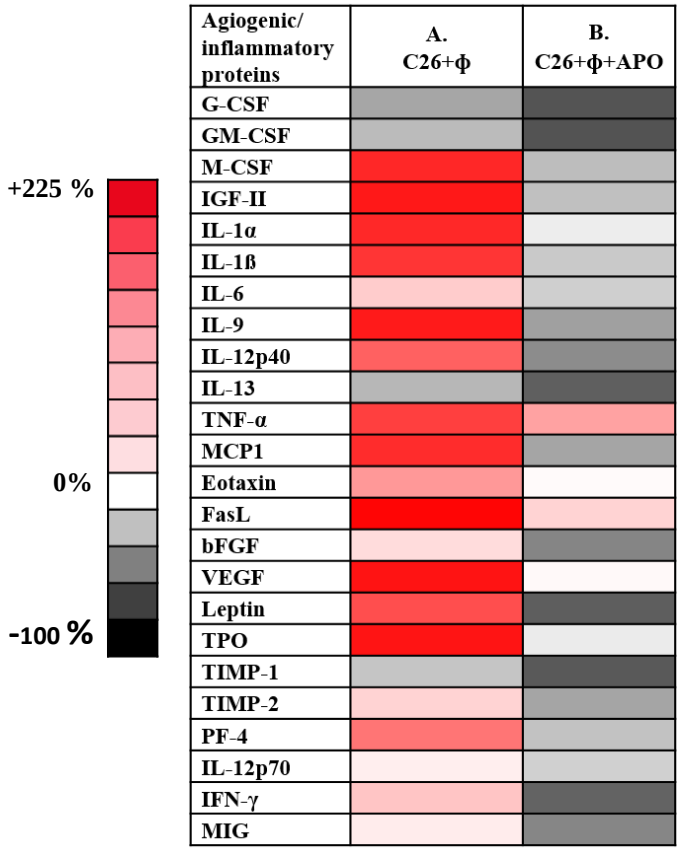

Figure 2. A. The effects of TAMs on the production of angiogenic proteins in the co-culture model. **B.** The involvement of TAMs NADPH-oxidase in the production of angiogenic proteins in the co-culture model**.** Data are expressed as average % of reduction of protein levels ranging from 0% (white) to -100% (black) or stimulation (+) of production of proteins ranging from 0% (white) to +225% (red) compared with the levels of the same proteins in monoculture of C26 cells. **C26+ϕ** – C26 cells co-cultured with macrophages; **C26+ϕ+APO** – C26 cells co-cultured with macrophages in the presence of apocynin for 48h.

3.3. The pro-oxidant effects of TAMs on C26 colon carcinoma cells

To determine whether the proliferative activity of TAMs on C26 colon carcinoma cells is related to their modulatory effects on oxidative stress, the levels of a general oxidative stress marker -MDA as well as catalytic activity of catalase and production of nonenzymatic anti-oxidant systems were assessed and shown in **Fig. 3A-C**. Moreover, the involvement of the main pro-oxidant enzyme in macrophages, NADPH oxidase (Pick 2014) in the maintenance of the proliferative levels of oxidative stress in tumor microenvironment has been addressed (**Fig. 4A-D**). Our results proved that TAMs are important in the generation of tumor oxidative stress since MDA levels were significantly enhanced (2-fold higher, $P<0.05$) in the lysates from co-culture of C26 cells and macrophages compared to their levels in C26 cell lysates (**Fig. 3A**). These data are also supported by the results regarding the higher amount of nonenzymatic anti-oxidant systems in co-culture lysates compared to C26 cell lysates (2 times higher production in co-culture lysates compared to their production C26 cell lysates, $P=0.04$) (**Fig. 3C**). As catalase activity was only slightly increased and not statistically significant in the presence of macrophages (**Fig. 3B**, $P=0.61$), it seems that the main protective mechanism against high levels of ROS in the co-culture model is mediated rather by nonenzymatic anti-oxidant systems than by catalase.

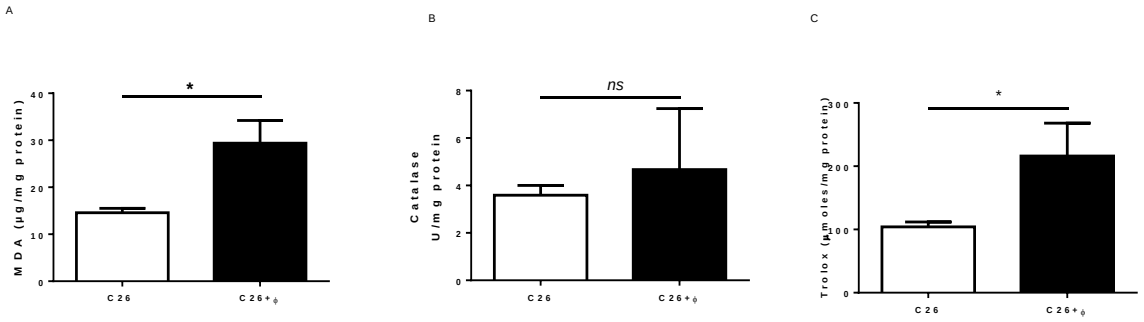

Figure 3. The effects of TAMs on the oxidative stress generated in the co-culture milieu.
A. Malondialdehyde (MDA) concentration in the cell lysates obtained from standard culture of C26 murine colon carcinoma cells (C26) and from co-culture of C26 cells with intraperitoneal macrophages (C26+ϕ) after 48h of incubation. **B**. Catalytic activity of catalase in the cell lysates obtained from standard culture of C26 murine colon carcinoma cells (C26) and from co-culture of C26 cells with intraperitoneal macrophages (C26+ϕ) after 48h of incubation. **C.** Total nonenzymatic anti-oxidant systems levels in the cell lysates obtained from standard culture of C26 murine colon carcinoma cells (C26) and from co-culture of C26 cells with intraperitoneal macrophages (C26+ϕ) after 48h of incubation. To compare the levels of MDA and nonenzymatic anti-oxidant defense systems and catalase activity in C26 cell standard culture and in cell co-culture unpaired *t*-test was used and the *P* value is indicated as follows: *ns* $P>0.05$; *$P<0.05$. The results represent the mean ± SD of two independent measurements.

To evaluate further the involvement of the TAMs-expressed NADPH-oxidase in the maintaining the C26 carcinoma oxidative stress TAMs co-cultivated with C26 cells were incubated

for 48h with an inhibitor of this enzyme (300 μM apocynin) and the oxidative stress parameters presented above were evaluated (**Fig. 4A-C**). As negative control standard culture of C26 cells treated with apocynin was used. No effects on the levels of MDA and nonenzymatic anti-oxidants and catalase activity were noted after incubation of C26 cells with apocynin (data not shown). Our data proved clearly the principal role of NADPH oxidase in the supporting the C26 carcinoma oxidative stress as the MDA levels in cell lysates obtained from co-culture treated with apocynin were reduced up to its levels in C26 cells cultivated alone (**Fig. 4A** compared to **Fig. 4A**, $P<0.08$). Neutralization of oxidative stress in co-culture microenvironment via NADPH oxidase suppression is also accompanied by the reduction of the production of nonenzymatic anti-oxidant systems (5 times lower amount after co-culture treatment with apocynin than their amount in cell co-culture with active NADPH oxidase, $P=0.0027$) (**Fig. 4C**).

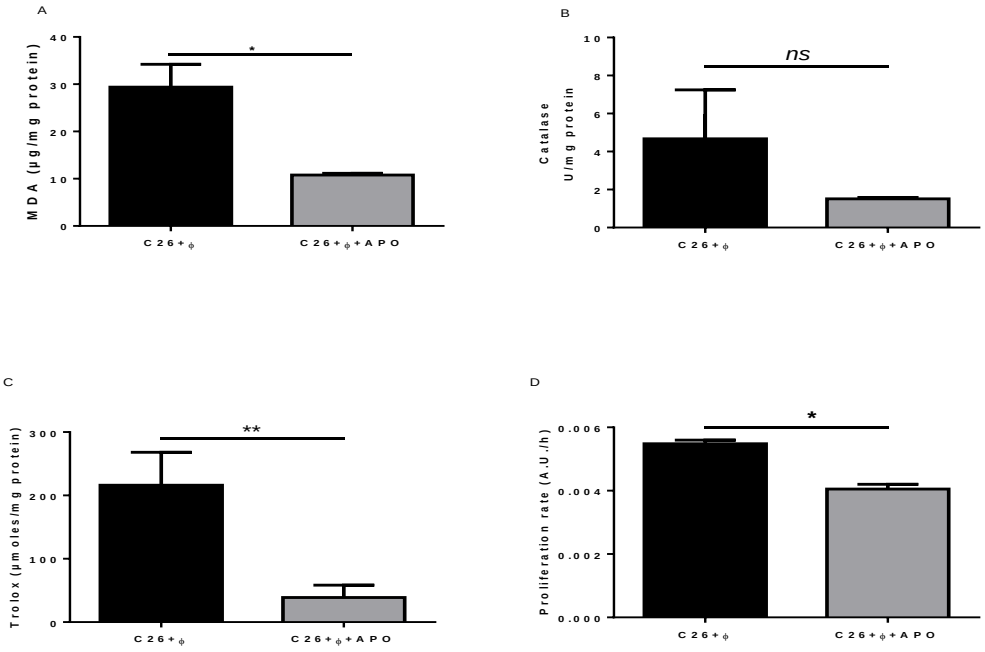

Figure 4. The involvement of TAMs NADPH oxidase in the oxidative stress generated in the co-culture of C26 murine colon carcinoma cells and macrophages. C26 cells co-cultured with macrophages in the absence of apocynin (C26+ϕ) and C26 cells co-cultured with macrophages in the presence of apocynin for 48h (C26+ϕ+APO) were compared with regard to the levels of MDA (**A**), catalytic activity of catalase (**B**), total nonenzymatic anti-oxidant systems amount (**C**), and proliferation rates of C26 cells (**D**). Data are shown as mean ± SD; n=3 measurements for MDA levels, catalase activity, and total nonenzymatic anti-oxidant systems assessment and n=6 replicates for proliferation rate. Unpaired *t*-test was used for statistical comparison and the *P* values are indicated as follows: *ns* $P>0.05$; * $P<0.05$; ** $P<0.01$.

Moreover, to link the role of NADPH oxidase to stimulatory effects of TAMs on C26 cells, the proliferation of these cancer cells under co-culture conditions in the presence of apocynin was tested. Our data demonstrated that the stimulatory effects of TAMs on C26 cell proliferation were annihilated after NADPH oxidase inhibition in macrophages. Thus, after cell co-culture treatment

with apocynin C26 cells proliferated similarly with C26 cells cultivated alone (**Fig. 4D** compared to **Fig. 1**, $P=0.06$).

3.4. The role of NADPH oxidase in the modulation of TAMs protumor actions on C26 cells

To evaluate whether the pro-oxidant effects of TAMs generated via activity of NADPH oxidase can be linked to potential stimulatory actions of TAMs on C26 cell proliferation, C26 murine carcinoma cells were cultured with macrophages in the presence of apocynin for 48h and then the production of different angiogenic proteins (**Fig. 2B**) were assessed. Our results have shown that C26 cells co-cultured with macrophages were treated with apocynin, the levels of all angiogenic proteins tested were reduced statistically significantly ($P<0.0001$) compared to their production in untreated cell co-culture lysates. On average, NADPH oxidase inhibition reduced by 63% the production of these proteins in apocynin-incubated cells compared to their production untreated cell co-culture. Except for TNF-α level that was slightly reduced (by 35%) all angiogenic and inflammatory protein levels were suppressed moderately (by 35-75%) to strongly (higher than 75%) after apocynin treatment (**Fig. 2B**).

In **conclusion**, our data supported the stimulatory role of TAMs on C26 murine colon carcinoma cell proliferation via anti-inflammatory, pro-angiogenic, and pro-oxidant effects on co-culture microenvironment. The main mechanism of the protumor function of TAMs in C26 tumor development is based on the maintenance of the physiological range of the oxidative stress and angiogenic capacity of C26 colon carcinoma milieu via NADPH oxidase activity. Moreover, in addition to this principal mechanism of action the anti-inflammatory and pro-angiogenic effects of TAMs on these cancer cells create a favorable microenvironment for C26 colon carcinoma development and progression. These findings provide new insights into tumor-targeted therapies based on TAMs re-education strategies.

Chapter IV. *In vivo* double targeting of C26 colon carcinoma cells and microenvironmental protumor processes using liposomal simvastatin

[This chapter was published as: Luput L, Licarete E, Drotar DM, Nagy AL, Sesarman A, Patras L, Rauca VF, Porfire A, Muntean D, Achim M, Tomuta I, Vlase L, Catoi C, Dragos N, Banciu M. In Vivo Double Targeting of C26 Colon Carcinoma Cells and Microenvironmental Protumor Processes Using Liposomal Simvastatin. J Cancer 2018; 9(2):440-449. doi:10.7150/jca.21560]

1. Introduction

Besides cholesterol lowering effects, SIM at very high doses possesses antitumor actions. Moreover our previous studies demonstrated that tumor-targeted delivery of SIM by using LCL

improved the therapeutic index of this drug in murine melanoma-bearing mice. To evaluate whether this finding can be exploited for future therapy of colorectal cancer, the antitumor activity and the underlying mechanisms of LCL-SIM efficacy for inhibition of C26 murine colon carcinoma growth *in vivo* were investigated. To find LCL-SIM dose with the highest therapeutic index, dose-response relationship and side effects of different LCL-SIM doses were assessed in C26 colon carcinoma-bearing mice. The underlying mechanisms of LCL-SIM versus free SIM treatments were investigated with regard to their actions on C26 cell proliferation and apoptosis (via tumor tissues immunostaining for PCNA and Bax markers), tumor inflammation and angiogenesis (using an angiogenic/inflammatory protein array. Our findings suggest that LCL-SIM antitumor activity on C26 colon carcinoma is a result of the tumor-targeting property of the liposome formulation, as free SIM treatment was ineffective. Moreover, LCL-SIM exerted significant antiproliferative and pro-apoptotic actions on C26 cells, notable suppressive effects on two main supportive processes for tumor development, inflammation and angiogenesis, and only slight anti-oxidant actions. Our data proved that LCL-SIM antitumor activity in C26 colon carcinoma was based on cytotoxic effects on these cancer cells and suppressive actions on tumor angiogenesis and inflammation.

2. Materials and methods

- LCL-SIM were prepared by using used **lipid film hydration** method. For this liposomal formulation were used 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Lipoid GmbH, Germany), N-(carbonyl-methoxypolyethylenglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Na-salt) (MPEG-2000-DSPE) (Lipoid GmbH, Germany), cholesterol (CHO) (Sigma-Aldrich, Germany), and SIM (Biocon Limited, India) in a molar ratio of 9.5:0.5:1:2.2. The liposomal SIM concentration was about 1300 µg/ml, corresponding to an encapsulation efficiency of 20%. Mean vesicles size of the liposomes was around 115 nm and the polydispersity values were lower than 0.15, both parameters showing a narrow size distribution and good potential for their passive accumulation in tumors (Alupei et al. 2015, Porfire et al. 2015).
- **C26 murine colon carcinoma cells** (Cell Lines Service GmbH, Eppelheim, Germany) were cultured in RPMI-1640 medium (Lonza, Group AG, Basel, Switzerland), supplemented with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), as monolayer at 37˚C in a humidified atmosphere containing 5% CO_2. **Colon**

carcinoma tumor model was generated by subcutaneous inoculation of 10^6 C26 cells in the right flank of 6-8 weeks-old male Balb/c mice (Cantacuzino Institute, Bucharest, Romania).

- **Tumor volume** was determined according to the formula: $\mathbf{V = 0.52}a^2b$, where *a* is the smallest and *b* is the largest superficial diameter. Experiments were performed according to the national regulations and were approved by university animal experiments ethical committee (registration no.**31375/06.04.2015**).
- To assess the **optimal dose of SIM incorporated in LCL** with the highest therapeutic index on C26 colon carcinoma, mice were injected *i.v.* (in caudal vein), twice at days 8 and 11 after tumor cell inoculation with the following doses of LCL-SIM: 2.5, 5, 7.5, and 10 mg/kg. Tumor size was measured daily and mice were sacrificed at day 16 after tumor induction, when first tumors from the control group reached the volume of 2000 mm^3 that represents the safe volume to prevent C26 carcinoma metastasis (Oft et al. 1998).
- To assess the effects of LCL-SIM *versus* free SIM on **tumor growth**, LCL-SIM and free SIM were administered in caudal vein at a dose of 5 mg/kg at days 8 and 11 after tumor cell inoculation. Tumor volume was measured daily and calculated as described above. On day 12, mice were sacrificed and tumors were measured and collected.
- To evaluate the cytotoxicity of treatments on tumor tissue, an **immunohistochemical analysis** was performed for two markers, the proliferating cell nuclear antigen (PCNA) and the Bcl-2-associated X protein (BAX). The data were expressed as mitotic and apoptotic index represented by percentage of PCNA-positive cells (brown stained nuclei) (Jin et al. 2004), respectively percentage of Bax-positive cells (brown stained cytoplasm) from the total number of counted cells (Jansson and Sun 2002).
- After mice were sacrificed, tumors were isolated, weighed and pooled **tumor tissue lysates** for each group and instant frozen in liquid nitrogen, than incubated with lysis buffer with protease and phosphatase inhibitors cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration of the supernatant was measured using biuret method (Gornall et al. 1949).
- The expression levels of angiogenic proteins in tumor tissue were investigated by performing a screening for 24 proteins involved in angiogenesis using **RayBio® Mouse Angiogenic Cytokine Antibody Array kit** (RayBiotech Inc., Norcross, GA, USA) as described previously (Banciu et al. 2006).
- Data from different experiments were reported as **mean ± SD**. Statistical comparisons of the overall effects of different treatments on tumors were evaluated by **one-way ANOVA with**

Dunnett's post-test for multiple comparisons. The tumor volume doubling time (DT) was estimated by using an exponential tumor growth equation. The differences between the effects of different treatments on angiogenic factor production were analysed **by two-way ANOVA with Bonferroni correction for multiple comparisons** using GraphPad Prism version 6 for Windows, GraphPad Software (San Diego, CA). A value of $P< 0.05$ was considered significant.

3. Results and discussion

3.1. Antitumor activity of LCL-SIM: dose-response relationship

To evaluate the relationship between different doses of LCL-SIM and their antitumor activity, mice received two *i.v.* injections of indicated doses, on day 8 and 11 after tumor induction. The tumor volumes were measured until day 16 when first tumors from the control group reached the maximum volume of 2000 mm^3. The results were shown as tumor volumes at day 16 in **Fig. 4A** and areas under the tumor growth curves (AUTC) in **Fig. 5B**.

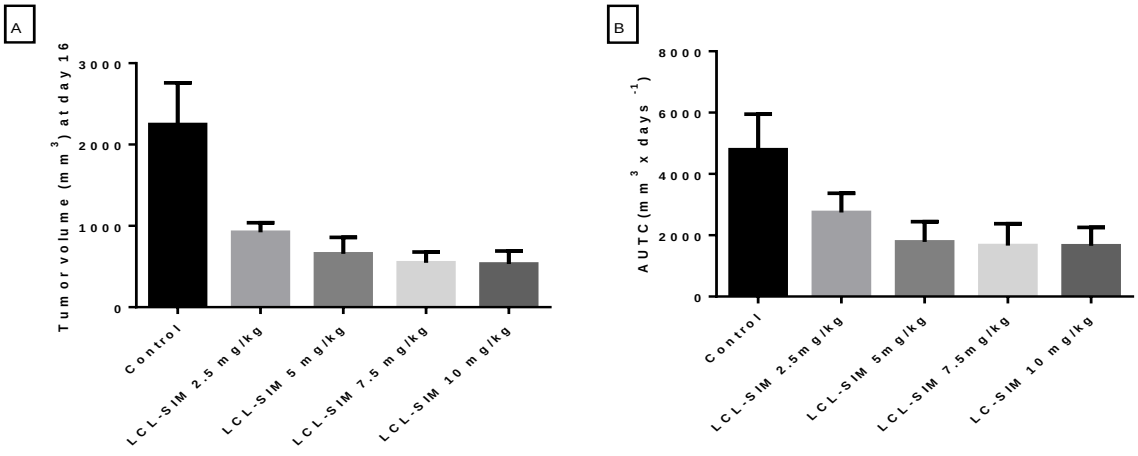

Figure 5. Antitumor activity of different doses of LCL-SIM. Mice received two *i.v.* injections of either formulation at day 8 and day 11, after tumor cell inoculation. The data were reported at day 16 after cell inoculation (the day when first tumors from the control group reached the maximum volume of 2000 mm^3). **A. Tumor volumes at day 16. B. Areas under the tumor growth curves (AUTC) until the day 16 after tumor induction.** The results were expressed as mean ± SD of five mice; Control - untreated group (group treated with PBS); 2.5 mg/kg LCL-SIM - group treated with 2.5 mg/kg LCL-SIM at days 8 and 11 after tumor cell inoculation; 5 mg/kg LCL-SIM - group treated with 5 mg/kg LCL-SIM at days 8 and 11 after tumor cell inoculation; 7.5 mg/kg LCL-SIM - group treated with 7.5 mg/kg LCL-SIM at days 8 and 11 after tumor cell inoculation; 10 mg/kg LCL-SIM - group treated with 10 mg/kg LCL-SIM at days 8 and 11 after tumor cell inoculation.

For all doses tested the values of tumor volumes as well as AUTC were much smaller than those obtained in control group and a significant negative correlation between dose and response

was noted (Spearman correlation coefficient of -1, *P*=0.01). Notably, the maximal antitumor activity was already achieved at a dose of 5 mg/kg of SIM. Thus, at this dose, tumor volumes were reduced with more than 70% (**Fig. 5A**) and the deceleration of tumor growth at day 16 after tumor induction was about 62% (**Fig. 5B**) compared with the growth of tumors from the control group

3.2. Effect of LCL-SIM *versus* free SIM on tumor growth

To compare the effects of 5 mg/kg LCL-SIM and the same dose of free SIM on C26 colon carcinoma-bearing mice, treatments were administered at day 8 and day 11 after tumor cell inoculation. The antitumor activities were evaluated by measuring the tumor volumes at day 12 (the day when mice were sacrificed) (**Fig. 6A**) and AUTC (**Fig. 6B**). Notably, tumor volumes from LCL-SIM-treated group were 50% smaller as compared with those noted from controls (*P*<0.01, **Fig. 6A**) while free administration of SIM did not affect this tumor growth parameter (**Fig. 6A**). In line with tumor volume data, AUTC were significantly reduced after LCL-SIM treatment as compared to free drug-treated tumors or controls (P<0.05, **Fig. 6B**). Altogether these data suggested that the antitumor activity of LCL-SIM was enabled by tumor-targeting capacity of the liposomes that augmented intratumor statins accumulation and thereby amplified the inhibitory effects of SIM on C26 colon carcinoma.

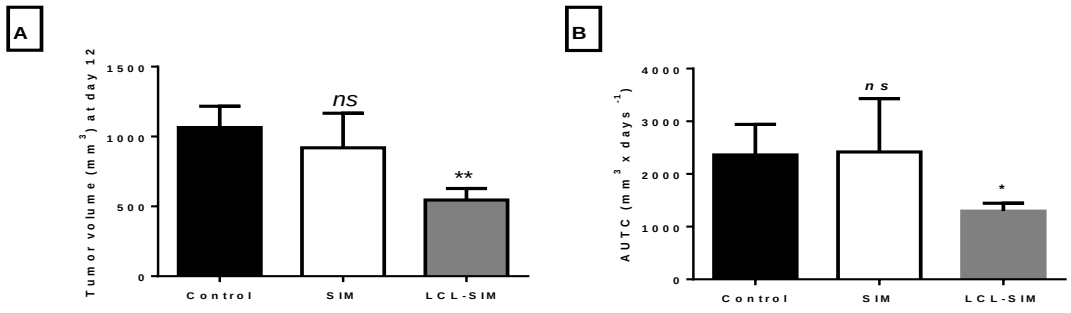

Figure 6. Antitumor activities of LCL-SIM and free SIM in C26 colon carcinoma-bearing mice. Mice received two *i.v.* injections of either formulation at day 8 and day 11, after cell inoculation. The data were reported at day 12 after tumor cell inoculation (the day when mice were sacrificed) **A. Tumor volumes at day 12 in different treatment groups. B. Areas under the tumor growth curves (AUTC) until day 12.** The results were expressed as mean ± SD of five mice; Control - untreated group (group treated with PBS); SIM - group treated with 5 mg/kg free SIM at days 8 and 11 after tumor cell inoculation; LCL-SIM - group treated with 5 mg/kg LCL-SIM at days 8 and 11 after tumor cell inoculation. To compare the effects of each treatment on tumors *in vivo* with controls (tumors in mice treated with PBS), one-way ANOVA with Dunnett's Test for multiple comparisons was used and the *P* values are indicated as follows: *ns*, not significant (*P*>0.05); *, *P*<0.05; **, *P*<0.01.

3.3. Cytotoxic effects of LCL-SIM on tumor tissue

To link the antitumor activity of LCL-SIM with its direct cytotoxic effects on C26 colon carcinoma cells, the proliferation as well as apoptosis-related molecules were assessed by immunohistochemical analysis of tumors after different treatments and the results were shown in **Fig. 7A-F**. Thus, tumors were evaluated immunohistochemically for the expression of proliferating cell nuclear antigen (PCNA) (**Fig. 7A-C**) as well as of Bax apoptotic protein (**Fig. 7D-F**). Our data suggested that the antitumor activities of different treatments correlated negatively with mitotic index (Spearman correlation coefficient =-0.82, *P*=0.0001) and positively with apoptotic index (Spearman correlation coefficient = 0.82, *P*=0.0014). More precisely, LCL-SIM-treated tumors presented much lower proliferative potential by 50-60% compared to either control tumors or SIM-treated tumors (**Fig. 7A-C**). After LCL-SIM administration, the intratumor expression of the apoptotic protein Bax increased slightly by 15% compared to its production in SIM-treated tumors and moderately by 36% compared to the same protein expression in control tumors (**Fig. 7D-F**).

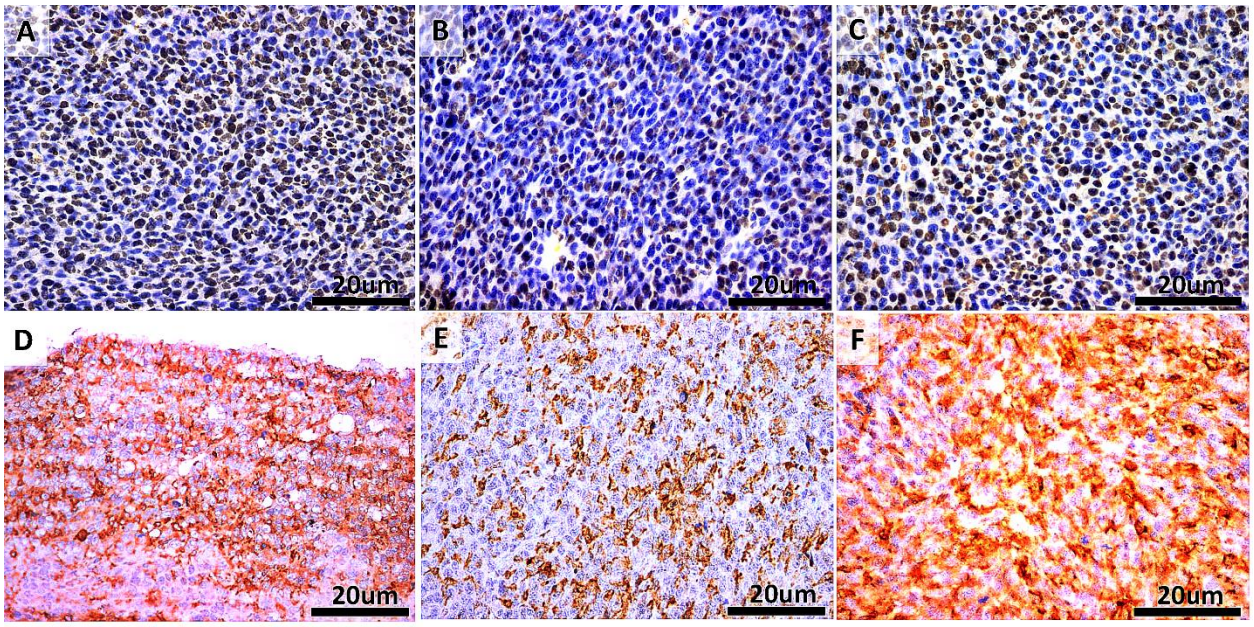

Figure 7. Immunohistochemical analysis of the cytotoxic effects of different treatments on C26 murine colon carcinoma *in vivo*. A-C. PCNA expression in tumors. Cells that present nuclear staining in brown are PCNA- positive cells. Sections were counterstained with Gill 2 hematoxylin. **D-F. Bax expression in tumors.** Cells that present cytoplasmic staining in brown are Bax - positive cells. Sections were counterstained with Gill 2 hematoxylin. **A** and **D**-Control (untreated group); **B** and **E**- group treated with 5 mg/kg SIM at days 8 and 11 after tumor cell inoculation; **C** and **F**- group treated with 5 mg/kg LCL-SIM at days 8 and 11 after tumor cell inoculation. Scale bars of 20 μm and original magnification of 400*X*.

3.4. Effects of LCL-SIM on tumor angiogenesis

To link the antitumor activity of LCL-SIM in C26 colon carcinoma with its effects on tumor angiogenesis we investigated the effects of liposomal administration of SIM and free SIM on the production of 24 proteins involved in angiogenesis and inflammation from tumor tissue, using a protein array. The effects of LCL-SIM and free SIM on the C26 tumor production of the pro-angiogenic proteins were presented in detail in **Table 1**.

Table 1. Effects of LCL-SIM and free SIM on pro-angiogenic protein production in C26 tumors.

Pro-angiogenic proteins	Percentage of inhibition (-) and stimulation (+) of pro-angiogenic proteins levels in the C26 colon carcinoma after different treatments compared to their control levels	
	SIM	**LCL-SIM**
G-CSF	-52.89 ± 0 (****)	-83.11 ± 2.85 (****)
GM-CSF	-71.22 ± 0 (****)	-69.66 ± 6.82 (****)
M-CSF	27.57 ± 3.84 (*ns*)	20.77 ± 6.84 (*ns*)
IGF-II	9.32 ± 0.15 (*ns*)	-6.13 ± 5.71 (*ns*)
IL-1α	1.13 ± 17.06 (*ns*)	-22.08 ± 0.14 (*ns*)
IL-1ß	-79.20 ± 9.78 (****)	-76.02 ± 1.36 (****)
IL-6	-66.88 ± 15.64 (****)	-91.25 ± 0.74 (****)
IL-9	-32.48 ± 4.33 (*)	-32.1 ± 10.56 (*)
IL-12p40	57.37 ± 34.13 (****)	3.34 ± 6.29 (*ns*)
IL-13	-29.18 ± 10.33 (*)	-26.8 ± 2.68 (*ns*)
TNF-α	-72.38 ± 1.94 (****)	-70.96 ± 3.9 (****)
MCP-1	85.36 ± 15.36 (****)	73.66 ± 9.5 (****)
Eotaxin	-86.46 ± 0 (****)	-88.94 ± 5.67 (****)
FasL	-11.8 ± 7.52 (*ns*)	-60.38 ± 23.6 (****)
bFGF	55.32 ± 24.32 (****)	-59.77 ± 18.41 (****)
VEGF	-66.08 ± 0.17 (****)	-71.34 ± 1.46 (****)
Leptin	-42.69 ± 13.14 (***)	-48.25 ± 9.8 (***)
TPO	86.08 ± 0.46 (****)	23.96 ± 4.5 (*ns*)

*The protein levels in tumors after different treatments were compared to protein levels in control tumors. The results represented the mean ± SD of two independent measurements. ns, not significant, P>0.05; *, P<0.05; **, P<0.01.***; P<0.001; ****, P<0.0001. TPO, thrombopoetin.*

The levels of most of the pro-angiogenic proteins were reduced significantly in case of liposomal and free SIM administration compared to their control levels. Nevertheless, the overall reduction of pro-angiogenic proteins was stronger and statistically significant after LCL-SIM treatment compared with that induced by free SIM administration. On average, the effect of LCL-SIM on pro-angiogenic protein levels was 22% higher than the effect of free SIM on the same protein production (P<0.0001) (**Table 1**).

In the case of anti-angiogenic proteins, both treatments exerted similar strong stimulatory effects on the levels of TIMP-1 and PF-4 (75-165% stimulation compared to control levels of these proteins) and moderate reducing effects on the levels of TIMP-2 (45-65% reduction compared to control protein production) (**Table 2**). Moreover, SIM administration stimulated the production of IL-12 subunit p70 (IL-12p70) (by 104%) and monokine induced by interferon-γ (MIG) (by 55%) while LCL-SIM exerted notable reducing effects (by 50%) on the intratumor production of IFN-γ and MIG (**Table 2**).

Table 2. Effects of LCL-SIM and free SIM on anti-angiogenic protein production on C26 tumors.

Anti-angiogenic proteins	Percentage of inhibition (-) and stimulation (+) of anti-angiogenic proteins levels in the C26 colon carcinoma after different treatments compared to their control levels	
	SIM	**LCL-SIM**
TIMP-1	75.52 ± 14.27 (****)	99.89 ± 1.2 (****)
TIMP-2	-44.11 ± 3.05 (***)	-64.33 ± 3.99 (****)
PF-4	162.56 ± 39.74 (****)	120.91 ± 11.48 (****)
IL-12p70	103.64 ± 24.41 (****)	25.03 ± 3.37 (*ns*)
IFN-γ	-8.48 ± 5.57 (*ns*)	-50.37 ± 0.16 (****)
MIG	54.74 ± 8.52 (****)	-49.78 ± 4.21 (****)

*The protein levels in tumors after different treatments were compared to protein levels in control tumors. The results represented the mean ± SD of two independent measurements; ns, not significant, P>0.05; ***; P<0.001; ****, P<0.0001.*

Taken together, our data proved that the incorporation of SIM in LCL offers an increased antitumor activity in C26 murine colon carcinoma-bearing mice. One of the future issues is to further identify the potential of this treatment to "re-educate" tumor-associated macrophages to fight against cancer.

Chapter V. Liposomal simvastatin sensitizes C26 colon carcinoma to the antitumor effects of liposomal 5-fluorouracil *in vivo*

[This chapter was submitted to publication in the journal PLoS ONE as: Lavinia Luput, Alina Sesarman, Alina Porfire, Marcela Achim, Dana Muntean, Tibor Casian, Laura Patras, Valentin Florian Rauca, Denise Minerva Drotar, Ioana Stejerean, Ioan Tomuta, Laurian Vlase, Nicolae Dragos, Manuela Banciu, Emilia Licarete. Liposomal simvastatin potentiates the antitumor effects of liposomal 5-fluorouracil on C26 colon carcinoma in vivo]

This study investigated the antitumor efficacy of a novel targeted combined therapy of CRC based on the administration of LCL-SIM described in the previous study, combined with 5-FU – the major chemotherapeutic agent used in CRC therapy, also encapsulated in LCL. The main molecular mechanisms underlying the antitumor activity of the two therapeutic agents were assessed. The results of this study revealed the potential of LCL-SIM to sensitize the C26 colon carcinoma cells to the antitumor actions of LCL-5-FU. The combined therapy exerted inhibitory effects on angiogenesis of C26 colon carcinoma and antioxidant effects. Thus, our data demonstrated the potential of this combined therapy to become a future tumor-targeted therapy in CRC.

Chapter VI. Conclusions

- **The first study** revealed that TAMs support proliferation of C26 colon carcinoma cells in an oxidative stress-dependent manner that potentiate the angiogenic capacity of the tumor microenvironment.
- **The second study** demonstrated that the antitumor effects of LCL-SIM exerted on C26 murine colon carcinoma-bearing mice were based on the suppression of tumor angiogenesis and inflammation as well as significant antiproliferative and pro-apoptotic actions on C26 colon carcinoma cells, being tightly linked with the passive targeting capacity of LCL to tumor microenvironment.
- **The third study** proved the potential of the LCL-SIM to sensitize the C26 colon carcinoma cells to LCL-5-FU administration. Altogether, our data demonstrated the potential of the combined therapy based on the administration of LCL-SIM and LCL-5-FU to become a successful colorectal cancer targeted therapy.

Selected references

Aebi H. 1984. Catalase in vitro. Methods Enzymol 105:121-126.

Alberts AW, et al. 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc Natl Acad Sci U S A 77:3957-3961.

Albini A, Tosetti F, Benelli R, Noonan DM. 2005. Tumor inflammatory angiogenesis and its chemoprevention. Cancer Res 65:10637-10641.

Alupei MC, Licarete E, Patras L, Banciu M. 2015. Liposomal simvastatin inhibits tumor growth via targeting tumor-associated macrophages-mediated oxidative stress. Cancer Lett 356:946-952.

Askarizadeh A, Butler AE, Badiee A, Sahebkar A. 2019. Liposomal nanocarriers for statins: A pharmacokinetic and pharmacodynamics appraisal. J Cell Physiol 234:1219-1229.

Balkwill F, Mantovani A. 2001. Inflammation and cancer: back to Virchow? Lancet 357:539-545.

Balkwill FR, Capasso M, Hagemann T. 2012. The tumor microenvironment at a glance. J Cell Sci 125:5591-5596.

Banciu M, Schiffelers RM, Fens MH, Metselaar JM, Storm G. 2006. Anti-angiogenic effects of liposomal prednisolone phosphate on B16 melanoma in mice. J Control Release 113:1-8.

Bellosta S, Ferri N, Bernini F, Paoletti R, Corsini A. 2000. Non-lipid-related effects of statins. Ann Med 32:164-176.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68:394-424.

Bulbake U, Doppalapudi S, Kommineni N, Khan W. 2017. Liposomal Formulations in Clinical Use: An Updated Review. Pharmaceutics 9.

Cazin JL, Gosselin P, Cappelaere P, Robert J, Demaille A. 1992. Drug resistance in oncology: from concepts to applications. J Cancer Res Clin Oncol 119:76-86.

Condeelis J, Pollard JW. 2006. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 124:263-266.

Erel O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 37:277-285.

Gabizon AA. 2001. Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. Cancer Invest 19:424-436.

Gazzerro P, Proto MC, Gangemi G, Malfitano AM, Ciaglia E, Pisanti S, Santoro A, Laezza C, Bifulco M. 2012. Pharmacological actions of statins: a critical appraisal in the management of cancer. Pharmacol Rev 64:102-146.

Gopalan A, Yu W, Sanders BG, Kline K. 2013. Simvastatin inhibition of mevalonate pathway induces apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 signaling pathway. Cancer Lett 329:9-16.

Gornall AG, Bardawill CJ, David MM. 1949. Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751-766.

Grothey A, Sargent DJ. 2016. Adjuvant Therapy for Colon Cancer: Small Steps Toward Precision Medicine. JAMA Oncol 2:1133-1134.

Haley B, Frenkel E. 2008. Nanoparticles for drug delivery in cancer treatment. Urol Oncol 26:57-64.

Hanahan D, Coussens LM. 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21:309-322.

Herbeuval JP, Lelievre E, Lambert C, Dy M, Genin C. 2004. Recruitment of STAT3 for production of IL-10 by colon carcinoma cells induced by macrophage-derived IL-6. J Immunol 172:4630-4636.

Hindler K, Cleeland CS, Rivera E, Collard CD. 2006. The role of statins in cancer therapy. Oncologist 11:306-315.

James AD, et al. 2015. The Plasma Membrane Calcium Pump in Pancreatic Cancer Cells Exhibiting the Warburg Effect Relies on Glycolytic ATP. J Biol Chem 290:24760-24771.

Jansson A, Sun XF. 2002. Bax expression decreases significantly from primary tumor to metastasis in colorectal cancer. J Clin Oncol 20:811-816.

Jin W, Gao MQ, Lin ZW, Yang DX. 2004. Multiple biomarkers of colorectal tumor in a differential diagnosis model: a quantitative study. World J Gastroenterol 10:439-442.

Karatas F, Karatepe M, Baysar A. 2002. Determination of free malondialdehyde in human serum by high-performance liquid chromatography. Anal Biochem 311:76-79.

Knox JJ, Siu LL, Chen E, Dimitroulakos J, Kamel-Reid S, Moore MJ, Chin S, Irish J, LaFramboise S, Oza AM. 2005. A Phase I trial of prolonged administration of lovastatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck or of the cervix. Eur J Cancer 41:523-530.

Kodach LL, et al. 2011. Statins augment the chemosensitivity of colorectal cancer cells inducing epigenetic reprogramming and reducing colorectal cancer cell 'stemness' via the bone morphogenetic protein pathway. Gut 60:1544-1553.

Licarete E, Sesarman A, Banciu M. 2015. Exploitation of pleiotropic actions of statins by using tumour-targeted delivery systems. J Microencapsul 32:619-631.

Lin EY, Pollard JW. 2007. Tumor-associated macrophages press the angiogenic switch in breast cancer. Cancer Res 67:5064-5066.

Longley DB, Harkin DP, Johnston PG. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3:330-338.

Maeda H, Bharate GY, Daruwalla J. 2009. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. Eur J Pharm Biopharm 71:409-419.

Malam Y, Loizidou M, Seifalian AM. 2009. Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends Pharmacol Sci 30:592-599.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23:549-555.

Matsumura Y, Maeda H. 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46:6387-6392.

Noy R, Pollard JW. 2014. Tumor-associated macrophages: from mechanisms to therapy. Immunity 41:49-61.

Oft M, Heider KH, Beug H. 1998. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol 8:1243-1252.

Pick E. 2014. Role of the Rho GTPase Rac in the activation of the phagocyte NADPH oxidase: outsourcing a key task. Small GTPases 5:e27952.

Pollard JW. 2004. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4:71-78.

Porfire A, Tomuta I, Muntean D, Luca L, Licarete E, Alupei MC, Achim M, Vlase L, Banciu M. 2015. Optimizing long-circulating liposomes for delivery of simvastatin to C26 colon carcinoma cells. J Liposome Res 25:261-269.

Schointuch MN, Gilliam TP, Stine JE, Han X, Zhou C, Gehrig PA, Kim K, Bae-Jump VL. 2014. Simvastatin, an HMG-CoA reductase inhibitor, exhibits anti-metastatic and anti-tumorigenic effects in endometrial cancer. Gynecol Oncol 134:346-355.

Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van Damme J, Mantovani A. 2000. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. J Immunol 164:762-767.

Sica A, Schioppa T, Mantovani A, Allavena P. 2006. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer 42:717-727.

Sirtori CR. 2014. The pharmacology of statins. Pharmacol Res 88:3-11.

Solinas G, Germano G, Mantovani A, Allavena P. 2009. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 86:1065-1073.

Stine JE, Guo H, Sheng X, Han X, Schointuch MN, Gilliam TP, Gehrig PA, Zhou C, Bae-Jump VL. 2016. The HMG-CoA reductase inhibitor, simvastatin, exhibits anti-metastatic and anti-tumorigenic effects in ovarian cancer. Oncotarget 7:946-960.

Swartz MA, Iida N, Roberts EW, Sangaletti S, Wong MH, Yull FE, Coussens LM, DeClerck YA. 2012. Tumor microenvironment complexity: emerging roles in cancer therapy. Cancer Res 72:2473-2480.

Thibault A, et al. 1996. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin Cancer Res 2:483-491.

Torchilin VP. 2000. Drug targeting. Eur J Pharm Sci 11 Suppl 2:S81-91.

Violette S, Poulain L, Dussaulx E, Pepin D, Faussat AM, Chambaz J, Lacorte JM, Staedel C, Lesuffleur T. 2002. Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. Int J Cancer 98:498-504.

Yang C, Liu HZ, Fu ZX, Lu WD. 2011. Oxaliplatin long-circulating liposomes improved therapeutic index of colorectal carcinoma. BMC Biotechnol 11:21.