JBUON 2019; 24(1): 106-115
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Antiproliferative effects of propofol and lidocaine on the colon adenocarcinoma microenvironment

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Summary

Purpose: *Certain anesthetic interventions may influence the postoperative outcome in surgical cancer patients. Our study investigated the antiproliferative effects of propofol and lidocaine in two colon cancer cells lines, fibroblasts and in co-cultures.*

Methods: *The antiproliferative effects of concentrations of propofol and lidocaine were assessed in HCT-116 and RKO cell lines, in fibroblasts (CCD-18Co) and in co-culture system.*

Results: *Both propofol (2-4 mcg/ml) and lidocaine (2-4 μM) inhibited significantly colon cancer cell proliferation ($p<0.05$). Caspase-8, heat-shock proteins (HSP-27 and HSP-60), insulin growth factor (IGF)-II, insulin growth factor binding proteins, p53 protein and survivin were significantly differentially expressed in malignant cells and in fibroblasts exposed to lidocaine.*

Conclusion: *Lidocaine and propofol selectively inhibited colon cancer cells proliferation. Antiproliferative effects were tumor-, dose- and time-dependent and may be at least partially explained by activation of apoptosis protein pathways. Further studies are necessary to confirm the clinical impact of our data.*

Key words: *antiproliferative, colon cancer, lidocaine, propofol, tumor microenvironment*

Introduction

It is increasingly recognized that perioperative anesthetic interventions may influence postoperative outcome, recurrences and metastasis in cancer patients undergoing surgery [1-3]. Among these interventions, total intravenous anaesthesia [4] and local anaesthetics administered either as regional anaesthesia [5], by intravenous (i.v.) infusion [6] or by infiltration [7,8] play a key role. Colon cancer surgery is a good example of surgical intervention where local anaesthetics may be administered either as regional analgesia (epidural analgesia) or as i.v. infusion (lidocaine). Intravenous lidocaine is currently recognized as an intervention that significantly reduces postoperative acute and chronic pain, accelerates resumption of postoperative paralytic ileus and speeds recovery [9,10].

In the last years, several *in vitro* studies have reported antiproliferative effects of lidocaine on different types of cancer cells (breast, colon, tongue or melanoma) [8,11,12]. Most of the results showed that these effects were time- and dose-dependent [8,13]. Identified mechanisms responsible for the

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Received: 10/06/2018; Accepted: 23/07/2018

antiproliferative effects include inhibition of sodium channel (VGSC) [14], DNA demethylation [15,16], inhibition of Src signalling pathways [17] or activation of caspases and the regulation of mitogen-activated protein kinase (MAPK) signaling pathways [18,19]. In the last years tumor microenvironment gained attention due to the fact that this may influence tumor development and treatment efficiency [20].

Our study aimed to investigate the antiproliferative effects of both propofol and lidocaine in clinical concentrations by using a co-culture system with the two different colon cancer cell lines (HCT-116 and RKO) and one of colon fibroblasts (CCD-18Co cells). *In vitro* assessment was carried out by flow cytometry-based assays. Western blot assay was done to identify cellular mechanims responsible for these effects. Encouraged by our previous results in identifying p53 as a potential mechanism for the antiproliferative effects of lidocaine in human hepatocarcinoma [21], we aimed at confirming the role of p53 in the antiproliferative effects in colon cancer.

Methods

Cell lines

Our study used two colon cancer cell lines -HCT-116 and RKO colon adenocarcinoma cells- and CCD-18Co colon normal fibroblasts, all purchased from ATCC (Manassas, USA). All cells were maintained using Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal calf serum (FCS) and 1000 U/mL Penicillin/Streptomycin (P/S). Both HCT-116 and RKO cells expressed the CFMDA green fluorescent protein (GFP), while CCD-18Co cells expressed the DDAO red protein.

Flow cytometry analysis of co-cultures for cell growth analysis

The co-culture systems (HCT-116-CFMDA-CCD-18Co-DDAO / RKO-CFMDA-CCD-18Co-DDAO) were cultured in the medium conditions mentioned above at a confluence of 60-70%. The dynamics (representing the ratio between cell mitosis and cell death) of the two cell sub-populations were analyzed at 1, 3 and 8 days at the Research Center for Functional Genomics and Translational Medicine – Iuliu Hatieganu University of Medicine and Pharmacy using a FACS Canto II flow cytometer.

Cells were detached from the culture flask using trypsin/EDTA for 15 min before being centrifuged for 5 min at 21°C, 1000 rpm. The trypsin was removed, cells were counted, and 500,000 cells were resuspended in 500 µl of phosphate buffered saline (PBS), with 7-aminoactinomycin D (7-AAD).

Protein array and Western blotting

To investigate the proteins involved in antiproliferative effects of lidocaine in the two colon cell lines and in co-culture system, we used a human apoptosis antibody array membrane (Abcam, USA). Adhered cells were lysed using Laemmli buffer (Biorad, Hercules, CA, USA) supplemented with 10% protease inhibitor complete EDTA free (Roche) and the final protein concentration was measured using a BCA Protein Assay kit (Pierce, Rockford, MA, USA). Thirty µg of cell lysates were then electrophoresed on 10% polyacrylamide gells (Biorad) before being transferred to Immobilon PSQ membranes (Millipore, Bedford, MA, USA). Membranes were blocked with blocking buffer (TBS with 5% skim milk and 0.1%Tween-20), before being incubated with the primary antibody at 4 °C overnight. After washing of the membrane with washing buffer 5 times for 5 min each time at room temperature, the membranes were incubated with the secondary antibody (HRP-conjugated goat anti-rabbit IgG). The image was finally analyzed using an enhanced chemoilluminiscence-plus reagent (GE Healthcare, Buckinghamshire, UK) and the densitometry of the result was analyzed on the western blotting image using the J image software.

Statistics

Statistical analyses were performed using R (R Development core team, USA) and GraphPad Prism 5.0 (GraphPad Software INC, CA, USA). The obtained data was first examined for normality of distribution using the Shapiro-Wilk test. The distribution of all data was Gaussian; thus, data were analyzed using the t-test. For each concentration, we calculated the area under the curve (AUC) using the trapezoidal method. To evaluate the AUCs trend regarding the different concentrations of lidocaine and type of cell line, we used the two-way ANOVA test. A $p<0.05$ was considered significant.

Results

Co-culture and simulating the tumor environment niche

Before investigating the co-culture system, to properly simulate the tumor microenvironment niche, IC_{50} was calculated for each cell line, as shown in Figure 1. Our data showed that both lidocaine and propofol had antiproliferative effects.

In concentrations between 2-4 µg/mL propofol significantly inhibited colon cancer cell proliferation with an estimated IC_{50} at 48 hrs of 1038 µg/mL for RKO, an IC_{50} of 247.1 µg/mL in HCT-116 cells and IC_{50} of 1371 µg/mL in CCD-18Co cells. For malignant cell lines, IC_{50} was smaller in comparison with IC_{50} for fibroblasts ($p=0.0004$ and $p=0.002$, respectively).

For lidocaine, IC_{50} at 48 hrs of exposure was 948.6 µM in HCT-116 cells, 2197 µM in RKO cells and 1896 µM in CCD-18Co cells (Figure 1). Thus, for RCT-116 malignant cells, IC_{50} was at least 2-fold smaller in comparison to fibroblasts

(p=0.0021), showing that in these cells, for certain concentrations that might inhibit cancer growth, the surrounding healthy tissue may protect against dissemination.

On the other hand, these antiproliferative effects were not so expressed in RKO cells, as compared with HCT-116 cells, leading to the hypothesis that these effects are not only time- and dose-dependent but cell-dependent.

In the next step, two co-culture systems were set up: HCT-116-CFMDA-CCD-18Co-DDAO and RKO-CFMDA-CCD-18Co-DDAO, using the two different colon adenocarcinoma cell lines and one cell line of normal colon fibroblast. These two systems aimed to simulate, with all its limitations nevertheless, the colon microenvironment of invasion of the *muscularis mucosa* by the malignant cells. By staining cancer cells with CFMDA, we could detect green cancer cells, whereas by staining fibroblasts with DDAO, we had red fibroblasts. These 2 intracellular systems allowed the identification of the cell types after being co-cultured, by flow cytometry. The setting-up of the co-culture systems by flow cytometry is shown in Figure 2A-B. Figure 2A and B show the flow cytometry histograms, showing all the cellular populations, cultured both individually, as well as in co-culture. The data was extracted based on at least 100,000 analyzed cells. The detailed statistical data for the cell co-culture is shown in Figures 3A-C. Cells were cultured with lidocaine, using the IC_{50} concentrations shown in Figure 1.

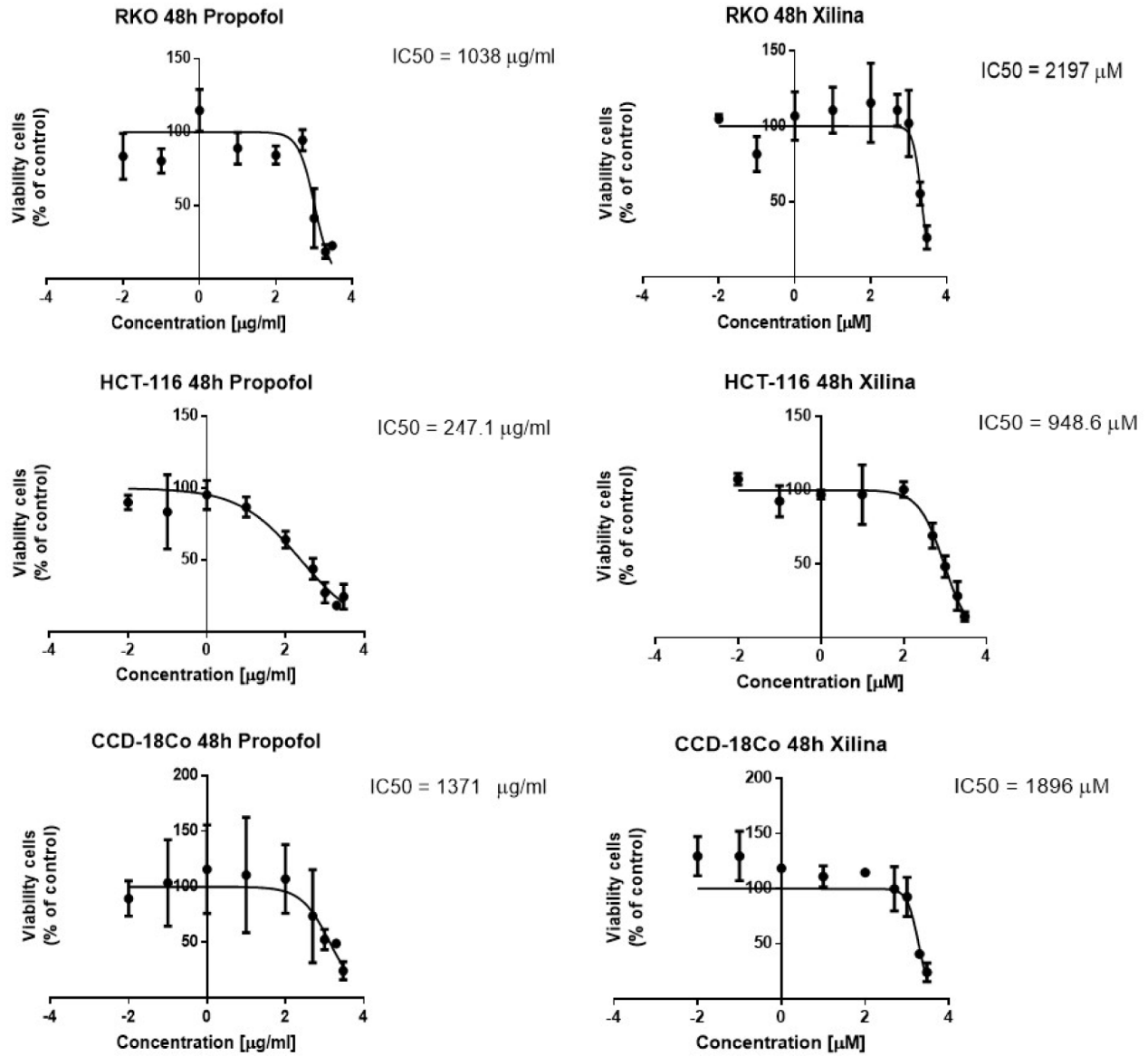

Figure 1. Preliminary determination of the IC_{50} on cell viability of both propofol (HCT116 vs CCD-18Co, p=0.0004 and RKO vs CCD-18Co, p=0.002) and lidocaine (HCC116 vs CCD-18Co, p=0.0021 and RKO vs CCD-16Co, p=0.0006) on all three cell lines, HCT116 and RKO cancer cells and cancer-associated fibroblasts (CCD-16Co).

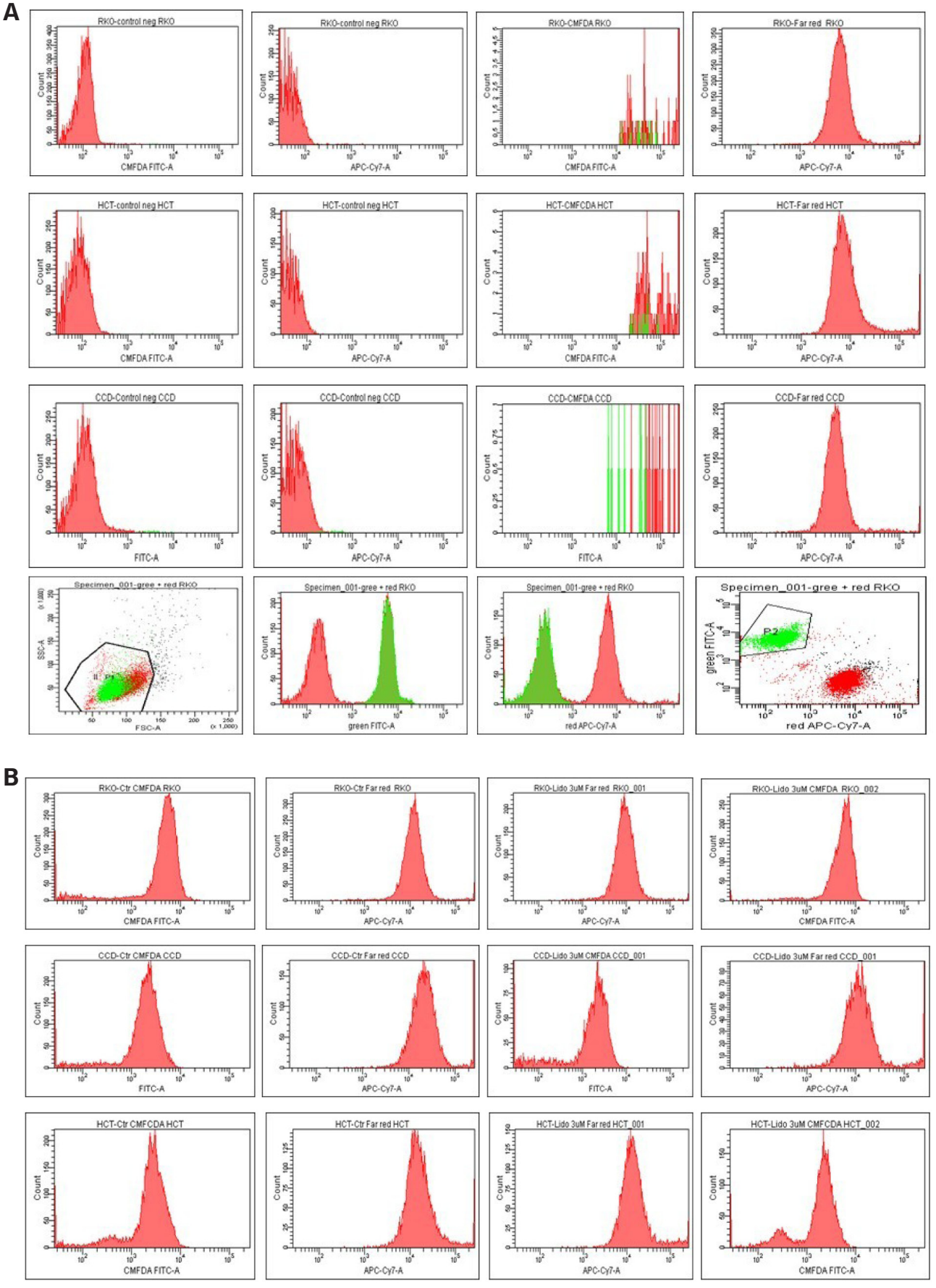

Figure 2. Setting up of the co-culture system analyses by flow cytometry. Figures **2A** and **B** show the flow cytometry histograms, showing all the cellular populations, cultured both individually, as well as in co-culture. The data resulted from at least 100,000 analyzed cells.

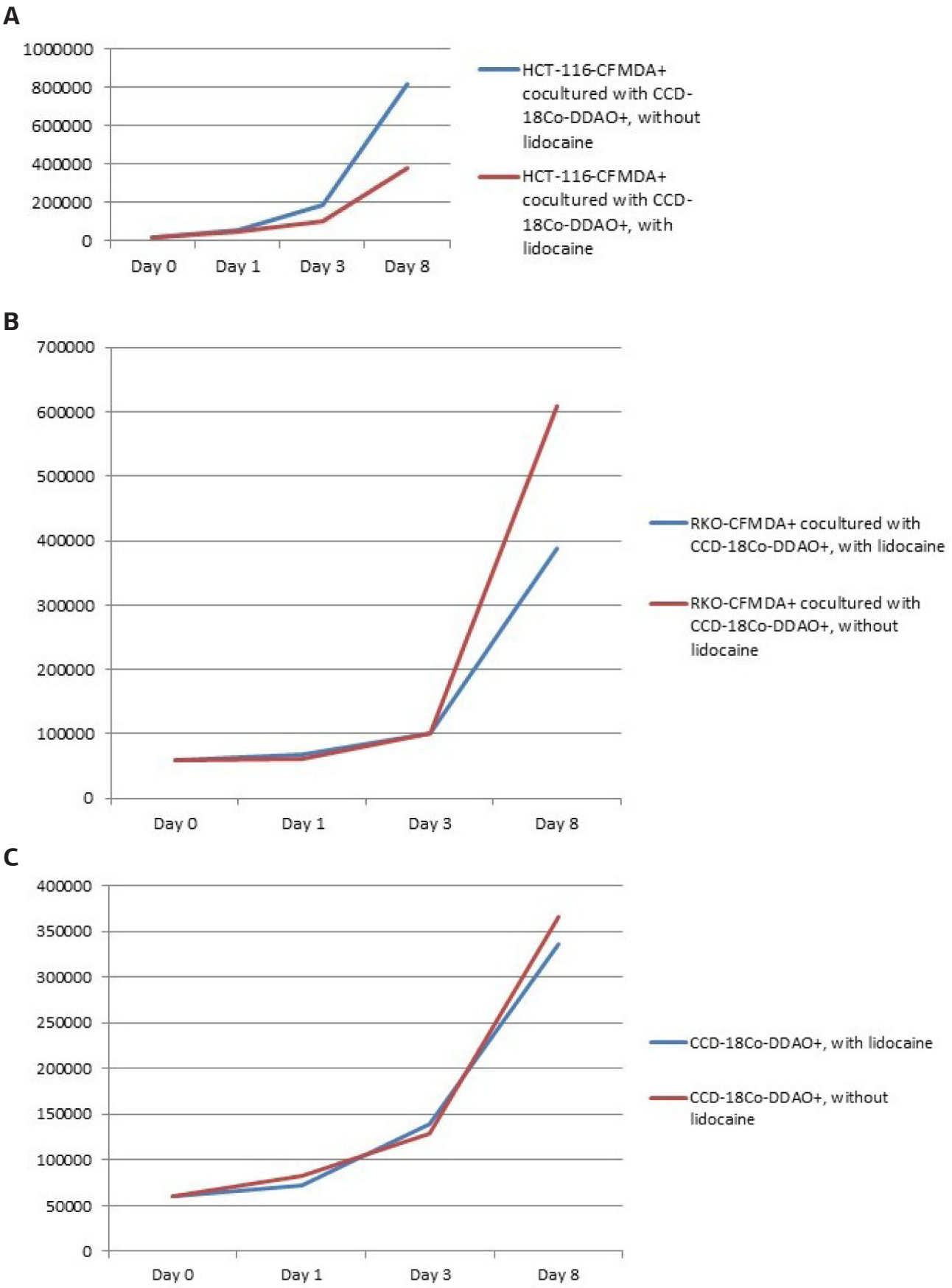

Figure 3. Detailed analysis of the cellular co-culture systems, with and without lidocaine, allowing the assessment of how different cells behave *in vitro* and thus simulating the desmoplastic stroma niche. Figure **3A** shows the data for HCT-116 cancer cells (p=0.0002), Figure **3B** shows the data for RKO cancer cells (p=0.0004) and Figure **3C** shows the data for CCD-18Co cancer-associated fibroblasts (p=0.0136).

We cultured green cancer cells (CFMDA positive) with red fibroblasts (DDAO positive) and counted the cells on days 0, 1, 3 and 8. The total cancer cell populations were significantly reduced in cell cultures treated with lidocaine (in concentration of 948.6 μM/ml) as shown in Figures 3A-B ($p<0.05$), whereas in fibroblasts no differences were noted in cultures exposed to lidocaine and in those without ($p>0.05$). It is interesting that lidocaine reduced cancer cell count starting with day 2 and the process was time-dependent. Thus, if the total cell population is differentially influenced by lidocaine, the influence is mostly in malignant cells.

Assessment of the protein-based pathways by which lidocaine could inhibit cancer cell proliferation

After the functional assays previously described showing that lidocaine acts on the malignant cell and not on the surrounding microenvironment (the fibroblasts represent the surrounding desmoplastic

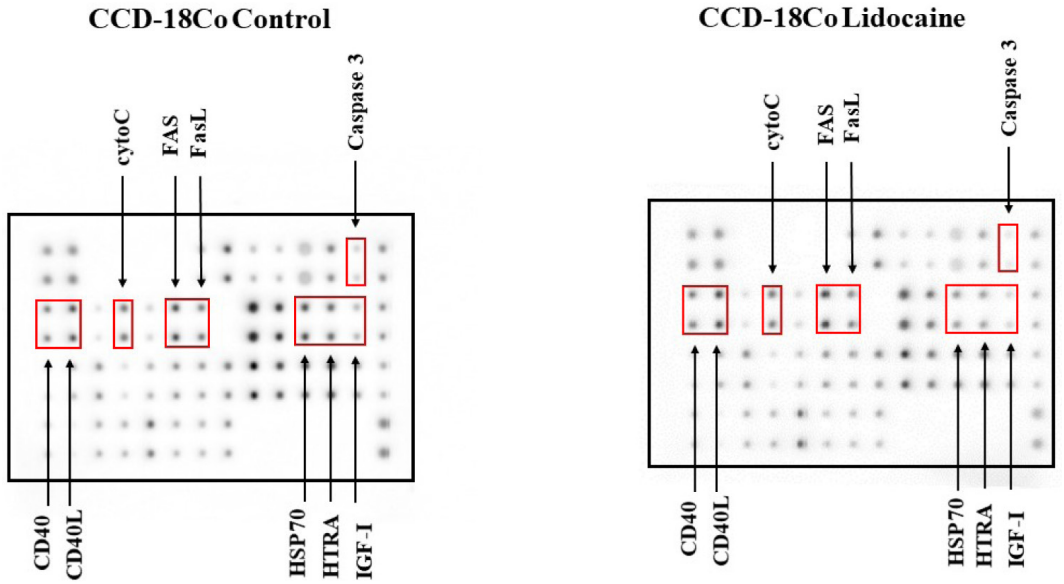

Figure 4. Western blotting-based analysis for the proteins involved in cell death (both apoptosis and necrosis), assessing the effects of lidocaine on the tumor-associated fibroblasts (CCD-18Co cell line).

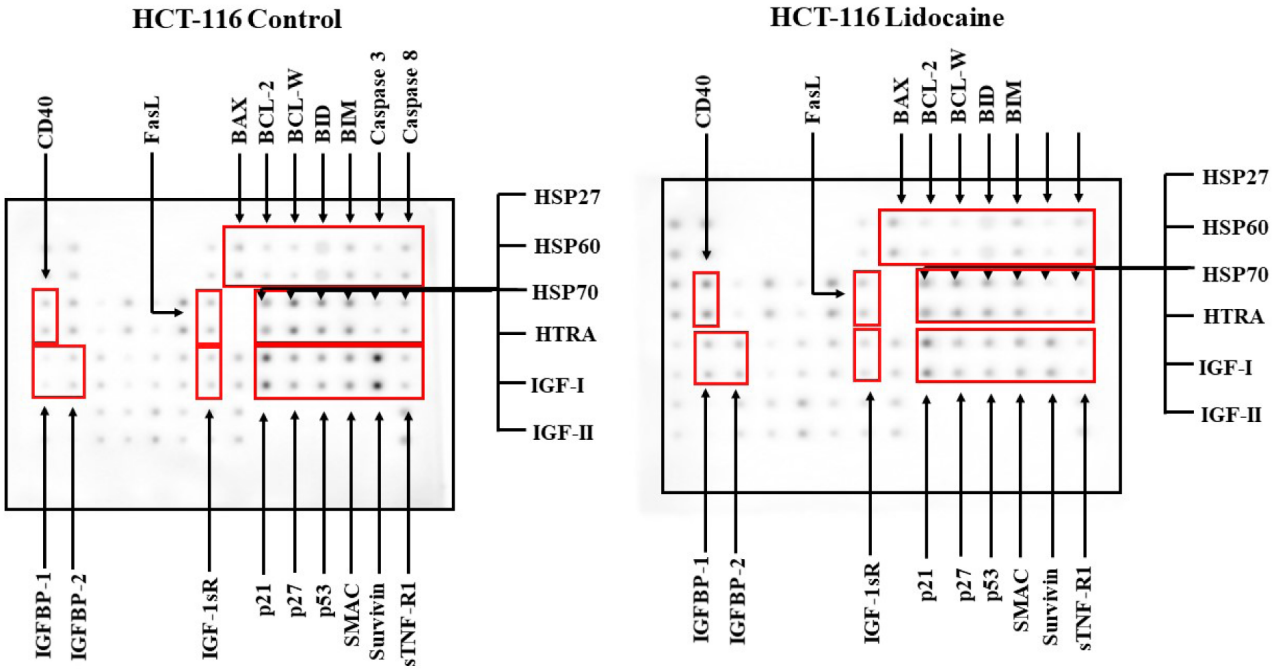

Figure 5. Western blotting-based analysis for the proteins involved in cell death (both apoptosis and necrosis), assessing the effects of lidocaine on the cancer cells (HCT-116 cell line).

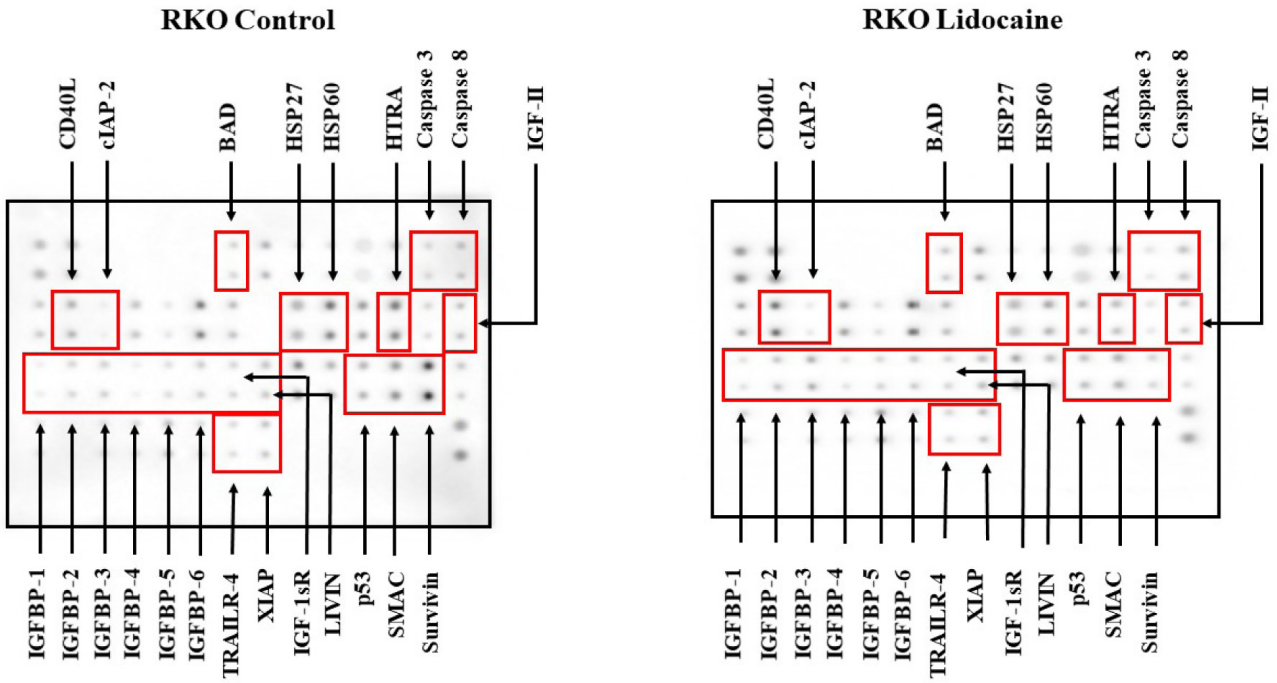

Figure 6. Western blotting-based analysis for the proteins involved in cell death (both apoptosis and necrosis), assessing the effects of lidocaine on the cancer cells (RKO cell line).

stroma), we used a western blotting-based protein array to investigate whether lidocaine acts by using molecular pathways related to apoptosis. The results are shown in Figures 4-6.

As it can be seen in Figures 4-6, we compared cell cultures (2 colon cancer cell lines and fibroblasts) with and without lidocaine. By using ImageJ analysis of the protein intensity, we compared the level of each study protein between the different cell lines, with special attention on comparing fibroblasts with malignant cells (Table 1).

Thus, we identified Caspase-8, heat-shock proteins (HSP-27 and HSP-60), insulin growth factor (IGF)-II, insulin growth factor binding protein (IGFBP)-1, IGFBP-2, IGF-1sR, p53 protein, second mitochondria-derived activator of caspase (SMAC) and survivin, as significantly differentially expressed between fibroblasts and malignant cells exposed to lidocaine (in concentration of 948.6 μM/ml).

As can be seen in Figures 4-6 and in Table 1 most of the proteins involved in cellular apoptosis were activated by lidocaine in cancer cells as compared to fibroblasts. However, there were differences between the two cell lines in terms of lidocaine's effects.

Discussion

In the last years several studies have shown that both propofol and especially local anesthetics - i.v. lidocaine - have antiproliferative effects in different cancer cells [7,8,11-13]. Questions have been raised on the potential mechanisms responsible for these anticancer effects.

For propofol, the mechanisms responsible for the antiproliferative effects include decreased expression of extracellular matrix protein and invasiveness of colon cancer cells [22,23], inhibition of tumor angiogenesis [24,25], upregulation/downregulation of microRNA expression, apoptosis and decreasing of HIF1α [26-28].

In case of local anesthetics, Na^+ channels blocking action seems to be the main mechanism of anticancer effects [14], but other mechanisms are also involved [15-18].

Thus DNA demethylation [15,16], modulating ectodomain shedding of heparin-binding epidermal growth factor-like growth factor (HB-EGF) [7], inhibition of Src signaling pathways [17], mitogen-activated protein kinase pathway [19] have all been involved in the antiproliferative effects of lidocaine.

For both propofol and local anesthetics some of these antiproliferative effects have been confirmed in animal studies [13] or in retrospective studies on TIVA vs inhalation anesthesia in humans [4].

On the other hand, in the last years tumor microenvironment has gained attention due to its involvement in tumor development and implication in treatment efficiency [20,29,30]. A normal tumor microenvironment would normally be able to limit tumor development to adjacent tissues,

Table 1. Significance of the effects of lidocaine added to colon cancer cells and fibroblasts on the different protein pathways involved in apoptosis

Proteins	*p value*		
	CCD-18Co cells	*RKO cells*	*HCT-116 cells*
BAD	0.1415	0.0263*	0.0693
BAX	0.5289	0.5573	0.0466*
BCL-2	0.2018	0.5656	0.0161*
BCL-W	0.6135	0.3668	0.0262*
BID	0.1092	0.2253	0.0017**
BIM	0.3592	0.1112	0.0179*
CASPASE 3	0.0354*	0.0099**	0.0155*
CASPASE 8	0.3891	0.0233*	0.0173*
CD40	0.0194*	0.1233	0.0374*
CD40L	0.0232*	0.0177*	0.0709
cIAP-2	0.3298	0.0041**	0.2218
FasL	0.0045**	0.4096	0.0021**
HSP27	0.2578	0.0088**	0.0484*
HSP60	0.159	0.0468*	0.0181*
HSP70	0.0351*	0.0729	0.0237*
HTRA	0.0318*	0.0180*	0.0156*
IGF-I	0.0325*	0.2878	0.0490*
IGF-II	0.1979	0.0117*	0.0197*
IGFBP-1	0.4547	0.0009***	0.0062**
IGFBP-2	0.1528	0.0066**	0.0132*
IGFBP-3	0.285	0.0004***	0.1282
IGFBP-4	0.096	0.0081**	0.2624
IGFBP-5	0.7446	0.0114*	0.1026
IGFBP-6	0.4444	0.0228*	0.6335
IGF-1sR	0.8231	0.0102*	0.0444*
LIVIN	0.9252	0.0336*	0.158
P27	0.2321	0.0685	0.0264*
P53	0.4186	0.0492*	0.0340*
SMAC	0.4025	0.0312*	0.0073**
SURVIVIN	0.0558	0.0017**	0.0031**
sTNF-R1	0.0992	0.2763	0.0149*

*p <0.05, **p <0.01, ***p <0.001

while a pathological one can even favor tumor development and spread, while blocking therapeutic action [20].

The aim of our study was to investigate potential apoptotic effects of propofol and lidocaine on two different types of colon cancer cells vs tumor microenvironment cells represented in our study by fibroblasts and to identify potential mechanisms of action responsible for these apoptotic effects in case of lidocaine. We choose to further investigate lidocaine's cellular mechanisms, taking in consideration that in cell cultures the antiproliferative effects of both drugs were most significant after 48 hrs and in terms of clinical application, i.v. lidocaine infusion may be recommended for 48 hrs postoperatively.

Our results showed that both propofol and lidocaine have antiproliferative effects in colon cancer HCT-116 and RKO cells. Propofol exhibits antiproliferative effects in concentration of 2-4 mcg/mL, as those frequently used during total intravenous anesthesia. For propofol IC_{50} was lowest in HCT-116, 247.1 μM, while in RKO cells IC_{50} was 1038 μM, both smaller than IC_{50} in CCD-18Co cells, suggesting that antiproliferative effects may be tumor-dependent. Similarly, Mammoto et al. reported that in concentration between 1-5 mcg/mL propofol decreased the invasion ability of different human cancer cells, effects that were tumor-dependent [31]. Tsuchiya et al. also reported that propofol has apoptotic effects in HL-60 cells by activating both cell surface receptors and mitochondrial pathways [32].

For lidocaine IC_{50} was 948.6 μM in HCT-116 cells while in RKO cells this concentration was 2147 μM, greater as compared with the one in CCD-18Co, suggesting the same that antiproliferative effects are tumor-dependent. For propofol, these effects were significant at clinically relevant plasma concentrations 2-4 mcg/mL after 48 hrs of exposure.

Similar results on the apoptotic effects of lidocaine in different types of cancer cells have been reported by others [8,11-13]. Thus Sakaguchi et al. reported that lidocaine may suppress the proliferation in human tongue cancer cells [8] in concentrations compatible with those reached during inflitration, while in more recent studies antiproliferative effects of lidocaine have been reported in breast and hepatocarcinoma cells in different concentrations [11,13].

It is interesting that in co-cultures in our study lidocaine did not affect fibroblasts' proliferation while significant antiproliferative effects were visible in cancer cells. These effects were most visible after day 2 of exposure to lidocaine leading to clinical implications, that postoperative i.v. lidocaine infusion should last at least 48 hrs.

Regarding possible mechanisms of action responsible for these effects, our western blotting assays showed that lidocaine significantly triggered those protein pathways related to apoptosis. Thus, caspase 8, HSP 27 and 60, IGF-II, IGFBP1 and 2, p53, SMAC, SURVIVIN were significantly influenced by lidocaine in both types of colon cancer cells, while there are other types of proteins that are selectively influenced by lidocaine depending on tumor type: Bax, Bcl-2 and p27. Similar results regarding some of these mechanisms have been

reported by others [11,33]. However, our study investigated simultaneously a whole range of proteins involved in apoptosis in two types of colon cancer cells, demonstrating that the whole chain of apoptosis pathway may be activated depending on the tumor type.

We, like others [22], believe that these are additional mechanisms of action implicated in lidocaine's antiproliferative effects that complete a whole mosaic of actions [34,42].

One of the major limitations of our study is the lack of validation on *in vivo* setting. The effects of lidocaine in malignant cells when surrounded by a strong desmoplastic reaction is a topic of great interest nowadays. We choose to investigate the mechanisms for lidocaine's antiproliferative effects due to 48-h interval when these effects were significant, taking in consideration the possibility of administering lidocaine i.v. infusion for 48 hrs postoperatively. We have attempted to simulate the colon cancer microenvironment *in vitro* by creating a co-culture system of malignant cells and tumor-associated fibroblasts, but as we know, the *in vitro* setting might sometimes be very different from the far more complex *in vivo* setting. However, an intact microenvironment may protect tumor from spreading; lidocaine preserved fibroblasts while decreasing cancer cells proliferation.

The next step for validating our hypothesis would be to investigate the effects of lidocaine on a murine model of colon carcinoma. We have already developed a clinical trial (NCT02786329) in patients with colorectal carcinoma recruiting patients.

In conclusion our study showed that both propofol and lidocaine selectively inhibited colon cancer cells proliferation without affecting tumor microenvironment (cancer-associated fibroblasts). These antiproliferative effects are tumor- dose- and time-dependent. For lidocaine these effects may be at least partially explained by activation of apoptosis protein pathways, that complete a whole mosaic of actions. Further studies in animals and prospective randomized studies in humans are necessary to confirm these data and to evaluate the clinical impact of these effects.

Acknowledgements

The current research was financed by an internal grant of the Iuliu Hatieganu University of Medicine and Pharmacy, Grant Director dr.Tiberiu Tat (PCD contract 7690/109/2016).

Conflict of interests

The authors declare no conflict of interests.

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