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Carnitines Slow Down Tumor Development of Colon Cancer in the DMH-Chemical Carcinogenesis Mouse Model

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ABSTRACT

Dietary agents are receiving much attention for the chemoprevention of cancer. While curcumin is known to influence several pathways and affect tumor growth in vivo, carnitin and its congeners play a variety of important metabolic functions: are involved in the oxydation of long-chain fatty acids, regulate acyl-CoA levels and influence protein activity and stability by modifying the extent of protein acetylation. In this study we evaluated the efficacy of carnitines in the prevention of cancer development using the 1,2,-dimethylhydrazine (DMH)-induced colon carcinogenesis model. We also assessed whether their combination was able to give rise to increased protection from cancer development. Mice treated with DMH were dosed orally with curcumin and/or carnitine and acylcarnitines for 20 weeks. At the end of the treatment colon samples were collected, and scored for multiple ACF and adenomas. We observed that carnitine and acyl-carnitines had same, if not higher, efficacy than curcumin alone in inhibiting the formation of neoplastic lesions induced by DMH treatment. Interestingly, the combination of curcumin and acetyl-L-carnitine was able to fully inhibit the development of advanced adenoma lesions. Our data unveil the antitumor effects of carnitines and warrant additional studies to further support the adoption of carnitines as cancer chemopreventative agents. J. Cell. Biochem. 114: 1665–1673, 2013.

KEY WORDS: CURCUMIN; CARNITINE; GROWTH ARREST; APOPTOSIS; ANIMAL MODEL; COLON CANCER

Cancer is one of the leading cause of death worldwide, with a growing number of cases every year due to aging of the population, increased impact of environmental factors and improved diagnosis [Ferlay et al., 2010]. Lung, breast, colorectal, stomach, and prostate cancers cause the majority of cancer deaths and important risk factors for cancer include tobacco use, unhealthy diet, physical inactivity, and the harmful use of alcohol. Although about 30% of cancer occurrence can be prevented by avoiding certain risk factors, it is well known that another proportion of cancers can be prevented by modifying lifestyle and introducing certain nutrients in the diet. Hence, several naturally occurring substances, or nutraceuticals, have been explored for their ability to prevent or slow down cancer.

It is estimated that one third of all cancers are preventable simply by modification of diet, maintenance of optimum body weight, and regular physical activity or reducing exposure to environmental pollution [Surh, 2003; Wogan et al., 2004]. Chemoprevention, by definition, is a means of cancer control by which the occurrence of the disease can be entirely prevented, slowed down, or reversed by the administration of one or more naturally occurring and/or synthetic agents. Among naturally occurring substances, curcumin from the rhizome of the *Curcuma longa* plant has been proven to

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have a wide range of beneficial properties, including anti-inflammatory, antioxidant, chemopreventive activity, and inhibition of the growth of neoplastic cells. Curcumin inhibits cancer development and progression, targeting multiple steps in the pathway of malignant transformation [Hatcher et al., 2008]. Cytotoxic effect of this potential nutraceutical has been observed in vitro in several cultured tumor cell lines of the colon [Chen and Xu, 2005; Watson et al., 2010], medulloblastoma [Lee et al., 2011], prostate [Mukhopadhyay et al., 2001], head and neck [Wilken et al., 2011], breast [Mehta et al., 1997], ovarian [Lin, 2007] cancer. When evaluated in models of carcinogen-induced tumorigenesis in forestomach, duodenum, and colon of mice, curcumin was effective in reducing the number of induced tumors [Huang et al., 1994]. Moreover, curcumin has been used to inhibit formation of early neoplastic lesions in APC-Min/+ mouse, a model of familial adenomatous polyposis (FAP) where animals carry an autosomal dominant heterozygous nonsense mutation of the mouse *Apc* gene, homologous to human germ-line and somatic *APC* mutations [Perkins et al., 2002].

L-carnitine (LC) and its short chain derivatives are essential cofactors for mitochondrial transport and oxidation of long chain fatty acids, have a role in the modulation of stress response and also act as scavengers of oxygen free radical in mammalian tissue [Izgut-Uysal et al., 2007]. Acyl-carnitines play a role in the metabolism regulating acyl-CoA levels and influence protein activity and stability by modifying the extent of protein acetylation. Acetyl-L-carnitine (ALC) has been shown to exert a dual role in cancer: (a) a neuroprotective role against chemotherapy-induced peripheral neuropathy [Pisano et al., 2003]; (b) a direct anti-tumor activity through the potentiation of platinum based therapy which at least in part involves an increase of tumor suppressor p53 protein acetylation and stability [Pisano et al., 2010].

Colorectal cancer (CRC) is the third most common malignancy in the world and, in spite of the recent development of targeted therapies the 5-year survival rate of metastatic colon cancer is still below 10% [Ferlay et al., 2010]. Mice which develop spontaneous tumors either by physical-carcinogen induction or by genetic engineering have contributed to our understanding of the molecular pathways responsible for the initiation, progression, and metastasis of cancer cells and have extended our knowledge of the mechanistic role that oncogenes and tumor suppressor genes have in these processes. In these models, the interactions between tumor cells and their microenvironment are potentially more representative of specific human tumor histotypes than transplanted xenografts because of their in situ and autochthonous origin [Sharpless and Depinho, 2006]. One of the models used to asses efficacy of new drugs on colon cancer is the 1,2,-dimethylhydrazine (DMH) carcinogenesis rodent model. DMH can specifically induce in rodents colon cancer with molecular and histopathological features similar to those observed in human sporadic colon cancer. Hence, this system has been extensively used in the study of the molecular biology, prevention and treatment of colon cancer. After administration, DMH is metabolized into azoxymethane (AOM) and then methylazoxymethanol (MAM) by CYP2E1, which causes DNA mutations [Fiala et al., 1977]. Mutation of K-Ras activates this pathway and its downstream PI3K/Akt and MAPK pathways while mutations of APC and/or β-catenin also prevent the latter from being degraded by GSK-3 and accumulation of β-catenin leads to cell proliferation. TGFβ, a pro-apoptotic protein, is inhibited leading to uncontrolled growth of colorectal cells. All of these changes form the basis of DMH-induced carcinogenesis. Pathogenesis of induced colon cancer can be observed by the progressive formation of lesions of growing complexity. Starting from aberrant crypt foci (ACF) into adenomas and then colon carcinomas. This model has been extensively used in studies to evaluate and compare the efficacy of several agents, natural and synthetic, in preventing colon cancer occurrence [reviewed in Corpet and Tache, 2002].

In this study we analyze the effects of LC and acyl-carnitines (ALC and propionyl-L-carnitine (PLC)), in vitro on colon cancer cell line and in vivo, on DMH-induced mouse colon carcinogenesis. We evaluate a possible synergism with curcumin in preventing/inhibiting cancer cell growth. In vitro data show that curcumin inhibits the growth of HT-29, a colon cancer cell line, in a dose-dependent manner, as previously indicated by the literature and that the addition of carnitines sensitize HT-29 cell line to the action of curcumin. In vivo data show that LC and acyl-carnitines are as effective as curcumin in slowing down the development of chemically induced CRC and we obtained some indication that carnitines may increase the efficacy of curcumin treatment. Our study opens some possibilities for curcumin and carnitines for their future use as cancer preventive agents.

MATERIALS AND METHODS

CELL CULTURE

HT-29 colon cancer cell line was obtained from the American Type Culture Collection (ATCC) and maintained at 37°C in a humidified incubator with 5% CO_2 in DMEM (Sigma–Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

REAGENTS

DMH was purchased from Sigma–Aldrich and 4 mg/ml solution was freshly prepared by resuspending DMH in saline solution and bringing pH to 7 by 10 M NaOH. Curcumin extract (Sigma–Aldrich cat:C1386) and pure curcumin (Sigma–Aldrich cat:C7727) were purchased from Sigma–Aldrich. For in vitro study, 100 mM stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. LC, acetyl-L-carnitine, PLC were supplied by Sigma-Tau (Pomezia, Italy) and 1 M solutions were freshly prepared in serum free DMEM. For in vivo study, curcumin extract was dosed as suspension in 0.5% methylcellulose, where also LC and acyl-carnitine were dissolved for combinatorial treatment. When dosed alone, LC and acyl-carnitine were dissolved directly in 0.5% methylcellulose sterile solution.

CELL PROLIFERATION ASSAYS

Inhibition of cell proliferation was measured by colorimetric WST-1 assay. HT-29 cells were plated in triplicate in 96-well flat-bottom plates at a density of 2,000 cells/well and incubated overnight. Cells were cultured for an additional 72 h in the presence of fully supplemented culture medium alone, the DMSO vehicle, curcumin

(100–0.02 μM), or carnitines (100–0.02 mM). At the end of the experiment, cell survival was determined by WST-1 assay (Roche) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader and data were used to calculate CC_{50}, concentration of agent at which the cell growth is inhibited by 50% CC_{50} values were determined by SigmaPlot and data points are presented as the average value ± the standard deviation (SD).

ANNEXIN V APOPTOSIS ASSAYS

HT-29 cells were plated in 6-well tissue culture plates at a density of 10^5 cells/well and incubated 12–18 h. Cells were then treated with curcumin at 10 μM or various forms of canitines at 10 mM, alone or in combination, for 72 h. Then, cells were collected, washed and stained with Annexin V Alexa488 according to the manufacturer's protocol (Molecular Probe). Briefly, cells were washed twice with binding buffer and incubated in 100 μl labeling solution containing 5 μl Annexin V Alexa488 conjugate in the dark for 15 min at room temperature. Cells were further diluted with 400 μl of binding buffer containing 1 μg/ml of propidium iodide (PI). The fluorescence of the samples was detected by flow cytometry on FACS Calibur (Becton Dickinson) and data analyzed by FCS Express (De Novo Software).

ANIMALS

The animals used were 6-week-old male Balb/c mice (Charles River Laboratories, Inc.). After acclimatization for 1 week, they were housed six to a plastic cage and fed on basal diet (4RF24, Mucedola S.r.l.) with water ad libitum, in an animal facility controlled at a temperature of 23 ± 2°C, 60 ± 5% humidity, and with a 12 h light and dark cycle. At the end of the treatment period and before necropsy, mice were euthanized by compressed CO_2 gas in cylinder as indicated in the AVMA (American Veterinary Medical Association) Panel on Euthanasia and according to the guidelines described in UKCCCR [1998]. The experiments were conducted according to EU Directive EC86/609 on the protection of animals used for experimental and other scientific purposes, which was ratified by Italian Legislation with DL no. 116/92 on February 19, 1992. Approval of the study has been obtained by Takis Ethical Committee (n. TKEC-2010-02).

EXPERIMENTAL DESIGN

After 1-week acclimatization, six mice per group were stratified according to their body weight and assigned to one of the 11 groups (Table II). To induce tumor lesions mice were treated with intraperitoneal injection of DMH at weekly dosage of 20 mg/kg BW, for 6 weeks. One week after 1st DMH injection, mice were dosed with curcumin extract at 500 mg/kg BW, and carnitines at 200 mg/kg BW, alone or in combination, in 0.5% methylcellulose, daily by oral gavages, 5 days per week, and treatment is extended until experiment completion. Animal weight was monitored weekly. Twenty weeks after 1st DMH injection, mice were bled, euthanized, and colon samples collected.

PATHOLOGICAL EVALUATION

After dissection, colon samples were open longitudinally, placed on a sheet of paper and fixed overnight in 4% buffered formaldehyde (Sigma–Aldrich) at 4°C, washed twice in PBS and stored in 70% ethanol at 4°C. For histopathological determination of chemically induced lesions, colons were washed with PBS and stained with 0.1% methylene blue (Sigma–Aldrich) solution for 10 min at room temperature. Excess dye was rinsed off with PBS and colon segments were placed with mucosal side up on a microscopic slide. Lesions were quantified by a pathologist and scored as double ACF formed by two ACF, multiple ACF formed by three or more ACF, or early and late adenomas. Distinction between early and late adenomas were based on stage of development of adenomas and on the experience of the pathologist, with late adenomas being bigger than 1.5 mm in size and showing the evidence of invasion of the submucosa layer. Gross lesions, where present, were sectioned and paraffin embedded. When no evident lesions were present, the whole colon was paraffin embedded. Sections were hematoxylin and eosin (H/E) stained and evaluated by a pathologist.

IMMUNOHISTOCHEMISTRY

After pathological evaluation of ACF, gross lesions were sectioned and paraffin embedded for immunohistochemical analysis. After rehydratation, tissue sections were incubated with primary antibody against β-catenin (rabbit monoclonal antibody diluted 1:200; Abcam) and binding visualized using a labeled streptavidin biotin kit (Dako), following manufacturer instruction. Membrane staining for β-catenin was interpreted as a normal staining pattern which was observed in all of the normal colon epithelial specimens. Cytoplasmic or nuclear staining for β-catenin was interpreted as an abnormal staining pattern.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM unless otherwise indicated. Differences between means of the groups were analyzed with the use of a two-tailed unpaired Student's *t*-test or two-way ANOVA test. When required, *P* values are stated in the figure legends.

RESULTS

INHIBITION OF IN VITRO PROLIFERATION OF COLORECTAL CANCER CELLS BY CURCUMIN IS ENHANCED BY CARNITINE

To determine the effect on cell proliferation by carnitines and their combination with curcumin, human colon cancer HT-29 cells were treated with increasing concentrations of curcumin extract in presence or absence of carnitines (10 mM). The effect of single carnitines was also evaluated. After 72 h of treatment, cell viability was assessed by WST-1 assay and absorbance at 450 nm was measured using an ELISA reader. CC_{50} values were determined and the shift was calculated as fold sensitization (CC_{50} of curcumin alone/curcumin plus carnitines treatment). In the presence of complete medium (10% FCS) only PLC and a mix containing it, was able to sensitize HT-29 to curcumin treatment (Fig. 1A). Since the presence of carnitine in the serum might be competing with or inhibiting the uptake of exogenous carnitines, we performed the assay also at low serum concentration. When the assay was performed in these conditions (0.1% FCS) all the tested carnitines displayed the ability to sensitize HT-29 cell to curcumin treatment (Fig. 1C and Table I). The fold sensitization ranged from 1.28 to 1.89

TABLE I. Fold Sensitization of Combinatorial Treatment in HT-29 Colon Cancer Cell Line

	0.1% FCS		10% FCS	
	Fold sensitization	CC_{50} (μM)	Fold sensitization	CC_{50} (μM)
Curcumin	–	11.0	–	15.5
Curcumin + LC	1.3	8.5	0.7	21.9
Curcumin + ALC	1.6	6.9	1.1	14.7
Curcumin + PLC	1.9	5.8	1.3	11.6
Curcumin + LC + ALC + PLC	1.3	8.6	1.4	11.4
Curcumin extract	–	8.5	–	11.2

LC, L-carnitine; ALC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.
CC_{50} is the concentration of curcumin at which the cell growth is inhibited by 50%. CC_{50} values were determined by SigmaPlot. Fold sensitization has been calculated as CC_{50} of curcumin alone/CC_{50} curcumin + carnitine.

TABLE II. Experimental Plan and Dosage Schedule

	Treatment	Dosage
Group 1	L-carnitine	200 mg/kg
Group 2	L-acetyl-carnitine	200 mg/kg
Group 3	Propionyl-L-carnitine	200 mg/kg
Group 4	LC-ALC-ALP	(66 + 66 + 66 mg/kg) 200 mg/kg total
Group 5	Curcumin	500 mg/kg
Group 6	Curcumin + LC	500 mg/kg + 200 mg/kg
Group 7	Curcumin + ALC	500 mg/kg + 200 mg/kg
Group 8	Curcumin + PLC	500 mg/kg + 200 mg/kg
Group 9	Curcumin + LC-ALC-PLC	500 mg/kg + (66 + 66 + 66 mg/kg) 200 mg/kg total
Group 10	Control	
Group 11	Not DMH treated	

Colon lesions were induced by weekly intraperitoneal injection of 1,2-dimethylhydrazine (DMH) at a dosage of 20 mg/kg for a total of six injections (6 weeks total). After 1 week from first DMH injection, six mice per group were allocated based on their weight and dosed as indicated by oral gavages, 5 days per week, for 20 weeks after first DMH treatment. At the end of the study mice were sacrificed and colons collected.

for all carnitines. These data suggest a potential cooperative effects of these substances.

EVALUATION OF CARNITINES AS ENHANCER IN CURCUMIN-INDUCED APOPTOSIS IN HT-29 CELL LINE

To study the mechanism of curcumin sensitization by carnitines, apoptosis induction by curcumin alone was first evaluated in a time-course experiment where HT-29 cells were treated with increasing amounts of curcumin for 24, 48, and 72 h. Cells were collected at the indicated time points and apoptosis was quantified by Annexin V/PI staining and FACS analysis. Maximal induction of apoptosis (Fig. 2) was observed at 72 h with 10 μM curcumin. Then apoptosis induction was evaluated in combination treatments in which HT-29 cells were treated with 10 μM curcumin or 10 mM carnitines, alone or in combination for 72 h. Carnitines had no significant effects on the percentage of apoptotic cells, with the exception of

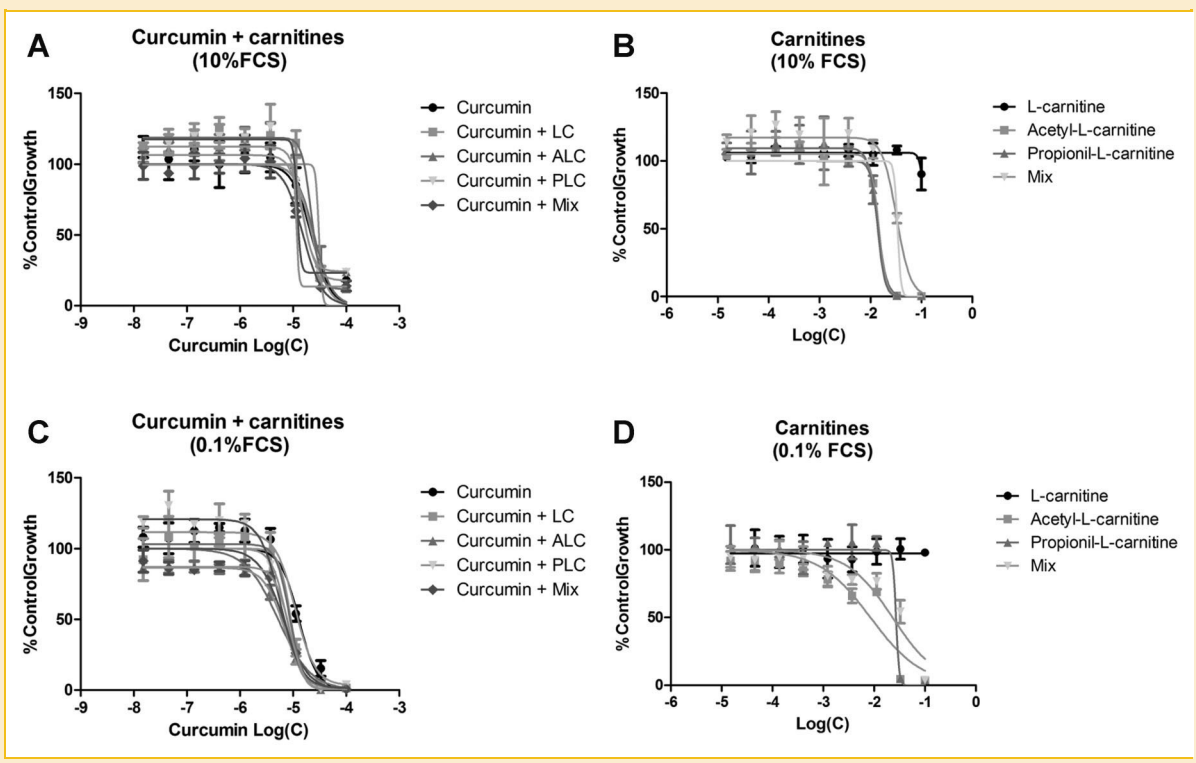

Fig. 1. Effect of curcumin and carnitines on HT-29 proliferation in standard (A,B) and low (C,D) serum conditions. A: Cells were treated with increasing amount of curcumin in the presence or absence of carnitines for 72 h. The addition of carnitines increases the efficacy of curcumin. B: Effect of single carnitines. Carnitines alone have different effect on HT-29 proliferation. C: Effect of increasing amount of curcumin in the presence or absence of carnitines for 72 h in low serum. D: Treatment with single carnitines in low serum conditions. The data shown here are an average ± SD of three replicate wells and are representative of three independent experiments.

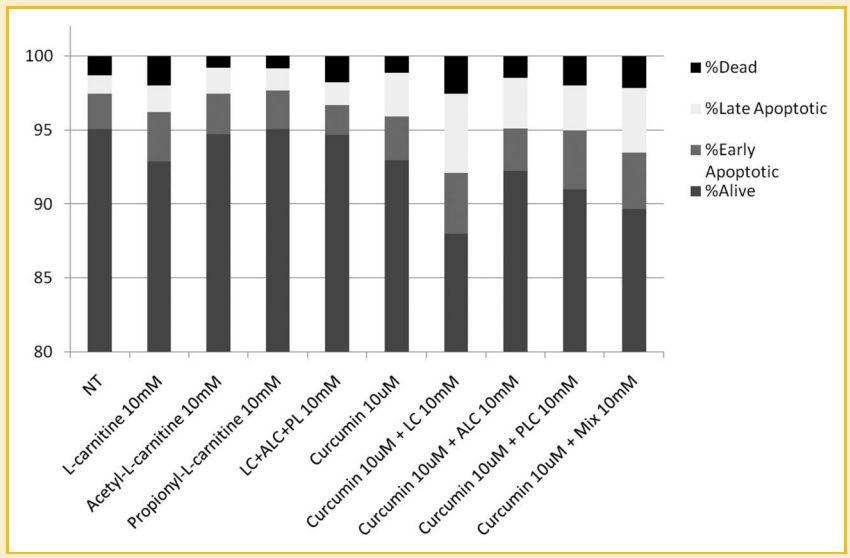

Fig. 2. Induction of apoptosis in HT-29. Apoptosis induction by curcumin and carnitine was evaluated by Annexin V/PI staining as described in the text. Data shown here are representative of three independent experiments.

some induction of apoptosis by LC. However, a sizeable increase of the apoptotic population was constantly observed with the combined treatment of carnitines and curcumin (Fig. 2). The most potent effect was seen, again, when cells were co-treated with curcumin and LC. All together these data indicate an additive effect of carnitines in increasing apoptosis in CRC cells when treated in vitro with curcumin.

EFFECT OF CURCUMIN AND CARNITINE, ALONE OR IN COMBINATION, ON DMH-INDUCED LESIONS

To assess the in vivo antitumor effects of curcumin and carnitines, colon cancer was induced in mice by use of the carcinogen DMH and animals were provided with daily oral administration of the compounds starting at 1 week after the first DMH injection (see the Material and Methods Section and Table II). Mice weight was monitored weekly through the entire duration of the study and no weight loss or differences among experimental groups were observed (not shown). Most of the animals were healthy throughout the duration of the experiment and no occurrence of death could be attributed to dosage or route of administration. At the end of the study (week 20), animals were euthanized and colon tissue was taken for lesion count and histopathological characterization.

Flat preparation of the colons were evaluated to quantify the development of both pre-cancerous (double and multiple ACF) and cancerous (early and late adenomas) lesions (Table III). This analysis confirmed the capability of curcumin to prevent cancer development. Most importantly carnitine and acyl-carnitines were able to prevent the formation of cancer lesions with a similar or even higher

TABLE III. Effect of Curcumin and Carnitines on DMH-Induced Colon Carcinogenesis in Balb/c Mice

	Lesions/animal (% inhibition)			
Experimental group	Double ACF	Multiple ACF	Early adenomas	Late adenomas
Vehicle	6.60 ± 0.37[a]	4.80 ± 0.89	4.80 ± 0.89	1.80 ± 0.45
L-carnitine	3.50 ± 0.41 (47)[c]	3.00 ± 0.47 (37)[b]	2.75 ± 0.20 (43)	0.75 ± 0.39 (58)
Acetyl-L-carnitine	4.50 ± 0.22 (32)[c]	3.17 ± 0.60 (34)	3.33 ± 0.67 (31)	0.83 ± 0.54 (54)
Propionyl-L-carnitine	4.67 ± 0.21 (29)[c]	3.33 ± 0.61 (31)	1.83 ± 0.60 (62)[e]	0.60 ± 0.37 (67)[e]
LC-ALC-PLC	4.00 ± 0.26 (39)[c]	2.67 ± 0.33 (44)[e]	2.67 ± 0.42 (44)[e]	0.17 ± 0.12 (91)[d]
Curcumin	4.00 ± 0.29 (39)[c]	3.80 ± 0.45 (21)	2.40 ± 0.55 (50)[e]	0.60 ± 0.22 (67)[e]
Curcumin + LC	5.00 ± 0.52 (24)[e]	2.67 ± 0.33 (44)[f]	2.33 ± 0.33 (51)[e]	0.67 ± 0.21 (63)[e]
Curcumin + ALC	4.17 ± 0.48 (37)[d]	3.33 ± 0.61 (31)	2.33 ± 0.71 (51)[e]	**0.00 ± 0.00 (100)**[f]
Curcumin + PLC	4.50 ± 0.72 (32)	3.50 ± 0.34 (27)	2.83 ± 0.31 (41)[e]	0.83 ± 0.31 (54)[e]
Curcumin + LC-ALC-PLC	4.60 ± 0.22 (30)[d]	5.40 ± 0.74 (0)	3.60 ± 0.37 (25)	1.20 ± 0.34 (33)

LC, L-carnitine; ALC, acetyl-L-carnitine; PLC, propionyl-L-carnitine.
[a]Mean ± SEM.
[b]% Inhibition from vehicle treated group is shown in parenthesis.
[c]Significantly different from vehicle treated group, $P < 0.0005$.
[d]Significantly different from vehicle treated group, $P < 0.005$.
[e]Significantly different from vehicle treated group, $P < 0.05$.
[f]Significantly different from curcumin treated group, $P < 0.05$.

efficacy than curcumin alone. In particular, the mixture of the three carnitines (LC, ALC, PLC) was efficacious in inhibiting the formation of lesions through all the steps of tumor development and progression: from double ACF, to multiple ACF and to early and late adenomas. Oddly, the efficacy of the mixture was reduced when dosed in combination with curcumin extract. Probably the combination was deleterious for the bioavailability of both carnitines and curcumin and further experiments are needed to confirm this hypothesis.

LC and acyl-carnitines were as efficacious as curcumin in prevention of double ACF, which are considered the first step of colon tumor formation. Maximal inhibition of double ACF was observed in the group treated with LC (47%) while curcumin extract inhibited double ACF formation of 39%. No increase in efficacy was observed in the combinatorial treatment of curcumin with each of the different forms of carnitine.

A significant inhibition of multiple ACF formations was observed only in the group treated with the mixture of the three carnitines. In the combinatorial treatment, the addition of LC significantly enhanced the efficacy of curcumin extract in preventing the formation of this type of lesions.

Early adenomas formation was significantly affected in the groups treated with curcumin, PLC, and carnitines mix in the single treatment settings, while no increase of efficacy was observed in the combinatorial treatment adding the different forms of carnitines to curcumin extract dosage. Statistical analysis revealed differences of efficacy among the different treatments at different tumor stages.

Late adenomas represent an advanced stage of oncogenic transformation which will inevitably evolve into invasive adenocarcinoma if not excised or treated efficaciously. Late adenomas were more strongly inhibited when mice were treated with curcumin alone, PLC alone, and the carnitines mix while in the combinatorial treatment a strong additive, if not synergic, activity was observed in the group treated with ALC and curcumin extract. In this group, no late adenomas were observed in any of the mice analyzed (Fig. 3).

PATHOLOGICAL EVALUATION ON LATE STAGE ADENOMAS

To assess the effect of curcumin and carnitines on the progression of late stage colon adenomas, H/E stained sections from gross lesions or whole colons were evaluated and tumors from each experimental group were analyzed. Macroscopically, the neoplasms induced by DMH treatment were of superficial or protrudent type. Histologically, they were tubular adenomas or tubular adenocarcinomas. Most of the adenocarcinomas invaded the submucosa layer of the colon and dysplastic lesions were also observed in the colonic mucosa surrounding the tumors (data not shown). Adenomas in mice treated with curcumin or canitines showed no macroscopic difference with untreated groups. No high grade adenomas or adenocarcinomas were found in the group treated with the combinatorial treatment (Table III). These data, taken together, confirm a strong additive, if not synergic, antitumor effect of curcumin and carnitines.

At the molecular level, rodent models of colon carcinogenesis display the same multiple genetic alterations found in humans. In particular, altered cellular localization of β-catenin protein was observed in dysplastic ACF, adenomas and adenocarcinomas, indicating that activation of Wnt signaling by accumulation of β-catenin is a major mechanism in the AOM-induced rodent colon carcinogenesis model, as occurs in human colon cancer [Takahashi et al., 2000]. Curcumin has been shown to inhibit proliferation and metastasis and to induce apoptosis in CRC by modulating several different signal pathways, including Wnt signaling [Sarkar et al., 2010]. We have analyzed β-catenin expression and localization in paraffin embedded colon section by IHC and found that also in our

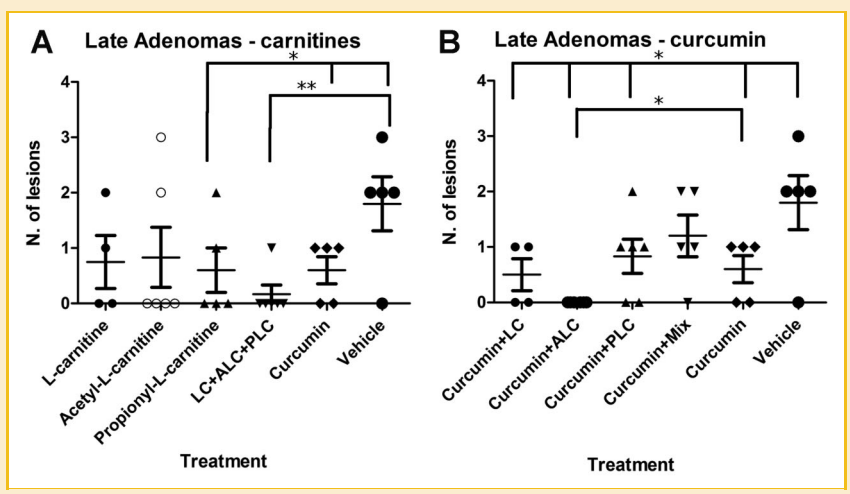

Fig. 3. Late adenoma scoring. A: Single treatment groups. Carnitines have similar or higher efficacy than curcumin alone in inhibiting adenoma formation. B: Combinatorial treatment. Acetyl-L-carnitine enhances the efficacy of curcumin in preventing the formation of adenomas. $^{*}P < 0.05$ versus vehicle or curcumin alone, $^{**}P < 0.005$ versus vehicle.

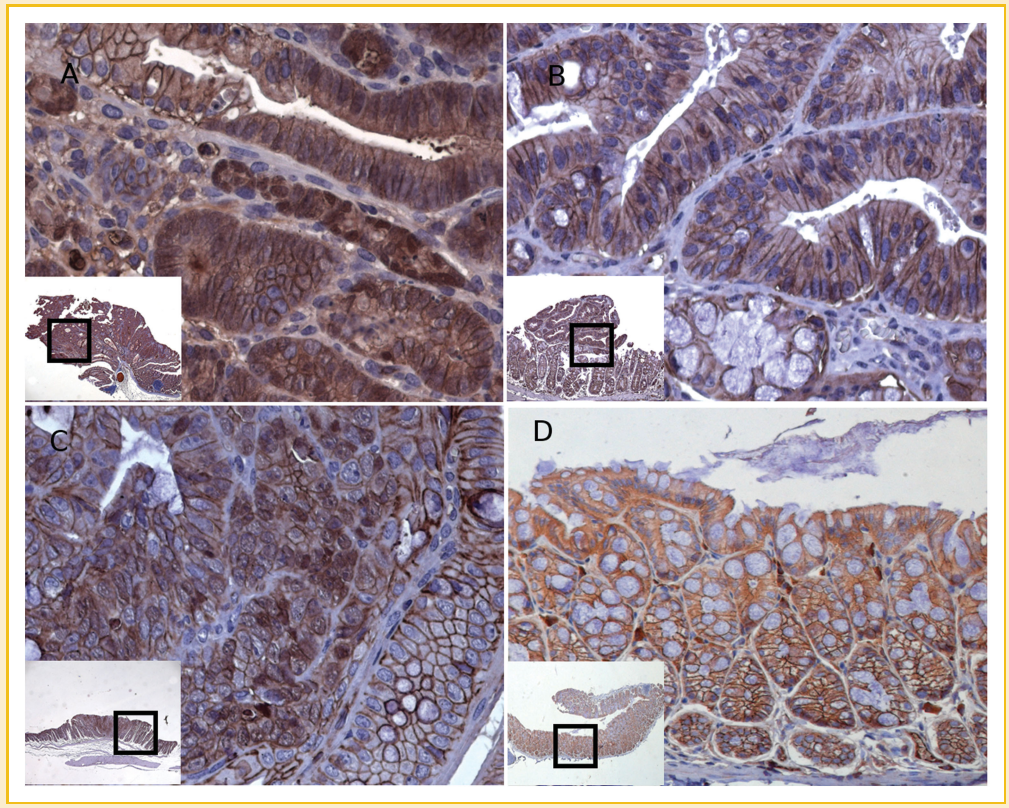

Fig. 4. Representative β-catenin immunohistochemistry of adenomas developed in the groups treated with acetyl-L-carnitine (A), curcumin extract (B), and vehicle alone (C). Nuclear/cytoplasmic accumulation β-catenin can be observed in the groups treated with acetyl-L-carnitine or vehicle alone, while aberrant accumulation of β-catenin is inhibited in curcumin treated groups (B). The group treated with the combination of the two drugs display correct localization of β-catenin expression (D) (400× magnification, inserts 25× magnification).

case curcumin treatment inhibited nuclear translocation and accumulation of β-catenin (Fig. 4).

DISCUSSION

Among nutraceuticals, curcumin has shown antitumor effects in a variety of models. It is believed that cancer arises from stem cells and/or progenitor cells through the dysregulation or acquisition of self-renewal. Interestingly, it was found that curcumin could inhibit mammosphere formation and could also decrease the amount of aldehyde dehydrogenase-positive cells in normal and malignant breast cells through the inhibition of Wnt signaling [Kakarala et al., 2010].

The carnitine system, consisting of LC, carnitine esters, specific enzymes and membrane transporters, plays a central role in energy metabolism [Bremer, 1983; Bieber, 1988; Steiber et al., 2004; Calabrese et al., 2006]. In particular, ALC is implicated in the regulation of the acetyl-coenzyme A (acetyl-CoA) level, resulting in an increased ratio of acetyl-CoA to CoA [Steiber et al., 2004; Calabrese et al., 2006]. At the nuclear level, ALC provides a source of acetyl groups for nuclear protein acetylation by histone acetyl-transferases [Madiraju et al., 2009]. Acetylation of the transcription factor p53 by histone acetyl transferase p300/CBP has been reported to enhance its transcriptional activity [Ito et al., 2001; Tang et al., 2008]. Pisano et al. have recently shown that ALC was effective in enhancing the antitumor potential of platinum compounds in wild-type p53 tumors. ALC alone and in combination with a histone deacetylase inhibitor, exhibited an outstanding antimetastatic activity.

In this study, we have evaluated the efficacy of carnitines and curcumin in colon cancer proliferation and development of spontaneous colon cancer by identification and quantitation of ACF in mouse colon whole mount since ACF are pre-neoplastic lesions considered specific biomarkers for colon carcinogenesis. We found that carnitines were able to potentiate curcumin antiproliferative effects in vitro (Fig. 1) and this effect was also associated to increased apoptotic index in HT-29 cell culture treated with the curcumin–carnitines mixture. In the Annexin V assay, a reduction of live cells, in parallel with an increase of dead cells population, was observed when carnitines where added to the curcumin treatment. In the in vitro apoptosis assay, LC showed a great potential in sensitizing colon cancer cells to curcumin treatment. Most interestingly, a significant impact on tumor initiation and progression was observed in DMH treated mice (Table III). The effect of single carnitine and acyl-carnitines treatment equal or

exceed the one with curcumin in the different stages of induced colon lesions. The mixture of the three carnitines (LC, ALC, PLC) was efficacious in inhibiting the formation of lesions through all the steps of tumor development and progression. Late adenomas, the lesions that have the higher probability to evolve into metastatic cancer, are mostly inhibited when treated with curcumin alone, or propionyl-L-carnitine alone, or carnitines mix. The most interesting data was observed when mice are treated with the combination of ALC and curcumin. Colon derived from these mice did not show any late adenomas lesions and histopathological analysis of the colons confirmed these observations (Table III). With the aim to determine the molecular events involved in the chemoprevention of chemically induced lesions, we have analyzed β-catenin and p53 expression and their localization. The absence of gross lesions in the group treated with the combination of ALC and curcumin, have limited our analysis only to the groups treated with the single agents. As expected, we have found that curcumin treatment alone interferes with the aberrant β-catenin localization in the nucleus or in the cytoplasm, which is one of the major driver of neoplastic transformation in the colon. Likewise we have analyzed the expression of p53 in the neoplastic lesion, but no differences have been observed between the groups treated with carnitines or curcumin and the non-treated group (data not shown). Our explanation is that, in this model, ALC is impacting tumor progression in other ways rather than stabilizing p53 expression. In fact, in vitro studies in a previous report have shown an increase of Caspase-3 activity in HT-29 cells when treated with palmitoyl-carnitine and LC [Wenzel et al., 2005], and in this case apoptosis seems to be induced by elevated ROS levels due to accelerated fatty acid oxidation.

In conclusion, in this study we show a preventive effect of carnitines in a pre-clinical model that resemble sporadic human CRC. Our findings, albeit preliminary, warrant further studies to elucidate the mechanisms of action of carnitines as cancer preventative agents also in the light of their excellent safety and tolerability profile.

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