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In vivo Evidence of γ-Tocotrienol as a Chemosensitizer in the Treatment of Hormone-Refractory Prostate Cancer

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Key Words
Tocotrienol · Vitamin E · Prostate cancer · Docetaxel · Chemotherapy

Abstract
γ-Tocotrienol (γT3) is known to selectively kill prostate cancer (PCa) cells and to sensitize the cells to docetaxel (DTX)-induced apoptosis. In the present study, the pharmacokinetics of γT3 and the in vivo cytotoxic response of androgen-independent prostate cancer (AIPCa) tumor following γT3 treatment were investigated. Here, we investigated these antitumor effects for PCa tumors in vivo. The pharmacokinetic and tissue distribution of γT3 after exogenous γT3 supplementation were examined. Meanwhile, the response of the tumor to γT3 alone or in combination with DTX were studied by real-time in vivo bioluminescent imaging and by examination of biomarkers associated with cell proliferation and apoptosis. After intraperitoneal injection, γT3 rapidly disappeared from the serum and was selectively deposited in the AIPCa tumor cells. Administration of γT3 alone for 2 weeks resulted in a significant shrinkage of the AIPCa tumors. Meanwhile, further inhibition of the AIPCa tumor growth was achieved by combined treatment of γT3 and DTX ($p < 0.002$). The in vivo cytotoxic antitumor effects induced by γT3 seem to be associated with a decrease in expression of cell proliferation markers (proliferating cell nuclear antigen, Ki-67 and Id1) and an increase in the rate of cancer cell apoptosis [cleaved caspase 3 and poly(ADP-ribose) polymerase]. Additionally, the combined agents may be more effective at suppressing the invasiveness of AIPCa. Overall, our results indicate that γT3, either alone or in combination with DTX, may provide a treatment strategy that can improve therapeutic efficacy against AIPCa while reducing the toxicity often seen in patients treated with DTX.

Introduction

Prostate cancer (PCa) is the most common type of noncutaneous cancer in developed countries. It is responsible for more male deaths than any other cancers, except for lung and bronchus cancer [1]. Most PCa present themselves as mixtures of androgen-dependent and androgen-independent cells during clinical diagnosis. They initially respond to androgen ablation therapy by undergoing programmed cell death (apoptosis) [2]. However, patients with advanced PCa develop a hormone-refractory disease that results in a fatal effect because of the

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growth of androgen-independent PCa (AIPCa) cells [3]. Until recently, the only chemotherapeutic drug that has shown improvement in the survival of patients, docetaxel (DTX), can only extend the overall survival by 2–2.5 months [4]. Furthermore, the treatment is associated with significant side effects. Therefore, an alternative methodology of enhancing the apoptotic response is necessary to develop new therapeutic drugs for the treatment of AIPCa.

Natural products have historically been rich sources of biologically active compounds for drug discovery [5]. Tocotrienols (T3) are important plant vitamin E constituents found in palm oil. Together with tocopherols (TCP), they provide a significant source of antioxidant activity to all living cells [6, 7]. This common antioxidant attribute reflects the similarity in chemical structure of T3 and TCP, which differ only in their side chain saturation. The common hydrogen atom from the hydroxyl group on the chromanol ring acts to scavenge the chain-propagating peroxyl-free radicals. Depending on the locations of methyl groups on their chromanol ring, T3 and TCP can be divided into 4 isomeric forms: alpha (α), beta (β), gamma (γ), and delta (δ).

Apart from the antioxidant property of T3, a recent in vitro study [8] has demonstrated that treatment of PCa cell lines (LNCaP, DU145 and PC3) with T3-enriched fraction resulted in significant decreases in cell viability and colony formation capability. More interestingly, T3-enriched fraction was able to selectively spare the normal human prostate epithelial cells, making it an ideal candidate for developing it as an anticancer agent. By testing individual vitamin E isomers using PCa cell lines, we recently determined that γT3 possesses the most intense anticancer activity [9] across various types of cancer cells [9–11]. In addition, we found that the antitumor effect was mediated by the suppression of NF-κB and epidermal growth factor receptor (EGFR) signaling pathways, and the downregulation of Id family proteins (Id1 and Id3) [9–11].

Although the in vitro effect of γT3 on PCa cells has been well documented [9], there is no evidence about the effect of γT3 on PCa cells in vivo. Here, we demonstrated that γT3 selectively accumulate in AIPCa, thereby profoundly inhibiting AIPCa growth in vivo. The antitumor effect of γT3 was enhanced when administered in combination with DTX. The in vivo effect of γT3 was associated with the activation of cancer cell apoptosis and suppression of cell proliferation. These findings warrant a future investigation of the therapeutic potential of γT3 in the treatment of AIPCa.

Materials and Methods

Cell Line, Cell Culture Condition and Chemicals

Human AIPCa cell line, PC3, was obtained from the American Type Culture Collection and was grown in RPMI 1640 (Invitrogen, Carlsbad, Calif., USA) supplemented by 1% penicillin streptomycin and 5% FBS (PAA Laboratories GmbH, Pasching, Austria) in humidified 95% air and 5% CO_2 at 37°C. DTX (Calbiochem, San Diego, Calif., USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, Mo., USA). Solvents such as heptane and ethyl acetate were bought from Tedia Company Inc. (Fairfield, Ohio, USA). D-Luciferin, butylated hydroxytoluene (BHT) and 10% neutral buffered formalin were obtained from Sigma Aldrich. γT3 was purified from crude palm oil using the Davos separation technology. Crude palm oil feed was purchased from KLK Berhad (Ipoh, Malaysia). Using the corresponding T3 isomers as the reference standard, the purity of γT3 was verified to be >97% by high-performance liquid chromatography (HPLC) percentage area and gas chromatography.

Establishment of PC3 AIPCa Xenograft Model

Bioluminescent PC3-Luc human AIPCa cell line was generated as previously described [12, 13]. Briefly, cDNA encoding the luciferase gene was cloned into the pLenti6/V5. The construct was co-transfected with the packaging mix into HEK293 cells, and lentiviruses were collected and used for infecting the PC3 cells. Transfectants were obtained as a pool (PC3-Luc) by selection with 10 μg/ml of blasticidin for 1 week. The animal experimental protocol was approved by the NACLAR (National Advisory Committee for Laboratory Animal Research) guideline for Singapore for proper and humane use of animals. Male BALB/c athymic nude mice (4–5 weeks old; 18–22 g) were purchased from the Jackson Laboratory (Bar Harbor, Me., USA). Mice were housed in department 1 of the Biological Resource Centre (Biopolis, Singapore) under standard conditions (20.8 ± 2°C; 55 ± 1% relative humidity; 12-hour light/dark cycle) with rodent diet (Harlan Laboratories Inc., Indianapolis, Ind., USA) and chlorinated reverse osmosis water supplied in a pathogen-free environment. Briefly, 1×10^6 PC3-Luc cells in 100 μl of serum free RPMI 1640 were injected subcutaneously into the flank of nude mice using a 1-ml syringe with a 26-gauge needle (Becton Dickinson, Franklin Lakes, N.J., USA). All surgical operations were performed under aseptic conditions.

Nude mice bearing similar tumor sizes of about 100 mm^3 (after 2 weeks inoculation) were selected and randomly divided into 3 groups (n = 5 per group): G1 – control (DMSO as vehicle); G2 – γT3 (50 mg/kg/day), and G3 – combined γT3-DTX treatment (50 mg/kg/day of γT3 and 7.5 mg/kg/day of DTX). The mice were weighed daily and the tumors measured using a Carbon Fiber Digital Caliper (Fisher Scientific, Pittsburgh, Pa., USA) at the same time. The tumor volume was calculated as $4/3 \times \pi \times (\text{mean diameter}/2)^3$. The mice were dosed 5 times a week for 2 weeks. After 10 days of treatment, the mice were euthanized by CO_2 inhalation. Blood samples were collected via cardiac bleeding, using a 25-gauge needle. Blood samples were incubated at room temperature for 30 min, followed by centrifugation at 4,400 rpm and 4°C for 30 min. Serum, as the supernatant, was separated from the plasma and stored at –80°C. The tumors, liver, kidney, spleen, lung and heart were harvested. Part of the tumors was fixed in 10% neutral buffered formalin solution. The remaining tumors and all the isolated organs were immediately immersed in liquid nitrogen and stored at –200°C.

Pharmacokinetics of γT3 in Mice

C57BL/6 black mice were purchased from the Jackson Laboratory. Forty 5-week-old mice were given a single-dose intraperitoneal injection containing 50 mg/kg of γT3. Five mice were sacrificed at different time points (10 and 30 min, and 1, 3, 6, 24, 48 and 72 h). Blood samples were collected via cardiac bleeding. To isolate the serum, the blood samples were incubated at room temperature for 30 min, followed by centrifugation at 4,400 rpm and 4°C for 30 min. The γT3 concentration in serum was analyzed using the HPLC method described below.

Acute Toxicity Test

The maximum tolerated dose (MTD) was determined by increasing doses for different groups of mice until the highest dose without any mortality was found. Briefly, 45 C57BL/6 black male mice (5 in each group) received single-dose intraperitoneal injections containing 50, 100, 200, 400, 600, 800, 1,000, 1,500 and 2,000 mg/kg of γT3 per 100 μl injection volume. The weight and survival of the mice were observed for 30 days, followed by euthanasia via CO_2.

γT3 Extraction from Serums, Tumors and Organs

The serums were thawed and sonicated in an ultrasonic bath (Lab Companion, Vernon Hills, Ill., USA) for 5 min, followed by vortexing for 10 s. 100 μl of serum was transferred into an Iwaki Pyrex glass tube (Jawa Tengah, Indonesia) containing 900 μl of water. For the tumor and organ preparations, the tissues were homogenized in 1 ml of water using borosilicate glass homogenizer (Fisher Scientific), followed by transferring them to the Pyrex glass tube. Then, 5 μl of δT3 with a purity of 99% (100 mg of δT3 dissolved in 1 ml of ethanol) was used as an internal standard solution and was spiked into the mixture. The tube was vortexed for 10 s and sonicated for 2 min. After this, 4 ml of the BHT solution (5 mg of BHT in 100 ml of heptane) was added into the tube to minimize the oxidation of target analytes. Liquid-liquid extraction was performed by vortexing vigorously for 10 s. After liquid-liquid extraction, the tubes were centrifuged at 4,000 rpm for 5 min in a Heraeus Multifuge 3-SR Centrifuge (Newport Pagnell, UK). Then, 3.9 ml of the organic layer was transferred into another Pyrex tube. The extraction was repeated and a second organic layer taken out and pooled together with the first layer. The organic solution was evaporated using a Buchi Rotavapor R-205 (Flawil, Switzerland), and the dried residue was reconstituted in 1.5 ml of heptane and filtered, followed by HPLC analysis.

Determination of γT3 Level by HPLC

A normal phase of the HPLC method was performed as a modification of the procedure previously reported [14, 15]. First, 10 μl of sample was injected into an Agilent 1100 Series HPLC System (Agilent, Santa Clara, Calif., USA). The chromatographic separation was carried out by a Zorbax Silica 60 (5 μm; 250 × 4 mm i.d.) analytical column. The mobile phase used was a mixture of heptane/ethyl acetate (90:10, v/v) at a flow rate of 1.0 ml/min. The absorbance of γT3 was monitored by a diode array detector set at an excitation wavelength of 290 nm and an emission wavelength of 360 nm.

Serum-Based Toxicity Assays

Ten C57BL/6 black mice were given 5 intraperitoneal dose injections per week containing 50 mg/kg of γT3 or DMSO blank. The mice were sacrificed by cardiac bleeding and the serum was extracted by the method described above. Serum levels of the biomarkers albumin, creatinine, alanine transaminase, aspartate aminotransferase, urea and alkaline phosphatase were then measured by the colorimetric-based detection kits purchased from Randox Laboratories Ltd. (Crumlin, UK).

Immunohistochemistry

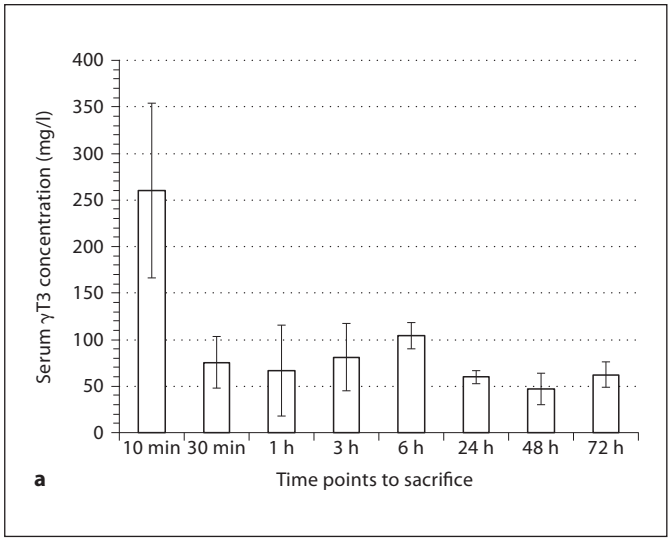
The tumor, liver, kidney, spleen, lung and heart of the mice were fixed in 10% neutral buffered formalin for 12 h. After fixation, the tissue samples were processed into paraffin blocks. Tissue sections were cut at a thickness of 5 μm using a Kedee microtome (China Jinhua Kedi Co. Ltd., Zhejiang, China), then deparaffinized in toluene and rehydrated in alcohol series. Endogenous peroxidase activity was blocked by treating the sections with 0.6% hydrogen peroxide in methanol for 20 min, followed by antigen retrieval treatment (Dako, Glostrup, Denmark). The sections were then incubated with peroxidase-blocking solution (Dako) for 1 h at 37°C to remove any nonspecific antigens. The specimens were incubated overnight at 4°C with primary rabbit polyclonal antibody against Snail (1:200), Id1 (1:250; Abcam, Cambridge, UK), cleaved caspase 3 and cleaved poly(ADP-ribose) polymerase (PARP; 1:50; Cell Signaling Technology Inc., Beverly, Mass., USA) and mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA), Ki-67 and E-cadherin (1:50; Santa Cruz Biotechnology, Santa Cruz, Calif., USA). After several rinses in TBS, the sections were incubated with Dako REAL™ EnVision™/HRP Rabbit/Mouse solution for 1 h at 37°C. The reaction was visualized by Dako REAL DAB+ chromogen. Mayer's hematoxylin (Dako) was used as counterstain. Standard inverted light microscopy (Nikon, Tokyo, Japan) was used to analyze the slides. To quantify the immunohistochemical reactivity, cells reacting positively to a target antibody were counted using a computer-based image analysis system (Stereo Investigator; MBF Bioscience, Williston, Vt., USA) on photomicrographs taken at a magnification of ×400 from 5 randomly selected fields per tumor. The percentage of positive cells was calculated as: number of positive cells/total number of cells × 100.

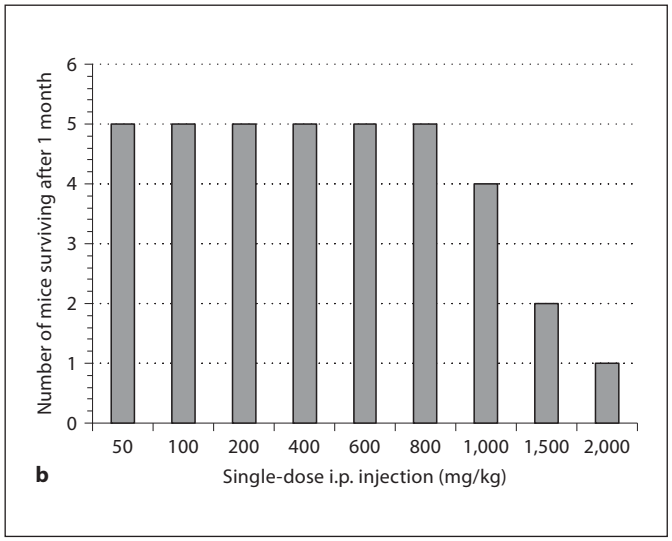
Bioluminescence Imaging

In vivo bioluminescence imaging of luciferase activity on the spontaneous AIPCa model was performed using the IVIS imaging system (Xenogen Corp., Hopkinton, Mass., USA) with the LivingImage acquisition and analysis software (Xenogen Corp.). D-Luciferin was dissolved to a concentration of 15 mg/ml in Dulbecco's phosphate-buffered saline, filter-sterilized and stored at –20°C. At the end of the treatment, the mice were given an intraperitoneal injection of luciferin solution (150 mg/kg body weight). Images were acquired 5 min after luciferin administration. The signal intensity was quantified as the sum of all detected photon counts within the region of interest from the tumors.

Statistical Analysis

Statistical data analyses were done using SPSS Manager. The data are presented as averages ± SD. The one-way analysis of variance (ANOVA) was used to compare the differences between the experimental groups (γT3 alone or combined γT3-DTX) and the control group (DMSO vehicle). $p < 0.01$ was considered as statistically significant.





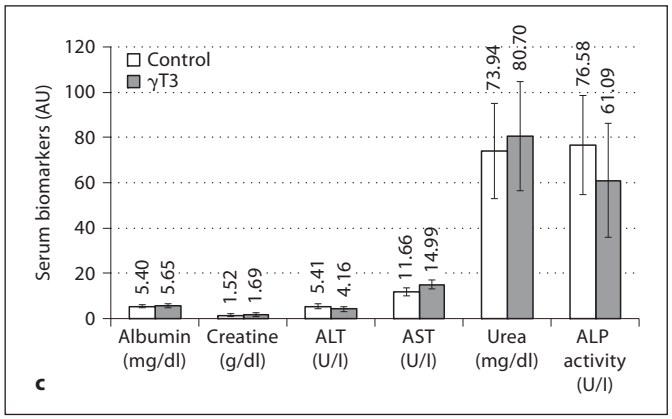


Fig. 1. Pharmacokinetics (**a**), acute toxicity (**b**) and serum biomarkers (**c**). **a** Forty 5-week-old C57BL/6 black mice received single-dose intraperitoneal injections containing 50 mg/kg of γT3. Five mice were sacrificed at different time points and the γT3 concentration was determined in serum. The level remained stable for at least 72 h. **b** Forty-five C57BL/6 black male mice (5 in each group) received a single intraperitoneal injection of 9 escalating doses of γT3. Survival after a 30-day observation period was determined. **c** Ten C57BL/6 mice received 5 intraperitoneal dose injections per week containing 50 mg/kg of γT3 or DMSO blank. After 1 month of treatment, several biomarkers were determined in serum.

Results

Pharmacokinetics and Acute Toxicity

We started by studying the pharmacokinetics of γT3 in serum after intraperitoneal administration. Mice were injected with 50 mg/kg of γT3 and blood was assayed for the γT3 concentration at different time points thereafter. As shown in the serum pharmacokinetic profile, the serum γT3 level decreased from 260 to 50 mg/l within 30 min after administration (fig. 1a). The level remained stable for at least 72 h.

To evaluate the acute toxicity, γT3 was injected intraperitoneally at 9 escalating doses for the determination of the MTD. The MTD is defined as the dose at which none of the 5 mice died within the 30-day observation period and at least 1 of the mice died at the next higher dose. As shown in figure 1b, the MTD was determined to be 800 mg/kg.

For mice having received 5 intraperitoneal dose injections per week (50 mg/kg γT3 or DMSO blank) for 4 weeks, there were no toxicological changes in any of the serum biomarkers examined (fig. 1c).

γT3 Inhibits the Growth of the PC3-Luc AIPCa Xenograft

Athymic nude mice were allografted with PC3-Luc cells and were divided into control (DMSO), γT3 and combined γT3-DTX treatment groups. The dosage for γT3 (50 mg/kg/day) was selected because it provided a significant antitumor effect to our nude mice without inducing the treatment-related mortality observed with higher doses (fig. 1b) [16, 17]. Similarly for DXT, the dosage was determined to be 7.5 mg/kg per week [12]. Tumor growth was monitored 5 times a week. There was no significant change in body weight throughout the entire study for all groups (fig. 2a). The AIPCa tumors in the

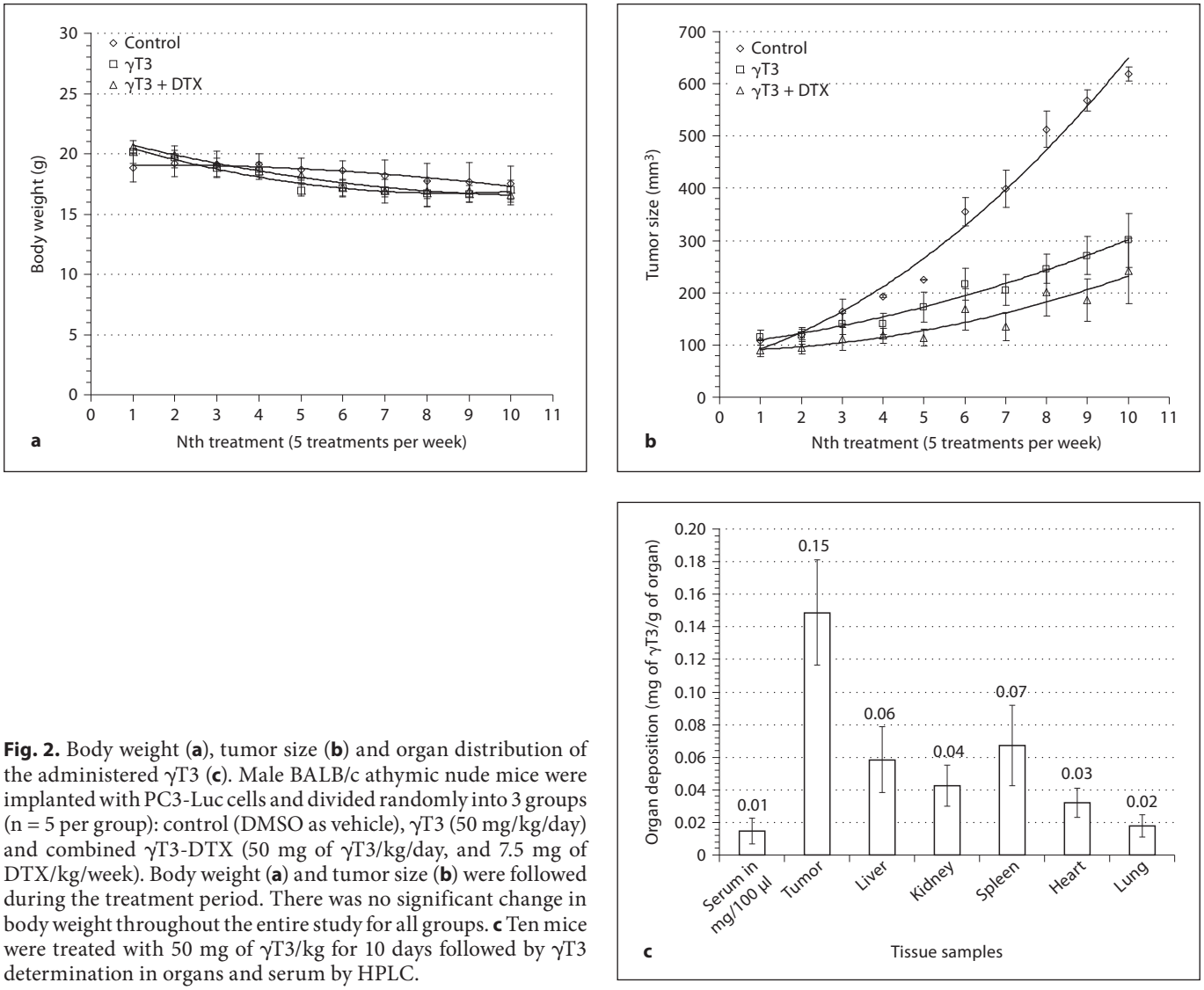


Fig. 2. Body weight (**a**), tumor size (**b**) and organ distribution of the administered γT3 (**c**). Male BALB/c athymic nude mice were implanted with PC3-Luc cells and divided randomly into 3 groups (n = 5 per group): control (DMSO as vehicle), γT3 (50 mg/kg/day) and combined γT3-DTX (50 mg of γT3/kg/day, and 7.5 mg of DTX/kg/week). Body weight (**a**) and tumor size (**b**) were followed during the treatment period. There was no significant change in body weight throughout the entire study for all groups. **c** Ten mice were treated with 50 mg of γT3/kg for 10 days followed by γT3 determination in organs and serum by HPLC.

control group grew rapidly, reaching an average volume of 620 ± 10 mm^3 by day 14 after the start of the treatment. In contrast, tumor growth on mice that were administered γT3 or combined γT3-DTX was profoundly inhibited, with tumor volumes remaining at 300 ± 48 and 240 ± 62 mm^3, respectively (fig. 2b). These results indicate that γT3 and combined γT3-DTX had significant inhibitory effects on AIPCa growth in vivo compared to the DMSO control group ($p < 0.002$).

Since the serum γT3 level dropped rapidly after administration, it is critical to understand if this was due to drug clearance or specific deposition to internal organs. We first determined the γT3 level of each of the vital organs from mice treated with 50 mg/kg of γT3 per day for 10 days by HPLC. As shown in figure 2c, the spleen and liver were determined to have the highest levels of γT3 deposition at the end of the treatment period. γT3 was also detectable in heart, kidney and lung tissues. More importantly, the examination of the tumor tissues revealed that γT3 accumulated primarily within the tumors, reaching a concentration of 0.15 ± 0.03 mg of γT3 per gram of wet weight (fig. 2c). This was at least 2-fold the amount detected in other internal organs. These results suggest that γT3 selectively deposited in AIPCa tumor tissues, which may help to explain why γT3 can exert significant antitumor activity at dosages that are associated with no observable toxicity.

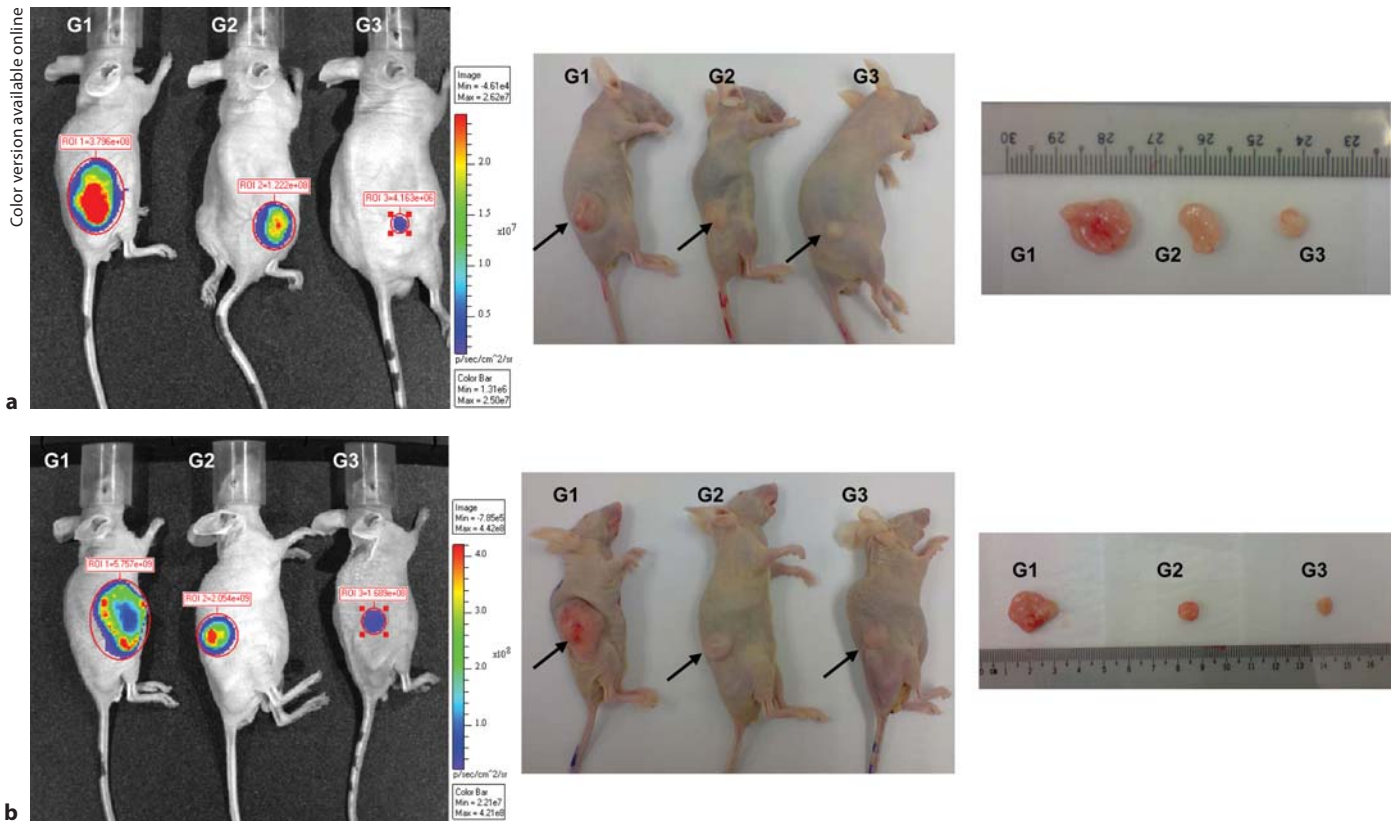


Fig. 3. In vivo imaging of PCa cells xenografted on male BALB/c athymic nude mice following drug treatment. For 2 repeated experiments, male BALB/c athymic nude mice were implanted with PC3-Luc cells and divided randomly into 3 groups (n = 10 per group). G1 = Control (DMSO as vehicle); G2 = γT3 (50 mg/kg/day); G3 = combined γT3-DTX treatment (50 mg of γT3/kg/day, and 7.5 mg of DTX/kg/week). The mice received intraperitoneal injections with D-luciferin solution (150 mg/kg body weight) 5 min before imaging, using the IVIS imaging system. The tumors in G1 grew rapidly by day 14 after the start of the treatment. In contrast, G2 and G3 showed a significant inhibitory effect on in vivo PCa growth.

In vivo Effect of γT3 on Cancer Cell Proliferation and Apoptosis

To confirm if the cytotoxic effect of γT3 was, as we reported in our in vitro studies [9], mediated by the inhibition of cell proliferation and induction of apoptosis, AIPCa tumor tissues were harvested from each treatment mouse group (fig. 3) and examined by immunohistochemistry. As shown in figure 4, PCNA, Ki-67 and Id1 expressions were downregulated after treatment with γT3 and combined γT3-DTX. Meanwhile, treatment with γT3 and combined γT3-DTX also activated the expression levels of cleaved caspase 3 and PARP (fig. 5), suggesting that more cells underwent apoptosis. These antiproliferation and proapoptosis observations are in agreement with the findings from our previous studies [9].

γT3 Antitumor Effect on Tumor Suppressor Gene

Downregulation of E-cadherin expression is one of the most frequently reported characteristics of metastatic cancers. Restoration of E-cadherin expression in cancer cells leads to suppression of metastatic ability [18, 19]. In AIPCa, downregulation of E-cadherin expression is correlated with high-grade tumors and poor prognosis [20], indicating their roles in PCa progression. Since γT3 was found to inhibit in vitro the invasion ability of PCa cells by upregulating E-cadherin expression, we then analyzed if γT3 could also affect E-cadherin levels in AIPCa tumors in vivo. E-cadherin expression in tumor sections from the control, γT3 and combined γT3-DTX groups of athymic nude mice was examined by immunohistochemistry, and the results showed that E-cadherin was upregulated after γT3 and combined γT3-DTX treatment (fig. 6a), whereas the repressor of E-cadherin, Snail [21],

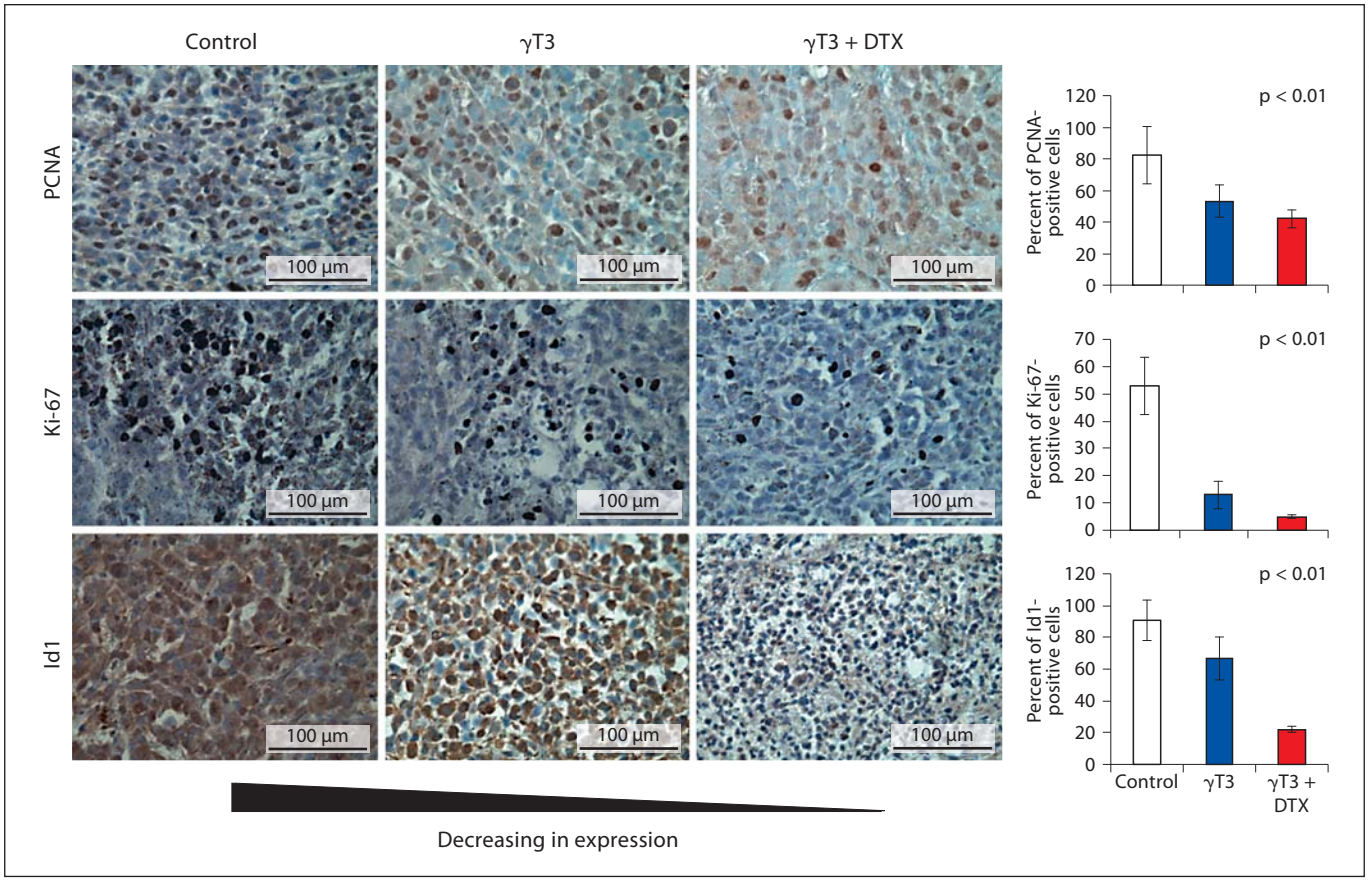


Fig. 4. γT3 antitumor effect on in vivo cell proliferation markers. Expression levels for PCNA, Ki-67 and Id1 (cell proliferation markers) were lower after the 2-week treatment with either γT3 or combined γT3-DTX (treated groups). The graphs (right) show the percentage of positively stained cells. One-way ANOVA was used to compare the differences between the means of treated groups versus the control DMSO group. Bars: means. Whiskers: SD. Photomicrographs: ×400.

was downregulated (fig. 6b). These data suggested that, in addition to the inhibition of AIPCa tumor growth, γT3 may possess in vivo antimetastatic activity.

Discussion

In this study, we showed that γT3 suppressed the growth of AIPCa in athymic nude mice. Studies of γT3 antitumor effects in vivo are limited by the lack of highly purified γT3 and the difficulties in delivering γT3 to tumor cells. Our laboratory has shown that the inhibitory effect of γT3 on PCa cell growth is specific to the fast proliferating cells in vitro [9]. Herein, we reported that the intraperitoneal route of γT3 administration is effective in suppressing AIPCa tumor growth.

The accumulation of γT3 in cancer cells is critical for its antitumor activities. Consistent with recent studies indicating high T3 deposition in hepatoma cells [22], pancreatic cells [16] and adipose breast tissues [23], we determined in our study that γT3 also accumulated in AIPCa tumors following intraperitoneal administration. Compared to the γT3 concentration determined in AIPCa tumors, the γT3 deposition in 5 vital organs (heart, liver, spleen, lungs and kidneys) was approximately half (fig. 2c). Similarly, other studies had reported T3 deposition in various tissues of rats, especially in adipose tissues, skin and heart [24]. Taken together, this evidence suggests that T3 is absorbed in, and distributed efficiently to, various internal organs in vivo [24, 25]. Nevertheless, the different levels of T3 deposition in the organs may be explained by several factors. First, the postabsorptive T3 metabolism affects its biological half-life [26, 27]. Second,

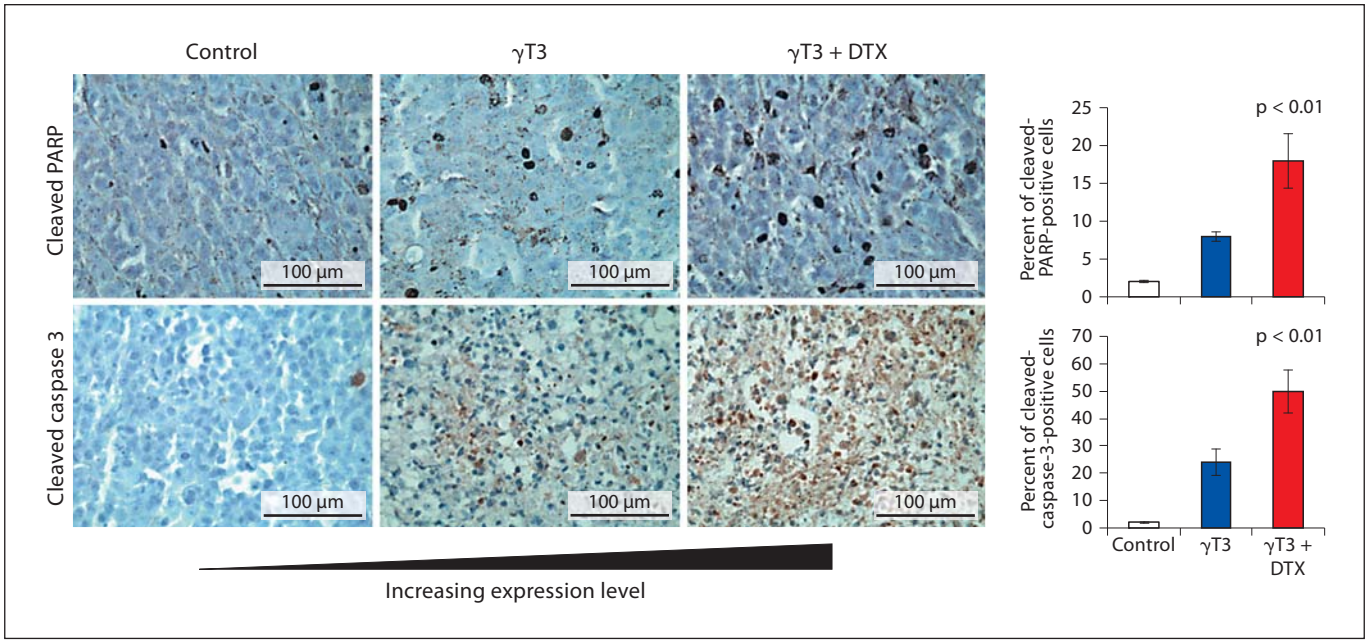


Fig. 5. γT3 antitumor effect on in vivo apoptosis markers. The expression levels for cleaved caspase 3 and PARP were higher after the 2-week treatment with either γT3 or combined γT3-DTX (treated groups). The graphs (right) show the percentage of positively stained cells. One-way ANOVA was used to compare the differences between the means of treated groups versus the control DMSO group. Bars: means. Whiskers: SD. Photomicrographs: ×400.

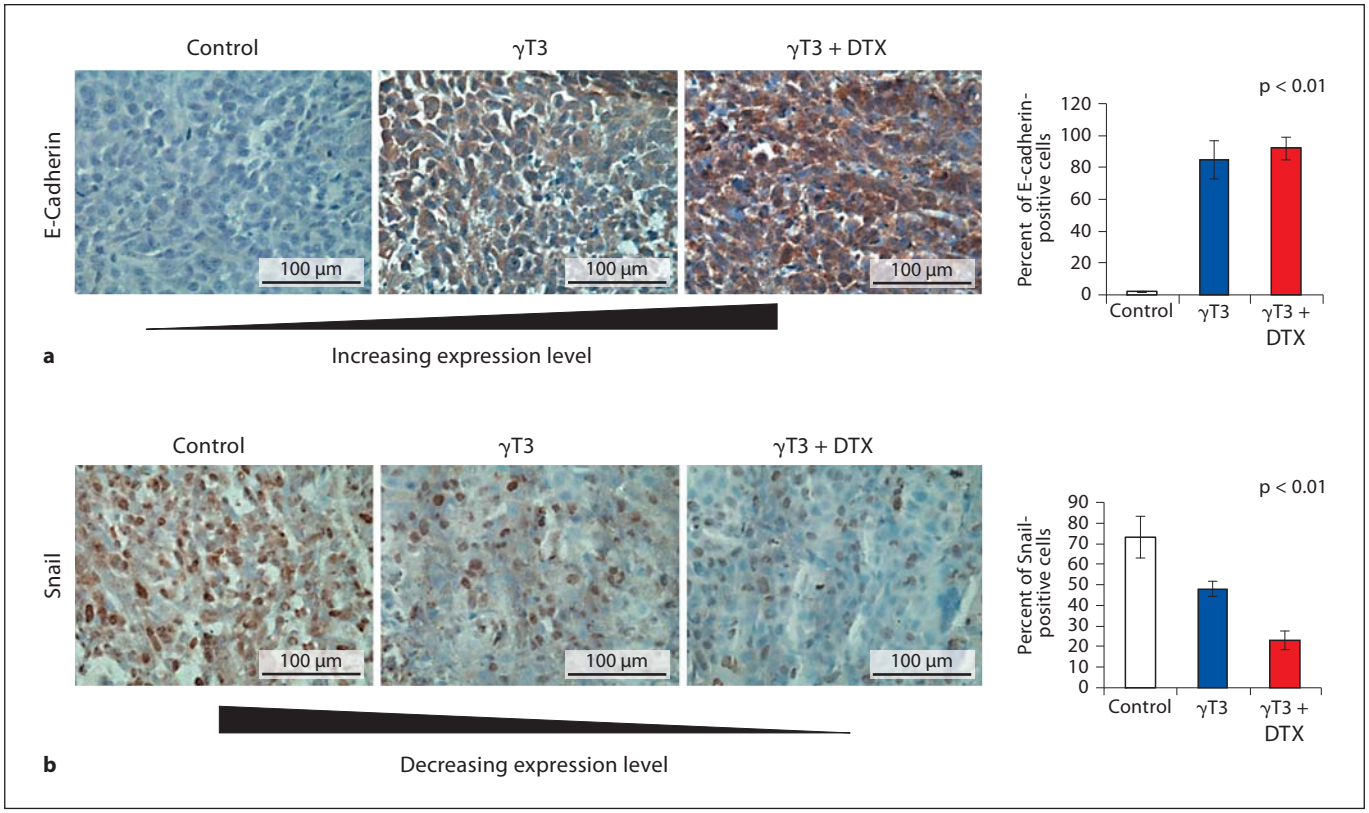
the level of adipose tissue that acts as a T3 reservoir affects the amount of T3 stored. For example, pancreatic tissue enclosed in the mesenteric adipose tissue may account for its high T3 deposition [16]. Third, strong cellular growth promotes T3 [9–11] and nutrient [28] uptake.

Although one study reported that negligible γT3 was detected in tissues of rat following intraperitoneal injection [29], the discrepancy between their findings and the majority of reports stated above was likely due to the method of T3 administration. In this study, DMSO was used to dissolve γT3 prior to intraperitoneal injection. In general, the peritoneum environment is less destructive to T3 compared to the upper gastrointestinal tract because the acidic environment of the latter may cause T3 to undergo biotransformation (oxidization) [30, 31]. Nevertheless, despite the deposition of γT3 in the vital organs, it has no observable side effects on body weight, normal organ weight and serum toxicity levels.

Despite many in vitro studies demonstrating antitumor effects of T3 [8–11, 32–40], there has been a limited number of studies determining whether similar inhibitory effects will be observed in an in vivo model. The hydroxyl moiety, found in all tocochromanol molecules which mediate the classical antioxidant properties of vitamin E, is generally believed to be unrelated to the antitumor activities of γT3 [9–11]. Previous in vitro studies, including ours, have shown that γT3 inhibits cancer cell growth by multiple mechanisms that include the induction of cell cycle arrest and apoptosis via regulation of Id1, NF-κB and Akt signaling pathways, or modulation of the cyclooxygenase activity [8, 9, 32–40]. The role of these processes in the antitumor effects of γT3 on AIPCa growth in vivo is yet to be elucidated.

In our previous in vitro study [9], where γT3 inhibited cell growth in androgen-dependent and androgen-independent PCa cell lines, we found that γT3 upregulated the E-cadherin gene, which is thought to inhibit cancer cell invasion, and metastasis [41]. In the same study, Id1, which is constitutively expressed by the AIPCa cell line PC3, was repressed by γT3 treatment leading to the suppression of NF-κB pathway molecules. In this study, we were able to further confirm these antitumor activities against AIPCa under in vivo conditions.

Because cell proliferation and apoptosis are critical processes for tumor growth, we investigated the modulation of these processes by γT3 using our AIPCa xenograft model. Consistent with previous findings [9], we observed a remarkable antiproliferative effect following in-



Color version available online

Fig. 6. γT3 antitumor effect on in vivo tumor suppressor gene (**a**) and its repressor (**b**). The expression levels for the tumor suppressor gene (E-cadherin) and its repressor (Snail) correlate inversely after treatment with either γT3 or combined γT3-DTX (treated groups). The graphs (right) show the percentage of positively stained cells. One-way ANOVA was used to compare the differences between the means of treated groups versus the control DMSO group. Bars: means. Whiskers: SD. Photomicrographs: ×400.

traperitoneal administration of γT3 and combined γT3-DTX, as evidenced by the repression of PCNA, Ki-67 and Id1 protein expression (fig. 4). In addition, apoptotic genes (cleaved caspase 3 and PARP) were also concomitantly activated in AIPCa tumors following intraperitoneal administration of γT3 and combined γT3-DTX. Several other in vitro studies have shown that γT3 triggers apoptosis in many cell lines including breast, prostate, colon, liver and gastric cell lines [32, 33, 42–48]. The exact mechanism responsible for γT3-induced apoptosis is not fully understood. Previous studies have suggested that γT3 may mediate apoptosis by modulation of the Id1/NF-κB signaling cascade [11, 32], or by activation of the caspase 3 and PARP proteins [42, 46, 49]. However, it is unclear whether these same mechanisms function under in vivo conditions. This study supports the process of apoptosis as an important mechanism contributing to the in vitro and in vivo antitumor activities of γT3 (fig. 5). The implication of these observations is that γT3 may be used in synergy with other antiproliferative agents against AIPCa.

Although tumor metastasis was not examined in this study, our finding that γT3 treatment resulted in enhanced expression of E-cadherin seemed to support that γT3 may have in vivo antimetastatic activity. Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis because it decreases cellular adhesion within the tissue, resulting in an increase in cellular motility. This, in turn, may allow cancer cells to cross the basement membrane and invade surrounding tissues [18, 50–52]. The exact interaction with γT3 remains to be investigated, but it may be a unique property of γT3 in phospholipid membranes. Since we have demonstrated in our previous study that γT3 can inhibit cancer cell invasion in vitro by induction of E-cadherin expression, this current finding provides strong

evidence to warrant further investigation of the antimetastatic effect of γT3 in vivo.

In addition, we questioned whether γT3 possessed synergistic interaction with DTX as a result of Id1 [9] and EGFR [9] gene suppression. Recently, novel therapeutic approaches that combined DTX with EGFR-targeting agents [53, 54] and an Id1-targeting agent [9–11] resulted in significantly higher proapoptotic effects. To this end, we compared the antiproliferative capability of γT3 treatment alone with combined γT3-DTX treatment. Remarkably, we found that combined γT3-DTX treatment resulted in a stronger suppression of cancer cell proliferation genes (fig. 4). The same combined treatment led to an enhancement of apoptotic responses, as indicated by the activation of cleaved caspase 3 and PARP (fig. 5). These findings suggest that the combined use of an inhibitor of EGFR-Id1 signaling with DTX could represent a more promising treatment strategy for patients with AIPCa.

Finally, we have demonstrated that γT3, a derivative of vitamin E, is capable of inhibiting AIPCa growth in vivo via the inhibition of cancer cell proliferation and induction of apoptosis. Although the number of animals investigated was insufficient (n = 10 per group) to achieve a reasonable assurance of validity, the results will nevertheless aid in the development of future preclinical and clinical studies on the antitumor effects of γT3 against AIPCa.

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Conflict of Interest

W.N.Y., N.Z. and Y.L.Y. are employed by Davos Life Science, which is a manufacturer of T3 based in Singapore.

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