The chemopreventive activity of the butyric acid prodrug tributyrin in experimental rat hepatocarcinogenesis is associated with p53 acetylation and activation of the p53 apoptotic signaling pathway

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The reversibility of non-genotoxic phenotypic alterations has been explored in order to develop novel preventive and therapeutic approaches for cancer control. Previously, it has been demonstrated that histone deacetylase (HDAC) inhibitor tributyrin, a butyric acid prodrug, to have chemopreventive effects on rat hepatocarcinogenesis. The goal of this study was to determine molecular mechanisms associated with the chemopreventive activity of tributyrin. Male Wistar rats were allocated randomly to untreated control group and two experimental groups. Rats in the experimental group 1 were treated with maltodextrin (3 g/kg body wt), and rats in experimental group 2 were treated with tributyrin (2 g/kg body wt) daily for 8 weeks. Two weeks after treatment initiation, rats from experimental groups were subjected to a ‘resistant hepatocyte’ model of hepatocarcinogenesis. Treatment with tributyrin resulted in lower HDAC activity and Hdac3 and Hdac4 gene expression, and an increase of histone H3 lysine 9 and 18 and histone H4 lysine 16 acetylation as compared with the experimental group 1. In addition to the increase in histone acetylation, tributyrin caused an increase in the acetylation of the nuclear p53 protein. These changes were accompanied by a normalization of the p53-signaling network, particularly by the upregulation of pro-apoptotic genes, and a consequent increase of apoptosis and autophagy in the livers of tributyrin-treated rats. These results indicate that the chemopreventive activity of tributyrin may be related to an increase of histone and p53 acetylation, which could lead to the induction of the p53 apoptotic pathway.

Introduction

Cancer is a complex multifactorial disease characterized by a variety of biologically significant and interdependent alterations, among which genetic and non-genetic, e.g. epigenetic, metabolic, and cell signaling, abnormalities play fundamental roles (1–5). It is becoming increasingly evident that cancer by itself can induce a range of non-genetic changes; however, a wealth of accumulated data indicate that carcinogen-induced distortion of the cellular phenotype, including the inability of cells to maintain and control properly the expression of genetic information, dysregulated cell signaling and intercellular communication, and evasion from cell death, are critical for tumor development. This suggests that preventing these cancer-related non-genotoxic abnormalities and/or their timely correction at early stages of the carcinogenic process are of great importance for the prevention of cancer development (6,7).

Numerous studies have demonstrated that many compounds, including bioactive food components, possess significant cancer chemoprotective effects (6,8). In previous studies, we demonstrated a potent chemopreventive effect of tributyrin, a butyric acid prodrug, on rat liver carcinogenesis (9,10). Specifically, using a classical ‘resistant hepatocyte’ model of hepatocarcinogenesis, we showed that treatment of male Wistar rats with tributyrin during the initiation and promotion stages of hepatocarcinogenesis reduced the size of placent al glutathione S-transferase (GSTP) foci, a sensitive marker for initiated liver cancer precursor lesions (11,12), by 50% and significantly inhibited their progression to preneoplastic nodules (9). However, the tributyrin treatment did not affect the number of GSTP-positive foci, indicating that the cancer-preventing activity of tributyrin is associated with inhibiting of the promotion stage of carcinogenesis. This suggestion was further confirmed by de Conti et al. (10), who demonstrated that treating rats with tributyrin during the promotion stage of liver carcinogenesis strongly inhibited the progression of GSTP-positive foci into preneoplastic nodules. It is well established that the progression of GSTP-positive foci into GSTP-positive nodules is a valid marker of liver carcinogenesis (11–13).

The observed cancer-inhibitory activity of tributyrin has been attributed to its ability to inhibit cell proliferation and induce growth arrest and apoptosis (9,10,14,15); however, the precise molecular mechanisms of the tributyrin action remained largely unknown. Based on these considerations, the goal of this study was to elucidate the underlying molecular mechanisms associated with the chemopreventive activity of tributyrin on rat hepatocarcinogenesis.

Materials and methods

Animals and experimental design

Male Wistar rats (50 g) were obtained from the Faculty of Pharmaceutical Sciences of the University of São Paulo (São Paulo, Brazil) breeding facility, housed in a temperature-controlled (24°C) room with a 12 h light-dark cycle and given ad libitum access to water and industrialized food (Purina Nutrimentos Ltd, Paulinia, Brazil). Rats were allocated randomly to an untreated control group and two experimental groups. Five rats in the control group were not submitted to any experimental procedure. Rats in the experimental group 1 were treated with maltodextrin (3 g/kg body wt) and rats in experimental group 2 were treated with tributyrin (2 g/kg body wt) daily for 8 weeks. Two weeks after treatment initiation, rats from both experimental groups were subjected to a ‘resistant hepatocyte’ model of hepatocarcinogenesis. Treatment with tributyrin daily for 8 weeks (7). Rats from control and experimental groups were euthanized under light ether anesthesia by exsanguination 8 weeks after the initiation of tributyrin treatment. The in-life portion of this study, tissue collection protocols and results of histopathological and immunohistochemical staining analyses, including p53, cell proliferation and apoptosis evaluation, are detailed in Kuroiwa-Trzmielina et al. (9). All experimental procedures were carried out in accordance with the animal study protocol approved by the Faculty of Pharmaceutical Sciences of the University of São Paulo Ethics Committee for Animal Research.

Determination of histone deacetylase activity

Total liver tissue lysates were prepared by homogenization of 30 mg of tissue in 500 μl of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM ethylenediaminetetraacetic acid). The lysates were centrifuged and the total protein content was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Histone deacetylase activity was determined using the histone acetyltransferase assay kit (Cayman Chemical Company, Ann Arbor, MI). Extracts were incubated in a 100 μg sample volume with 50 mM Tris–HCl, pH 7.4, 5 mM MgCl2, 1 mM ZnCl2, 100 μg of ATP-regenerating system, 50 μM [3H]acetyl-coenzyme A, 50 μg of histone H4 (single acetylation site at K16) and 100 μg of recombinant HDAC1. The reaction was started by the addition of the histone substrate and was terminated after 10 min at 37°C using 1 ml of 10% trichloroacetic acid. The reaction mixtures were then centrifuged at 13,000 g for 10 min at 4°C followed by addition of 1 ml of 10% trichloroacetic acid to the supernatant and precipitation with 1 ml of cold ethanol. The precipitated reaction products were collected by centrifugation at 13,000 g for 10 min at 4°C and washed 3 times with 1 ml of 10% trichloroacetic acid and once with cold ethanol. The pellets were then resuspended in 2 ml of deionized water and applied to a pre-washed Whatman cellulose thin layer chromatography plate. The plates were developed in a solvent system containing 20% isopropanol, 20% acetic acid and 60% water and were exposed to a phosphor screen to allow detection of radiolabeled spots. The radioactivity of the spots was determined using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). The amount of radiolabeled histone deacetylation was calculated as the difference between the activity in the absence and presence of the HDAC inhibitor trichostatin A. The data were analyzed by ANOVA followed by Tukey–Kramer post hoc test. A value of P < 0.05 was considered significant.
acid: 1 mM phenylmethylsulfonyl fluoride; 1 µg per ml each of aprotinin, leupeptin and pepstatin; 1 mM NaF, 1 mM NaVO₃ and 1 mM NaF) and incubated at 4°C for 30 min, followed by centrifugation at 10 000×g at 4°C for 20 min. The protein level was measured using the Bio-Rad Protein Assay kits (Bio-Rad Laboratories, Hercules, CA). The histone deacetylase (HDAC) activity in the livers was determined using HDAC Activity Assay kits (Abcam, Cambridge, MA) according to the manufacturer’s protocol. The HDAC activity in each sample was measured in triplicate and expressed per milligram of protein.

Western blot analysis of histone modifications

Acid extraction of histones was performed as described in Tryndyak et al. (17). Total histone extracts were resolved on a 15% denaturing polyacrylamide gel (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The status of histone H3 lysine 9 (H3K9), H3 lysine 18 (H3K18) and histone H4 lysine 16 (H4K16) acetylation and histone H3 lysine 4, histone 3 lysine 27 and histone 4 lysine 20 trimethylation in the livers of animals was analyzed by western blot analysis. All primary antibodies were purchased from Millipore Corporation (Billerica, MA) and diluted 1:1000. Alkaline phosphatase-coupled donkey anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for visualization. The signal intensity was analyzed by ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis of proteins

Total liver tissue lysates were separated into cytoplasmic fractions and pellets (nuclear fractions) were homogenized in 50 mM Tris–HCl, pH 7.4 (50 mM NaCl; 1 mM phenylmethylsulfonyl fluoride; 1 mM ethylenediaminetraacetic acid; 1% sodium dodecyl sulfate; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 1 µg per ml each of aprotinin, leupeptin and pepstatin; 1 mM NaF, 1 mM NaVO₃ and 1 mM NaF). Extracts containing equal quantities of proteins were separated by sodium dodecyl–polyacrylamide gel electrophoresis on 8–15% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were probed with primary antibodies against p53 (1:500; Cell Signaling Technology, Danvers, MA), acetyl p53 (Lys373 and Lys382) (1:500; Millipore Corporation), microtubule-associated protein 1 light chain 3 (LC3B; 1:1000; Cell Signaling), p53 E3 ubiquitin protein ligase (MDM2; 1:500; Santa Cruz Biotechnology), beclin 1 (BECN1; 1:1000; Cell Signaling) and β-actin (1:500; Santa Cruz Biotechnology). Alkaline phosphatase-coupled donkey anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology) were used for visualization. Images are representative of three independent immunoblots. Signals were quantified using ImageQuant 5.1 Software.

RNA isolation and quantitative reverse transcription–PCR array analysis

Total RNA (n = 4 from each diet group) was extracted from liver tissue using miRNAeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Complementary DNA was synthesized from 5 µg of total RNA using RT First Strand cDNA synthesis kits (Qiagen). Expression of the genes involved in the p53 signaling network was determined by reverse transcription (RT)–PCR arrays using a RT² Profiler PCR Array–Rat p53 Signaling Pathway Array (Qiagen) according to the manufacturer’s protocol. The relative level of messenger RNA for each gene was determined using the 2−ΔΔCt method (18). The results are presented as fold change for each messenger RNA in liver of rats of control group relative to control rats and tributyrin group relative to control group.

Quantitative RT–PCR

Total RNA (2 µg) was reverse transcribed using random primers and high-capacity cDNA archive kits (Applied Biosystems, Foster City, CA). The following TaqMan gene expression assays (Applied Biosystems) were used for quantitative RT–PCR: Hdac1 (Rn01525918_m1), Hdac2 (Rn01193634_g1), Hdac3 (Rn00584926_m1), Hdac6 (Rn01464246_m1), Hdac8 (Rn01528283) and Hdac8 (Rn01419046). Reactions were performed in a 96-well assay format using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Each plate contained experimental genes and a housekeeping gene Gapdh (Rn01775763_g1), and all samples were plated in duplicate. The relative amount of each messenger RNA transcript was measured using the 2−ΔΔCt method (18).

Statistical analyses

Results are presented as mean ± SD. Data were analyzed by one-way analysis of variance, with pair-wise comparisons being made by the Student–Newman–Keuls method. When necessary, the data were natural log transformed before conducting the analyses to maintain a more equal variance or normal data distribution. P-values < 0.05 were considered significant.

Results

Effect of tributyrin on hepatic HDAC activity and HDAC gene expression in preneoplastic livers

It is well documented that tributyrin acts as epigenetic modifier by inhibiting the functioning of HDACs. In view of this, we determined HDAC activity in the livers of control rats and rats from the experimental groups. Figure 1 shows that the preneoplastic livers of rats from the experimental group 1 were characterized by a marked, 3-fold, increase in HDAC activity as compared with the age-matched control rats. In contrast, the HDAC activity in the livers of rats treated with tributyrin did not differ from the values in control rats and were significantly lower as compared with the experimental group 1.

In addition to the increased HDAC activity, Figure 2 shows that the expression of HDAC genes, including HDac1, HDac3, HDac4, HDac6 and HDac8, was significantly increased in the preneoplastic livers of the experimental group 1 rats. This finding corresponds to previous reports of an upregulation of numerous HDACs (19–23) especially HDAC3 and HDAC4 (21,23) in human hepatocellular carcinoma. Treatment of rats with tributyrin decreased the expression of HDac3 and HDac4, two of the most upregulated HDAC genes in the preneoplastic livers of the experimental group 1 rats.

Effect of tributyrin on histone modifications in preneoplastic livers

Next, we examined the status of several histone lysine acetylation marks, including acetylation of histone H3K9, H3K18 and H4K16. Figure 3 shows that the extent of histone H3K9, H3K18 and H4K16 acetylation did not differ between the livers of experimental group 1 and control rats (Panel A). Tributyrin treatment resulted in a moderate but significant increase, 1.20 and 1.25 times, respectively, in hepatic histone H3K9 and H3K18 acetylation as compared with the control and experimental group 1 rats (Panel A). The most substantial changes were found in the level of histone H4K16ac in the livers of tributyrin-treated rats, which were 1.51 times greater from the values in the livers of rats from control and experimental 1 groups. In contrast, hepatic histone lysine methylation marks did not differ in rats from experimental groups 1 and 2 (Panel B).

Effect of tributyrin on the level of p53 protein in preneoplastic livers

Figure 4A shows the level of total p53 protein and acetylated form of p53 protein in the nuclear fraction in livers of control rats and rats from experimental groups 1 and 2. The level of total nuclear p53 protein in the livers of rats from the experimental group 1 and
tributyrin-treated rats was significantly increased. In contrast, the level of acetylated form of p53 protein in the nuclear fraction in the experimental group 1 rats was 1.6 times lower, which resulted in a substantial reduction of acetylated p53/p53 protein ratio as compared with the control rats. The level of either total p53 protein or its acetylated form in the nuclear fraction of rats treated with tributyrin did not differ from the control rats; however, the level of acetylated p53 in tributyrin-treated rats was significantly, 1.8 times, higher as compared with the experimental group 1 rats.

The level of p53 protein in the nuclear and, especially, in cytoplasmic fractions in the preneoplastic livers in rats from the experimental group 1 was significantly greater as compared with control rats (Figure 4B). Specifically, the level of cytoplasmic p53 protein was 1.8 times greater than in control rats. This led to an increase in ratio of cytoplasmic and nuclear forms of p53 protein in the livers of experimental group 1 rats. In contrast, the level of p53 protein in the cytoplasmic fraction in rats treated with tributyrin did not differ from the control rats. Importantly, the level of p53 protein in the cytoplasmic fraction in tributyrin-treated rats was significantly lower than in the experimental group 1 rats.

Similar to previous findings (24), the subcellular p53 alterations in the livers of the experimental group 1 rats were accompanied by a marked increase, 2.6 times, in the level of MDMD2 protein, a main regulator of the stability, subcellular localization and transcriptional activity of the p53 protein (25), as compared with the control rats (Figure 4C). In contrast, the level of MDMD2 in the livers of tributyrin treated rats increased 1.6 times only; however, despite this increase, the level of MDMD2 in the livers of tributyrin-treated rats was significantly lower than in the experimental group 1 rats.

Accumulating evidence indicates that p53 protein plays a major role in regulating autophagy (26,27). In view of this, we analyzed the level of LC3B and BECN1 proteins, a known markers of autophagy (28,29), in the livers. Figure 4D shows that the level of LC3B protein in the livers of rats from experimental groups 1 and 2 did not differ from values in control rats; however, in the livers of rats treated with tributyrin, it was significantly higher than in the experimental group 1 rats. The level of BECN1 protein in the livers of experimental groups 1 and 2 rats was significantly increased as compared with control rats (Figure 4D); however, in the livers of rats treated with tributyrin, the level of BECN1 was markedly greater than in rats from experimental group 1 (Figure 4D).

**Fig. 2.** Expression of HDAC genes in the livers of rats from control and experimental groups. The expression of HDac1, HDac2, HDac3, HDac4, HDac6 and HDac8 genes was determined by quantitative RT–PCR as detailed in ‘Materials and methods’. The results are presented as an average fold change in the expression of each gene in the livers of rats from experimental groups 1 and 2 relative to that in control rats, which were assigned a value 1. *Significantly different from the control rats; †Significantly different from the experimental group 1 rats. Data are presented as mean fold change ± SD (n = 4).

**Discussion**

Accumulating evidence has demonstrated the importance of non-genotoxic events in the progression of liver carcinogenesis and suggests that targeting and correcting these alterations is a promising avenue for the prevention of liver cancer. Previously, we demonstrated that tributyrin prevents rat liver carcinogenesis through normalizing the altered balance between cell proliferation and apoptosis, one of the fundamental characteristics of liver carcinogenesis (9,10); however, there was insufficient knowledge to clarify the underlying molecular mechanisms associated with the tumor suppressing activity of tributyrin.

In this study, we show that when rats undergoing hepatocarcinogenesis are treated with tributyrin, there is normalization of hepatic HDAC activity and expression of HDAC genes, which were profoundly elevated during carcinogenesis, and that this leads to an increased acetylation of histone lysines. To elucidate further the mechanisms responsible for activation of apoptosis and restoration of balance between cell death and cell proliferation in the livers of tributyrin-treated rats, we investigated effect of tributyrin on the p53 protein functions, one of the most deregulated pathways in liver carcinogenesis (30). The results of our study demonstrated a marked increase in cytoplasmic p53 protein in the preneoplastic livers of the experimental group 1 rats. Recent evidence indicates that p53, in addition to its classic tumor-suppressor functions, plays an important role in the regulation of autophagy. Depending of the subcellular location, p53 may either inhibit or activate autophagy (27,31). Specifically, it has been demonstrated that cytoplasmic p53 suppresses autophagy (27,31), and this inhibition may induce neoplastic cell transformation and promote cancer progression (32–34). In contrast, treatment with tributyrin normalizes the subcellular distribution of p53. These findings corresponded to our previous observations that showed a substantially reduced staining number of preneoplastic lesions with the cytoplasmic p53 staining in the livers of tributyrin-treated rats (9). Importantly, this tributyrin-induced normalization of the subcellular distribution of p53 was accompanied by an induction of autophagy in the livers of rats treated with tributyrin. This was evidenced by a marked upregulation of LC3B and BECN1 proteins in the livers of tributyrin-treated rats.
These findings correspond to the well-established fact of the major role of p53 in the induction of autophagy (27, 31). Furthermore, it has been demonstrated that an upregulation of p53-mediated autophagy and BECN1 signaling pathways inhibited the viability of human hepatocellular carcinoma HepG2 cells (35).

Additional evidence indicating the improper functioning of p53 in the preneoplastic livers of control rats was a decrease of p53 acetylation, which plays a critical role in the functional activity of p53 (36). The findings of an elevation of the cytoplasmic level of p53 and a marked loss of p53 acetylation in the preneoplastic livers correspond...
to previous reports of an impaired p53 pathway in enzyme-altered liver preneoplastic foci during rat hepatocarcinogenesis (37,38). Treatment of rats undergoing hepatocarcinogenesis with tributyrin was able to prevent these p53 alterations and block the tumorigenic progression of preneoplastic GSTP-foci. The results of our study suggest that tumor suppressing activity of tributyrin may be attributed to its ability to affect p53 acetylation by lowering the expression of Hdac3 and Hdac4 genes. This suggestion is supported by a wealth of data demonstrating a critical role of inhibition of class I HDAC, including HDAC1, HDAC2 and HDAC3, in p53 acetylation and increasing apoptosis in cancer cells (39–42). Importantly, our finding demonstrates that inhibitors of HDACs may be used not only for cancer treatment but also for the prevention of cancer development.

It is well established that acetylation, in addition to its key role in the maintenance of the functional activity of p53, is indispensable for p53 transcriptional activity (36). Indeed, a decrease in nuclear-acetylated p53 in the preneoplastic livers of the experimental group 1 rats was accompanied by a profound deregulation of the entire p53-signaling pathway, which was characterized by upregulation of antiapoptotic genes and downregulation of pro-apoptotic genes. Specifically, the expression of antiapoptotic Ccnb2, Cdc2, Sfn, Prc1, and Pttg1 genes that were significantly upregulated in the preneoplastic livers of the experimental group 1 rats did not differ from the values in the livers of control rats. At the same time, treatment with tributyrin significantly increased the expression of a number of the p53-dependent pro-apoptotic and antiproliferative genes, including Btg2, Gadd45a, E2f3 and Tnf (47–49). These findings correspond to our previous observation showing an activation of apoptosis in the livers of tributyrin-treated rats (9,10).

In addition to deregulated expression of antiapoptotic and pro-apoptotic genes, Sestrin2 (Sesn2), a p53 target, was markedly downregulated in the preneoplastic livers of the experimental group 1 rats. Recently, Sestrin2 has been identified as a critical link between p53 and the mammalian target of rapamycin pathway (49). Specifically, p53-driven Sestrin2 induction was able to override oncogenic mammalian target of rapamycin activation (50,51). In view of this, the 2.7-fold downregulation of Sesn2 in the preneoplastic livers of the experimental group 1 rats is another mechanism promoting the progression of GSTP-positive foci. In contrast, the 2.1-fold upregulation in the livers of tributyrin-treated rats is indicative of efficient tumor suppression.

In conclusion, the results of this study demonstrate that the underlying molecular mechanisms of tumor-suppressing activity of tributyrin on rat hepatocarcinogenesis are associated with its ability to prevent cancer-linked deacetylation of p53, which assures the proper functional activity of p53 and functioning of the p53-signaling...
network. This prevents evasion of apoptosis and disruption of a balance between apoptosis and cell proliferation, which block the carcinogenic progression of hepatocellular necrosis.

The views expressed in this article do not necessarily represent those of the United States Food and Drug Administration.

Conflict of Interest Statement: None declared.

References


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*aSignificantly different from control group, p < 0.05.

*bSignificantly different from experimental group 1, p < 0.05.

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