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RESEARCH ARTICLES

# Chemopreventive effects of the dietary histone deacetylase inhibitor tributyrin alone or in combination with vitamin A during the promotion phase of rat hepatocarcinogenesis☆

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**Abstract**

The chemopreventive effects of tributyrin (TB) and vitamin A (VA), alone or in combination, were investigated during the promotion phase of rat hepatocarcinogenesis. Compared to diethylnitrosamine control rats, TB and TB+VA-treated rats, but not VA-treated rats, presented a lower incidence and mean number of hepatocyte nodules and a smaller size of persistent preneoplastic lesions (pPNLs). In addition, TB and TB+VA-treated rats exhibited a higher apoptotic body index in pPNL and remodeling PNL, whereas VA-treated rats presented only a higher apoptotic body index in remodeling PNL. None of the treatments inhibited cell proliferation in PNL. TB and TB+VA-treated rats, but not VA-treated rats, exhibited higher levels of H3K9 acetylation and p21 protein expression. TB and VA-treated rats exhibited increased hepatic concentrations of butyric acid and retinoids, respectively. Compared to normal rats, diethylnitrosamine control animals exhibited lower retinyl palmitate hepatic concentrations. All groups had similar expression levels and exhibited similar unmethylated *CRBP-I* promoter region in microdissected pPNL, indicating that epigenetic silencing of this gene was not involved in alteration of retinol metabolism in early hepatocarcinogenesis. Data support the effectiveness of TB as a dietary histone deacetylase inhibitor during the promotion phase of hepatocarcinogenesis, which should be considered for chemoprevention combination strategies.


*Keywords:* Chemoprevention; Hepatocarcinogenesis; Tributyrin; Vitamin A; Epigenetic

## 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. The prognosis of patients with the disease is poor, with a 5-year recurrence rate exceeding 70% [1]. Thus, it is important to develop more effective strategies for the chemoprevention of HCC [2].

Epigenetic mechanisms such as DNA methylation and histone acetylation that are potentially reversible and susceptible to external stimuli are a good target for HCC chemoprevention [3]. The ability of dietary compounds to de-repress epigenetically silenced genes during carcinogenesis has important implications for cancer prevention and therapy [4–6]. Butyric acid (BA), which can be produced during dietary fiber fermentation by endogenous intestinal bacteria, exhibited anticarcinogenic effects by acting as a histone deacetylase inhibitor (HDACi) [7]. It has been already used in clinical trials for cancer treatment [8,9]; however, its short half-life limits its therapeutic application [10]. Recently, tributyrin (TB), a prodrug of BA [11] present in milk fat and honey [11,12], which possesses an increased half-life, has been shown to act as a chemopreventive HDACi when continuously administered during the initiation and promotion phases of hepatocarcinogenesis [13].

Retinoids are recognized as a powerful class of compounds for cancer control because of their essential role in normal cell proliferation and differentiation [14]. They may also have a place in HCC treatment and prevention [14,15]. When administered during initiation and promotion [16], as well as during progression [17] of hepatocarcinogenesis, vitamin A (VA; retinol) exhibited inhibitory activity. Retinol has been shown to modulate epigenetic mechanisms [18]; however, studies that have investigated that the

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implications of these effects for hepatocarcinogenesis chemoprevention are limited [17].

The most effective chemopreventive intervention would be at the promotion phase, which can be interrupted and, in some cases, reversed [19]. We sought to investigate the chemopreventive potential of TB or VA when administered alone, specifically during the promotion phase of rat hepatocarcinogenesis. Furthermore, since a combination of HDACi and retinoids has been proposed as a promising epigenetic anticancer strategy [20,21] and has been evaluated in clinical trials [22], we also investigated the effects of combining both compounds. Evaluated parameters included preneoplastic lesion (PNL) development, cell proliferation, apoptosis and hepatic BA and retinoid levels, as well as histone H3K9 acetylation, p21 expression and cellular retinol-binding protein (CRBP-I) promoter methylation and expression as molecular epigenetic targets.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

2-Acetylaminofluorene, diethylnitrosamine, 3,3-diaminobenzidine and diethyl pyrocarbonate were purchased from Sigma (St. Louis, MO, USA). The 98% 1,3di(butanoyloxy)propan-2-yl butanoate (TB) was purchased from Aldrich (St. Louis, MO, USA). Maltodextrin was purchased from Nestlé (São Paulo, Brazil). VA (retinyl palmitate, 97%) was purchased from BASF (Ludwigshafen, Germany). Corn oil was purchased from Mazola (São Paulo, Brazil), and the commercial diet was purchased from Purina (Campinas, Brazil). Anti-placental glutathione *S*-transferase (anti-GST-P; rabbit) antibody was purchased from Medical and Biological Laboratories Co. (Nagoya, Japan). Antiproliferating cell nuclear antigen (anti-PCNA; mouse) and anti-acetyl-histoneH3-Lys9 (rabbit) antibodies were purchased from Millipore (Billerica, MA, USA). Anti-p21 and anti-β-actin (mouse) antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Secondary biotinylated antibody and streptavidin–biotin–peroxidase complex (StrepABComplex/HRP Duet, mouse/rabbit) were purchased from Dako (Glostrup, Denmark). Illustra RNAspin Mini RNA Isolation kit was purchased from GE Healthcare (Chicago, IL, USA). The Wizard DNA Clean-Up System was purchased from Promega (Fitchburg, WI, USA). SuperScript II reverse transcriptase and the Platinum Taq DNA Polymerase kit were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals obtained were of the highest available quality.

### 2.2. Animals and experimental protocol

Male Wistar rats (~50 g) from a colony at the Faculty of Pharmaceutical Sciences of the University of São Paulo (São Paulo, Brazil) were maintained under standard environmental conditions. The animals had free access to industrialized food. With the exception of 10 rats that were not submitted for any experimental procedure (N group), 48 rats were used for the resistant hepatocyte (RH) model of hepatocarcinogenesis [23] as follows: animals received an intraperitoneal dose of diethylnitrosamine [20 mg/100 g body weight (b.w.)] for initiation. After a recovery period of 2 weeks, the initiated hepatocytes were selected/promoted by five intragastric doses of 2-acetylaminofluorene. The first four doses (2 mg/100 g b.w.) were administered on 4 consecutive days before a partial (2/3) hepatectomy was performed [24], and the remaining dose (0.5 mg/100 g b.w.) was administered on day 4 after the hepatectomy. One week after hepatectomy, the animals were treated for 5 consecutive weeks with the following: TB (TB group: 200 mg/100 g b.w.) daily; VA (VA group: 1 mg/100 g b.w.) dissolved in corn oil (0.25 ml/100 g b.w.) on alternate days or TB plus VA (TB+VA group: same TB and VA dosages). These doses were based on previous studies [13,16,17] that showed inhibitory actions by these compounds in rats submitted to the RH model. Animals receiving corn oil (0.25 ml/100 g b.w.) plus maltodextrin (300 mg/100 g b.w. - isocaloric to TB group) during the same period were used as controls (CO group). The animals of TB and VA groups also received corn oil (0.25 ml/100 g b.w.) or maltodextrin (300 mg/100 g b.w.), respectively (Fig. 1). The study was approved by the Faculty of Pharmaceutical Sciences of the University of Sao Paulo Ethics Committee for Animal Research (Protocol 25).

### 2.3. Visible hepatocyte nodule counting

After euthanasia, the liver from each animal was removed, weighed and grossly examined specifically on the surface for the presence of visible hepatocyte nodules of varied sizes. Hepatocyte foci were considered PNLs when they were smaller than one hepatic lobule, whereas hepatocyte nodules comprised spherical PNL that were larger than one or more hepatic lobules [25].

### 2.4. Double-staining Immunohistochemistry for GST-P and PCNA

Serial histological sections of liver samples fixed in methacarn solution were stained with an anti-PCNA antibody (1:400) and 3,3-diaminobenzidine and were costained with anti-GST-P antibody (1:500) and Fast Red. Hepatic PNL that labeled uniformly and nonuniformly for GST-P was classified as persistent (pPNL) and remodeling (rPNL), respectively [26]. The Image Pro Plus program (Media Cybernetic, Bethesda, MD, USA) using an Olympus photomicroscope (Tokyo, Japan) connected to a microcomputer was used for the analysis that was blindly conducted. Quantification was conducted by scanning the entire slide. Specificity of staining was controlled by omitting the primary antibody or by substituting it for nonimmunized goat serum. In all cases, the control resulted in an absence of staining. The reproducibility of the methods was confirmed by staining multiple slide sections from the same animal.

### 2.5. Apoptosis evaluation

Hepatic apoptotic bodies (ABs) were quantified by fluorescence microscopy as described [27,28], using a Nikon microscope (Tokyo, Japan) equipped with an epifluorescence unit. Identification of ABs was confirmed using the morphological criteria established by Goldsworthy et al. [29]. Quantification of ABs was blindly conducted by scanning the entire slide.

### 2.6. H3K9 histone acetylation

Acid extraction of histones and Western blot for H3K9 acetylation analysis were performed as described by Kuroiwa-Trzmielina et al. [13]. To quantify band intensities, a Bio-Rad densitometer (Imaging Densitometer, Model GS-700; Bio-Rad, Los Angeles, CA, USA) with quantification software (Molecular Analyst; Bio-Rad) was used. As a loading control for the relative amounts of the protein of interest, Western blots were normalized with staining of the nitrocellulose membrane with Coomassie blue [13,30].

### 2.7. p21 Western blot analysis

Total protein extraction and Western blot for p21 analysis were performed as described by Kuroiwa-Trzmielina et al. [13]. To quantify the band intensities, the Bio-Rad densitometer (Imaging Densitometer, Model GS-700) with quantifying software (Molecular Analyst) was used. For equal loading control of the relative amount of the protein of interest, Western blots were normalized against the β-actin signal [13].

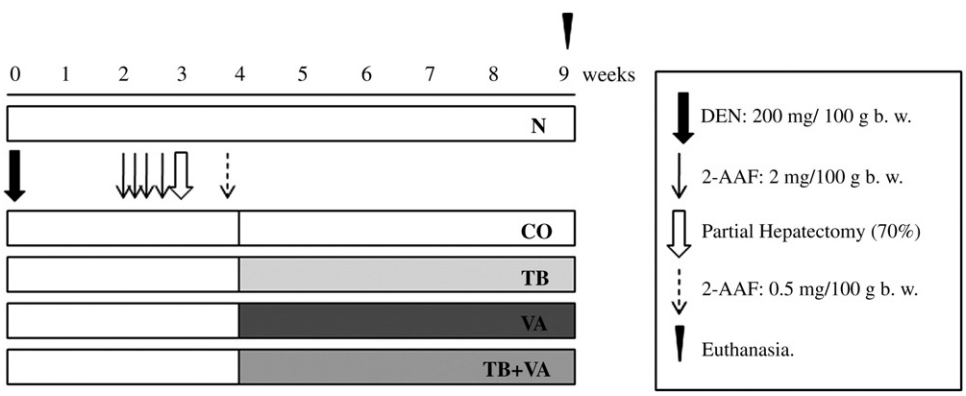


Fig. 1. Experimental design.

*2.8. Retinol, retinyl palmitate and BA hepatic quantification*

One gram of liver was used to determine the hepatic concentrations of retinol and retinyl palmitate [16] according to Stahl et al. [31] and Murray et al. [32]. The samples were analyzed in a chromatograph (model LC9A; Shimadzu, Tokyo, Japan) with a multisolvent pumping system, an auto injector (model SIL-6B) and a photodiode array UVVIS detector (model SPD-M6A), using a C18 reverse-phase column (CLC-ODS; 4.6 mm × 25 cm and 0.5 mm). Retinoids were detected at 325 nm, identified by comparison of their retention times to those of authentic standards and quantified by peak areas. BA was extracted according to Kuroiwa-Trzmielina et al. [13]. Samples and standards were injected into a GC-MS system (HP6890 model; Agilent, Palo Alto, CA, USA) equipped with a CP Wax 58 - FFAP (Varian 25 m × 0.32 m × 0.2 μm) column.

*2.9. Microdissection of pPNL*

For microdissection, 10-μm sections were cut from frozen liver on a cryostat. Sections were immediately stained with hematoxylin-eosin and dehydrated in graded ethanol and xylene [33]. All solutions were prepared with diethyl pyrocarbonate water. Microdissection was performed using a commercial microdissection device (Micro-Dissector; Eppendorf AG, Hamburg, Germany) connected to an inverted microscope (Axiovert 35M; Carl Zeiss, Oberkochen, Germany). One milligram each of pPNL and of the surrounding tissue was microdissected per animal.

*2.10. CRBP-I expression*

One hundred nanograms of total RNA extracted from microdissected pPNL and surrounding tissue with Illustra RNAspin Mini RNA Isolation kit were quantified using a Nanodrop spectrophotometer (Thermo Scientific, Rockford, IL, USA) and reverse transcribed with 1 unit of SuperScript II reverse transcriptase. This RNA input was chosen after preliminary experiments demonstrated a dose-dependent increase in CRBP-I RT-PCR product. Two microliters of complement DNA template were submitted to polymerase chain reaction (PCR) reaction with 5 μl of 10× reverse transcriptase (RT) PCR buffer (2.5 mM $MgCl_2$ final concentration), 1 μl of deoxyribonucleotides (dNTP) mix (10 mM of each dNTP), 1 μl of forward and reverse gene-specific primers (10 mM), 1.25 unit PlatinumTaq DNA Polymerase, and PCR grade water to make a total volume of 50 μl. The PCR conditions were as follows: RT reaction was performed at 50°C for 50 min, and initial PCR activation was performed at 95°C for 15 min, followed by 35 cycles (exponential phase of PCR reaction)—[94°C (denaturation) for 30 s, 56°C (annealing) for 75 s and 72°C (extension) for 30 s], with a 10-min final extension at 72°C. The PCR primers used were 5′-TTGTGGCCAAACTGGCTCCA-3′ (forward) for exon 1 and 5′-ACACTGGAGCTTGTCTCCGT-3 (reverse) for exon 3 of CRBP-I, which amplify a 320-base pair product [34]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a positive control [34]. PCR products were separated in 2% agarose gels, and DNA was visualized by ethidium bromide staining. Densitometric analysis of the bands was performed with Bio-Rad Molecular Analyst software.

*2.11. DNA extraction and bisulfite modification*

DNA extraction from microdissected pPNL was performed according to the phenol/chloroform protocol [35], with some modifications. One microgram of DNA (per sample) was denatured by the addition of 1 μl of herring sperm DNA (10 mg/ml) and 2 μl of NaOH (3 mol/l) and was heated to 50°C for 20 min. DNA was subsequently treated with 500 μl of a sodium bisulfite (2.5 mol/l)–hydroquinone (1 mol/l)–NaOH (2 mol/l) solution and heated to 70°C for 3 h in the dark. After this treatment, a Wizard DNA cleanup system was used for purification of the DNA, according to the manufacturer's instructions. The purified DNA was precipitated with 75 μl ammonium acetate (5 mol/l), 350 μl of 100% frozen ethanol and 1 μl of 20% glycogen (20 mg/ ml) and was incubated at 20°C overnight. After precipitation, DNA was centrifuged and dried at room temperature. DNA was resuspended with 50 μl of distilled, deionized water.

*2.12. Methylation-specific PCR*

Methylation-specific PCR amplification was performed according to Herman et al. [36], to analyze the methylation pattern of the promoter region of CRBP-I. Two microliters of complement DNA template was submitted to PCR reaction with 5 μl of 10× RT-PCR buffer (2.5 mM $MgCl_2$ final concentration), 1 μl of dNTP mix (10 mM of each dNTP), 1 μl of forward and reverse gene-specific primers (10 mM), 1.25 unit PlatinumTaq DNA Polymerase and PCR grade water to make a total volume of 50 μl. Primer sequences of CRBP-I for the unmethylated reaction were 5′-TGTTGGGAATT-TAGTTGTTGTTGTTTT- 3′ (forward) and 5′-TACCAAAACAACAACTACCAATACTACA-3′ (reverse), and for the methylated reaction, 5′-TTGGGAATTTAGTTGTCGTCGTTTC-3′ (forward) and 5′-AAACAACGACTACCGATACTACGCG-3′ (reverse). These primers were designed with Methyl Primer Express Software version 1.0 based on the rat CRBP-I promoter sequence [37]. The PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 59°C for 75 s and 72°C for 30 s, with a final extension of 72°C for 5 min [37]. The PCR products were separated in 2% agarose gels, and DNA was visualized by ethidium bromide staining.

*2.13. Statistical analysis*

Sigma Stat 2.0 (Jandel, San Rafael, CA, USA) program was used for the statistical analysis. When indicated, one-way analysis of variance (ANOVA) followed by Tukey test was used. Fisher's Exact Test was used when indicated. In all cases, a level of significance of $P<.05$ was applied.

## 3. Results

### *3.1. Body and liver weights and macroscopy and microscopy analysis of the liver*

Body weight and absolute and relative liver weights did not show significant differences between animals treated with VA, TB, TB+VA or CO during the promotion phase of hepatocarcinogenesis (data not shown). Table 1 presents the data on the incidence and mean number of visible hepatocyte nodules and the morphometric analysis of pPNL and rPNL of the CO, TB, VA and TB+VA groups. When compared to CO group, the TB and TB+VA groups, but not the VA group, exhibited a lower incidence ($P<.05$) and mean number ($P<.05$) of hepatocyte nodules and tended ($P<.09$) to present pPNL with smaller size compared to the CO group. There were no significant differences ($P>.05$) between the groups regarding the number of pPNL and rPNL, the size of rPNL and the area of liver section occupied by pPNL and rPNL.

### *3.2. Cell proliferation and apoptosis evaluation*

Fig. 2 shows the quantification of PCNA-stained hepatocytes/$mm^2$ and ABs/$mm^2$ in normal rat livers (N group), as well as in PNL areas of the CO, TB, VA and TB+VA groups. Cell proliferation and apoptosis indexes in the pPNL and rPNL of the CO group were higher ($P<.05$) than in hepatic tissue from the N group. These results agree with the information that during hepatocarcinogenesis, cell proliferation and apoptosis increase [13,30]. Compared to the CO group, no significant differences ($P>.05$) were observed in

Table 1
Quantification of visible hepatocyte nodules and morphometric analysis of GST-P–positive PNL of rats treated with TB and VA, alone or in combination, during the promotion phase of hepatocarcinogenesis

| Group/n | Incidence of nodules (%) | Mean number of nodules | Number of GST-P positive PNL per $cm^2$ | | Size of GST-P positive PNL ($mm^2$) | | Area of liver section occupied by GST-P positive PNL (%) | |
|---|---|---|---|---|---|---|---|---|
| | | | pPNL | rPNL | pPNL | rPNL | pPNL | rPNL |
| CO/10 | 70 | 18±4 | 36±3 | 31±4 | 0.16±0.04 | 0.09±0.04 | 6±3 | 3±1 |
| TB/11 | 18[a] | 1±1[b] | 45±7 | 37±4 | 0.08±0.01[c] | 0.05±0.01 | 4±1 | 2±1 |
| VA/12 | 33 | 7±1 | 38±1 | 34±4 | 0.26±0.90 | 0.13±0.03 | 10±3 | 5±1 |
| TB+VA/11 | 18[a] | 1±1[b] | 38±2 | 37±4 | 0.08±0.01[c] | 0.04±0.01 | 3±1 | 2±1 |

Values are represented as the mean±SEM. $n$=number of animals.
[a] Statistically different ($P<.05$) when compared to CO group according to Fisher's Exact Test.
[b] Statistically different ($P<.05$) when compared to CO group according to one-way ANOVA test followed by Tukey test.
[c] Tendency toward statistical difference ($P<.09$) when compared to the CO group according to one-way ANOVA test followed by Tukey test.

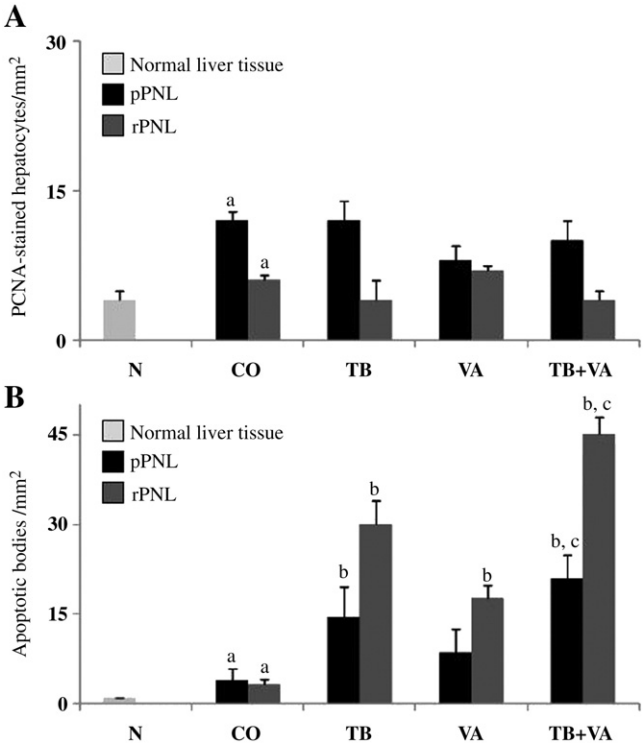


Fig. 2. Quantification of PCNA-stained hepatocytes (A) and hepatic ABs (B) of normal rats and rats treated with TB and VA, alone or in combination, during the promotion phase of hepatocarcinogenesis. pPNL, persistent PNLs. Values are represented as the mean±S.E.M. (N group, $n=0$; CO group, $n=10$; VA group, $n=12$; TB group, $n=11$; VA+TB group, $n=11$). Statistics were performed using one-way ANOVA followed by Tukey test. Statistically significant differences ($P<.05$) are represented by "a" when compared to N group, "b" when compared to PNL of the CO group and "c" when compared to PNL of the VA group.

the TB, VA and TB+VA groups regarding the cell proliferation index in pPNL and rPNL. Compared to the CO group, TB and VA+TB groups exhibited a higher ($P<.05$) ABs index in pPNL and rPNL, whereas the VA group exhibited a higher ($P<.05$) ABs index only in rPNL. Compared to the VA group, the TB+VA group exhibited a higher ($P<.05$) ABs index in pPNL and rPNL.

### 3.3. Histone acetylation and p21 expression

Fig. 3 shows the Western blot analysis of H3K9 acetylation and p21 expression, performed with protein extracts from normal rat livers and with the entire livers (nodules+nonnodular surrounding tissues) from animals from the CO, TB, VA and TB+VA groups. No differences ($P>.05$) were observed between the N and CO groups regarding H3K9 acetylation and p21 expression. Compared to the CO group, the TB and TB+VA groups, but not the VA group, exhibited higher levels (2.8- and 3.8-fold increases, respectively) of H3K9 acetylation; however, the differences reached statistical significance ($P<.05$) only for the TB+VA group. The TB and TB+VA groups, but not the VA group, both exhibited a 2.5-fold increase ($P<.05$) in p21 hepatic levels when compared to the CO group.

### 3.4. Hepatic concentrations of retinol, retinyl palmitate and BA

Table 2 shows the hepatic concentrations of retinol, retinyl palmitate and BA in the N, CO, TB, VA and TB+VA groups. Compared to the N group, the CO group exhibited lower ($P<.05$) concentrations of retinyl palmitate, but not ($P>.05$) of retinol. Hepatic concentrations of retinol and retinyl palmitate were higher ($P<.05$) in the VA and TB+VA groups, but not in the TB group, compared to the CO group. Compared to the VA group, the TB+VA group presented a lower ($P<.05$) concentration of retinyl palmitate, but not ($P>.05$) of retinol. No differences ($P>.05$) were observed between the N and CO groups regarding the BA concentration. Compared to the CO group, the TB and TB+VA groups, but not the VA group, exhibited higher ($P<.05$) BA concentration.

The TB+VA group exhibited a 40% reduction in BA hepatic concentration compared to the TB group.

### 3.5. CRBP-I expression and promoter methylation analyses in microdissected pPNL

Fig. 4 shows CRBP-I expression and promoter methylation analyses from the N, CO, TB, VA and TB+VA groups. No differences ($P>.05$) were observed in CRBP-I expression in normal rat livers, the surrounding

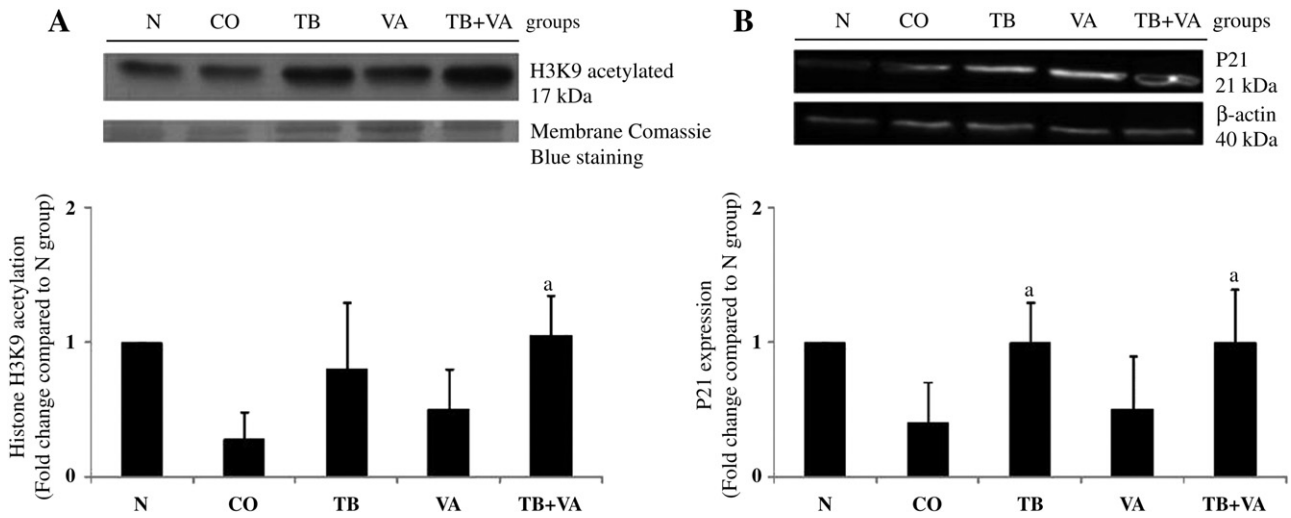


Fig. 3. (A) Western blot analysis of histone H3K9 acetylation and (B) p21 expression performed with hepatic proteins of normal rats and rats treated with TB and VA, alone or in combination, during the promotion phase of hepatocarcinogenesis. For histone H3K9 acetylation analysis equal protein loading was verified using membrane Coomassie blue staining. p21 expression was normalized against the β-actin signal. The results obtained are expressed as fold change compared to hepatic histone H3K9 acetylation levels (A) or p21 expression (B) in normal animals. Values are represented as the mean±S.E.M. Statistics were calculated by one-way ANOVA followed by Tukey test. Statistically significant differences ($P<.05$) are represented by "a" when compared to CO group. Liver samples of four animals from each group were analyzed. The blots show a representative sample from each group.

Table 2
Hepatic concentrations of retinol, retinyl palmitate and BA of rats treated with TB and VA, alone or in combination, during the promotion phase of hepatocarcinogenesis

| Group | Retinol (μg/g) | Retinyl palmitate (μg/g) | BA (μg/g) |
|---|---|---|---|
| N | 0.036±0.003 | 0.120±0.013 | 0.10±0.04 |
| CO | 0.035±0.005 | 0.076±0.013[a] | 0.23±0.20 |
| TB | 0.032±0.002 | 0.178±0.004 | 1.18±0.02[b] |
| VA | 0.264±0.013[b] | 5.808±0.486[b] | 0.25±0.10 |
| TB+VA | 0.240±0.059[b] | 2.946±0.730[b,c] | 0.69±0.10[b] |

Values are represented as the mean±S.E.M. Liver samples from 10 animals of each group were used for retinol and retinyl palmitate analyses, and liver samples from 6 animals of each group were used for BA analysis.
[a] Statistically different ($P<.05$) when compared to N group according to one-way ANOVA followed by Tukey test.
[b] Statistically different ($P<.05$) when compared to CO group according to one-way ANOVA followed by Tukey test.
[c] Statistically different ($P<.05$) when compared to VA group according to one-way ANOVA followed by Tukey test.

areas and pPNL areas of all groups. An unmethylated pattern at the CRBP-I promoter region at the evaluated loci was observed in microdissected normal rat hepatic tissue and in pPNL from all groups.

## 4. Discussion

Anticancer preclinical studies and clinical trials have evaluated the association of retinoids and synthetic HDACi [20–22]. Recently, a number of dietary compounds have been shown to act as HDACi [4,13], but the data are limited on their potential for combination therapy with retinoids. Thus, we investigated the chemopreventive effects of TB and the dietary retinoid VA alone and in combination in the promotion phase of the RH model.

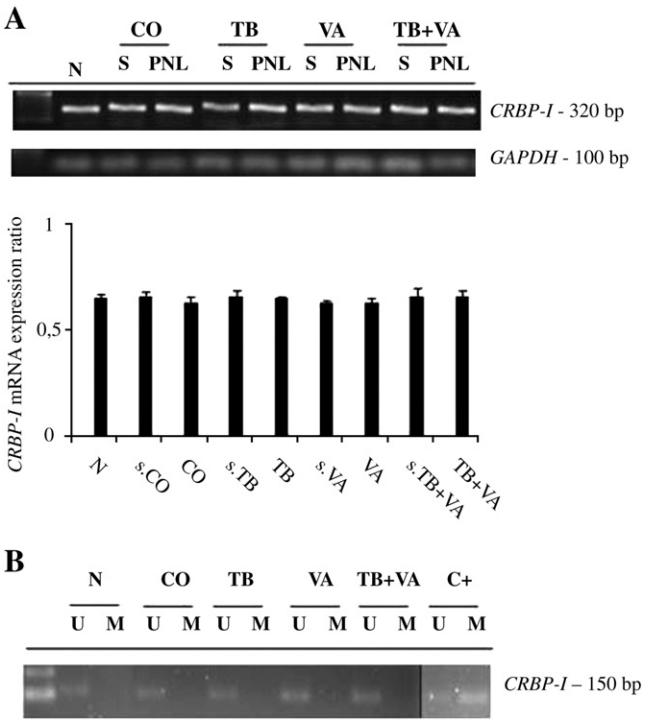


Fig. 4. Analysis of CRBP-I expression (A) and promoter methylation (B) in normal rats liver (N group) and pPNL microdissected from rats treated with TB and VA, alone or in combination, during the promotion phase of hepatocarcinogenesis. (A) Semiquantitative RT-PCR analysis of CRBP-I mRNA was performed with hepatic RNA of normal rats and hepatic pPNL, as well as from the surrounding (S) tissue. GAPDH expression was used as an input control. Results are expressed as the ratio of CRBP-I to GAPDH expression. Values are represented as the mean±S.E.M. (B) The presence of a visible PCR product in lanes "U" indicates the presence of the unmethylated CRBP-I gene. The presence of product in lanes "M" indicates the presence of methylated genes. Hepatic DNA treated in vitro with CpG-specific methylase from Spiroplasma (SssI methylase) was used as a positive control (C+) for CRBP-I promoter hypermethylation—delineated with a line. Normal rat liver (N group) was used as a negative control for methylation. Repeat methylation-specific PCR assays gave similar results. Liver samples of four animals from each group were analyzed. Gels show a representative sample from each group.

The use of experimental models of carcinogenesis could accelerate the discovery and development of potential chemopreventive strategies [38]. In this regard, the RH model has been shown to reproduce human HCC features [39] and to be a consistent bioassay for the assessment of chemopreventive agents, based on its capacity for producing hepatic PNL in a synchronized manner and for the ease of distinguishing the different stages of hepatocarcinogenesis [26,40]. Previously, continuous administration of TB during the initiation and promotion phases of the RH model resulted in chemopreventive effects [13]. In the present study, TB, alone or combined with VA, exhibited inhibitory effects when administered specifically during the promotion phase of this model, thus classifying it as a suppressing agent [41]. Because reducing initiation to zero is impossible [19], this reinforces TB potential for hepatocarcinogenesis chemoprevention.

In this study, TB induced apoptosis in pPNLs, which are sites of origin for HCC [26], and in rPNL, which tend to regress to tissue with a normal liver appearance [26]. VA induced apoptosis only in rPNL. Notably, the TB+VA combination appeared to exhibit additive effects on the induction of apoptosis in pPNL and in rPNL. None of the treatments inhibited cell proliferation in PNL. Studies conducted with cancer cell lines showed that treatment with retinoic acid in association with BA synergistically induced apoptosis [42]. This result provided the rationale for the development of retinoyloxymethyl butyrate, a prodrug of RA and BA that has been tested in acute promyelocytic leukemia cell lines [42]. A functional genetic screen demonstrated the involvement of the RA pathway in the proapoptotic effect of an HDACI [43]. In HCC cells, p21 expression was involved with apoptotic induction following treatment with a combination of an acyclic retinoid and the HDACi valproic acid [44]. In the present study, histone hyperacetylation and p21 expression were induced by TB and TB+VA, but not by VA treatment, which could reflect the transcriptional activation potential of TB acting as a HDACi [13] because p21 is up-regulated by HDAC inhibition [45]. p21 is also considered an RA-responsive gene because its promoter contains a retinoic acid responsive element [44].

Treatment with an HDACi induced acetylation in RARβ2 hypermethylated promoters in the presence of 13-*cis*-retinoic acid, leading to reexpression of RARβ2 in RARβ2-negative, retinoid-resistant melanoma cells [21]. This event resulted in an additive inhibitory effect on tumor cell growth in vitro and in vivo that was associated with p21 induction [21]. Although the TB and VA combination did not induce histone acetylation and p21 expression in an additive manner, other retinoids should not be excluded as potential partners of TB for epigenetic modulation during hepatocarcinogenesis.

Information on TB/BA absorption, metabolism and tissue distribution is limited [46]. TB and VA administration increased the hepatic concentration of BA and retinoids (retinol and retinyl palmitate), respectively, indicating effective absorption and distribution of both compounds to the liver. This result indicates that it is feasible to achieve hepatic levels sufficient for chemoprevention after oral consumption of TB and VA. Compared to animals that received only TB or VA, rats treated with these compounds combined presented decreased levels of BA and retinyl palmitate, but not of retinol, the free form of VA. This result could reflect the occurrence of eventual metabolic interactions of both compounds that did not appear to interfere with their chemopreventive effects. It was reported that although retinyl palmitate is the predominant ester of VA that is stored in the liver, dietary fatty acid composition can affect liver retinyl ester levels [47].

VA deficiency was observed in both experimental [48] and human HCC [49]. The lower hepatic retinyl palmitate concentration in the CO group compared to normal rat livers suggests that alterations in VA metabolism could occur in the initial phases of hepatocarcinogenesis. Epigenetic silencing of CRBP-I, an alteration described to be an evolutionarily conserved in some human and mouse cancers [50], could alter intracellular retinol transport and block retinyl ester formation [51]. However, in this study, no alterations in CRBP-I expression and promoter methylation were found in microdissected hepatic PNL, indicating that epigenetic silencing of this gene is not involved in the alteration of retinol metabolism in early rat hepatocarcinogenesis. Similarly, in human HCC, no difference was observed in CRBP-I expression, despite its reduced levels of VA when compared to normal liver tissue [52]. The retinyl palmitate decrease in liver tissue observed in CO group could be attributed to disruption of the lecithin/retinol acyltransferase gene. This enzyme catalyzes the esterification of retinol (VA) that is associated with CRBP-I in the liver and is found reduced in many types of cancer cells [53]. If epigenetic silencing of lecithin/retinol acyltransferase is involved in initial phases of hepatocarcinogenesis should be further evaluated.

It has recently been proposed that dietary agents are unlikely to act independently of each other but are more likely to function in specific combinations. Several lines of evidence that epigenetic-modifying dietary agents function with retinoids to impact cancer risk are consistent [54]. The original data of the present study support the effectiveness of TB as a dietary HDACi that should be considered for hepatocarcinogenesis chemoprevention combination strategies, including those with retinoids.

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