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Synergistic inhibition of cancer cell proliferation with a combination of δ-tocotrienol and ferulic acid

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ABSTRACT

Rice bran consists of many functional compounds and thus much attention has been focused on the health benefits of its components. Here, we investigated the synergistic inhibitory effects of its components, particularly δ-tocotrienol (δ-T3) and ferulic acid (FA), against the proliferation of an array of cancer cells, including DU-145 (prostate cancer), MCF-7 (breast cancer), and PANC-1 (pancreatic cancer) cells. The combination of δ-T3 and FA markedly reduced cell proliferation relative to δ-T3 alone, and FA had no effect when used alone. Although δ-T3 induced G1 arrest by up-regulating p21 in PANC-1 cells, more cells accumulated in G1 phase with the combination of δ-T3 and FA. This synergistic effect was attributed to an increase in the cellular concentration of δ-T3 by FA. Our results suggest that the combination of δ-T3 and FA may present a new strategy for cancer prevention and therapy.

1. Introduction

The consumption of certain whole foods (e.g., grains, fruits, and vegetables) is associated with reduced cancer risk, and their inherent safety makes them an appealing choice for widespread and long-term use in diverse populations [1]. Rice, *Oryza sativa*, is a staple food crop for more than half of the world's population. Given that the bran fraction of rice contains various bioactive components with chemopreventive activity, including tocotrienol (T3), ferulic acid (FA), γ-oryzanol, β-sitosterol, and squalene [2], many studies have investigated the cancer-fighting properties of dietary rice bran. In a prospective study, Tantamango et al. [3] reported an association between the consumption of certain foods and the risk of developing polyps. In addition to the protective effects of green vegetables, dried fruit, and legumes, the study found that consumption of brown rice was most strongly correlated with a reduced risk of polyp formation.

Vitamin E is the generic term for T3, which is distinguishable from tocopherol (Toc; well-known vitamin E) by the degree of saturation of its side chain. T3 has an isoprenoid side chain with three double bonds, whereas Toc contains a fully saturated phytyl tail. Vitamin E occurs naturally in eight forms: α-, β-, γ-, and δ-isomers of both Toc and T3 (Fig. 1A). T3 has recently gained attention due to its anti-oxidative [4], anti-hypercholesterolemic [5], and neuroprotective [6] effects, and its efficacy is superior to that of Toc. We previously demonstrated that T3 has potent anti-tumor activity, and that δ-T3 has the most potent anti-cancer activity (i.e., antiangiogenesis [7] and telomerase inhibition [8]) of the four T3 isomers.

FA (4-hydroxy-3-methoxy cinnamic acid) is a potent phenolic antioxidant (Fig. 1A). The antioxidative effects of FA are closely tied with its structural characteristics. Given its phenolic nucleus and unsaturated side chain, FA can readily form a resonance stabilized phenoxy radical, which accounts for its potent antioxidant activity [9]. FA is receiving widespread attention owing to its therapeutic effects against cancer [10], diabetes [11], cardiovascular disease [12], and neurodegenerative disorders [13]. The therapeutic potential of FA is largely attributed to its anti-oxidative effects.

Although rice bran is broadly beneficial for human health, very little is known about the synergistic effects of its components on inhibiting cancer cell proliferation. In the present study, we investigated a potential role for rice bran components, with particular emphasis on δ-T3 and FA, in the synergistic inhibition of human prostate carcinoma, human breast adenocarcinoma, and human pancreatic carcinoma cell proliferation.

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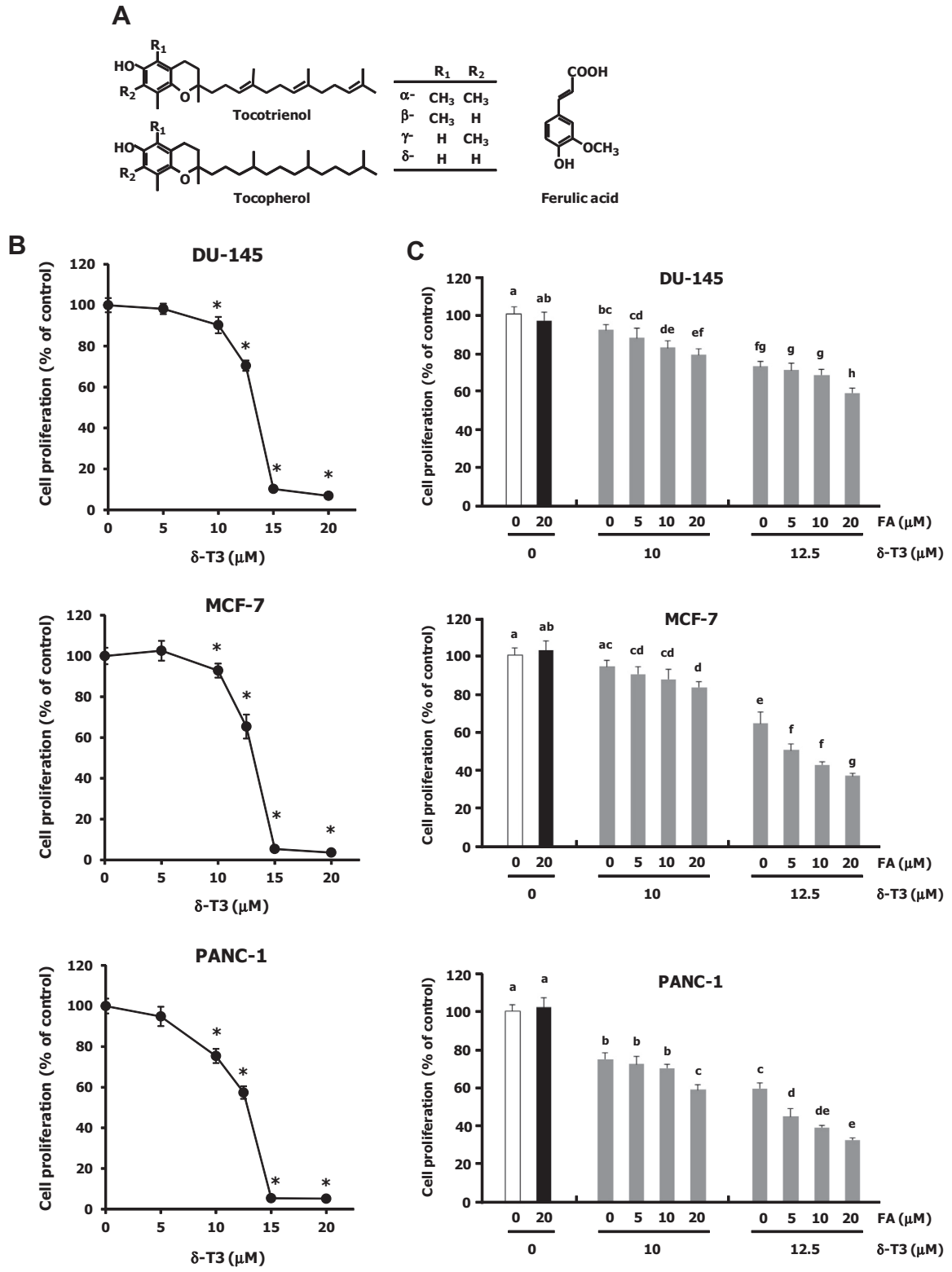

Fig. 1. Inhibition of cell proliferation by δ-T3 and the δ-T3 + FA combination. (A) Chemical structures of vitamin E and FA. (B) Human cancer DU-145, MCF-7, and PANC-1 cells grown in 96-well plates were treated with 5–20 μM δ-T3 or vehicle (control) for 72 h. Viable cells were evaluated with the MTT assay and are expressed as a percentage of the control. Values are presented as mean ± S.D. from six independent experiments; $^{*}P < 0.05$ compared with control. (C) Effects of 10–12.5 μM δ-T3 in combination with 5–20 μM FA on DU-145, MCF-7, and PANC-1 cells after 72 h. Values are presented as mean ± S.D. from six independent experiments. Values not sharing a common superscript are significantly different ($P < 0.05$).

2. Materials and methods

2.1. Materials

δ-T3 and FA were obtained from Eisai (Tokyo, Japan) and Wako (Osaka, Japan), respectively. RPMI 1640 medium (containing 0.3 mg/mL L-glutamine and 2.0 mg/mL sodium bicarbonate) was purchased from Sigma (St. Louis, MO), fetal bovine serum (FBS) was from Funakoshi (Tokyo, Japan), penicillin and streptomycin were from Gibco (Rockville, MD), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Dotite (Kumamoto, Japan). The primary antibodies for p21 and GAPDH were purchased from Cell Signaling Technologies (Danvers, MA).

2.2. Cell culture

DU-145 (human prostate carcinoma), MCF-7 (human breast adenocarcinoma), and PANC-1 (human pancreatic carcinoma) cells were provided by RIKEN BRC (Tsukuba, Japan). Cells were cultured in growth medium (RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. MTT assay

Test medium was prepared from growth medium supplemented with δ-T3 and/or FA (dissolved in ethanol at various concentrations). The final concentration of ethanol in the test medium was 0.1% (v/v). Cells were seeded onto a 96-well plate at a density of 4.0×10^3 cells/well in 100 μL of growth medium. After incubation for 24 h, the medium was removed, and cells were cultured in 100 μL of test medium. Cell proliferation was assessed with the MTT assay [14].

2.4. Analysis of T3

Cellular uptake and culture medium concentrations of δ-T3 were measured by high performance liquid chromatography (HPLC) with fluorescence detection [15]. In brief, after PANC-1 cells were cultured in test medium for 48 h, they were washed with PBS, suspended in 2 mL of water, and subjected to sonication for 90 s. Following this, 1 mL of 6% ethanolic pyrogallol and 200 μL of 10 μM ethanolic 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC; an internal standard) were mixed with the cell suspension. The sample mixture was further mixed with 0.2 mL of 60% aqueous KOH and incubated at 70 °C for 30 min. After cooling, 5 mL of hexane was added for extraction. Samples were centrifuged at 1000*g* for 5 min, and the upper hexane layer was collected and dried. The residue was dissolved in 200 μL of hexane, and a portion of the aliquot (10 μL) was subjected to HPLC. To measure the concentration of δ-T3 in cell culture medium, a 2 mL aliquot of the test medium was mixed with 1 mL of 6% ethanolic pyrogallol and 200 μL of 10 μM ethanolic PMC. The sample was extracted using hexane and subjected to HPLC as described above. Inersil SIL-100A-5 (4.6 × 250 mm; GL Science, Tokyo, Japan) was used as the HPLC column. A mixture of hexane/1,4-dioxane/2-propanol (1000:40:5, v/v/v) was used as the mobile phase. δ-T3 was detected and concentrations determined with an RF-10AXL FLD detector (excitation 294 nm, emission 326 nm; Shimadzu, Kyoto, Japan).

2.5. Cell cycle analysis

Cells treated with the test medium were harvested and washed with ice-cold PBS, then fixed with ice-cold 70% ethanol. Ethanol-fixed cells were treated with RNase (100 μg/mL in PBS) and stained with propidium iodide (50 μg/mL in PBS) in the dark for 30 min at 37 °C. Stained cells were transferred to FACS tubes and detected using flow cytometry (COULTER EPICS XL-MCL, Beckman Coulter, Brea, CA). The proportion of cells in each cell cycle phase was evaluated using ModFit LT 3.2 software (Verity Software House, Inc., Topsham, ME).

2.6. RNA isolation and quantitative real-time PCR

Total RNA was isolated using the NucleoSpin RNA II kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany). For real-time reverse transcription-PCR (RT-PCR), cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). The resulting cDNA was subjected to PCR amplification using SYBR Premix Ex Taq II (Takara) and gene specific primers for p21 and GAPDH (Takara). Quantitative PCR was performed with the Thermal Cycler Dice Real Time System (Takara). Thermal cycling conditions included an initial denaturation step at 95 °C for 10 s, which was followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

2.7. Western blot analysis

SDS–polyacrylamide gel electrophoresis was carried out, and proteins were transferred onto a polyvinylidene difluoride

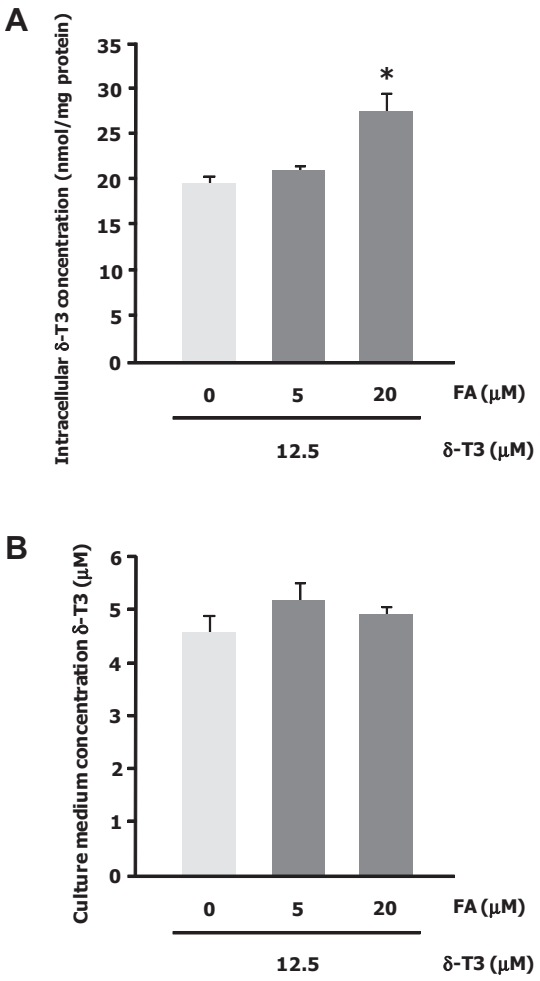

Fig. 2. Intracellular and culture medium concentrations of δ-T3 in PANC-1 cells. PANC-1 cells were treated with the indicated concentrations of δ-T3 and FA for 48 h. (A) Intracellular and (B) cell medium concentrations of δ-T3 were measured by HPLC. Values are presented as mean ± S.D. from three independent experiments; $^*P < 0.05$ compared with δ-T3 treatment.

membrane (Millipore, Billerica, MA). After blocking, the membrane was probed with primary antibodies for p21 and GAPDH, followed by secondary antibodies (anti-rabbit IgG) conjugated with horseradish peroxidase (Cell Signaling Technologies). Target proteins were visualized by the addition of Immobilon Western Chemiluminescent HRP Substrate (Millipore), and images were captured using the ChemiDoc MP system (Bio-Rad, Hercules, CA).

2.8. Statistical analysis

Data are presented as mean ± S.D. of at least three independent experiments. Data were evaluated by one-way analysis of variance (ANOVA), followed by individual comparisons with control using Dunnett's test. ANOVA, followed by the Tukey–Kramer test, was used to compare multiple groups. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Synergistic inhibition of DU-145, MCF-7, and PANC-1 proliferation by δ-T3 and FA

Before evaluating the synergistic effects of δ-T3 and FA, we examined the ability of δ-T3 or FA alone to inhibit the proliferation of three human cancer cell lines (DU-145, MCF-7, and PANC-1). δ-T3 inhibited the proliferation of these cell lines in a dose-dependent manner (Fig. 1B), with significant inhibition observed at concentrations of ⩾10 μM. δ-T3 at 15–20 μM for 72 h was highly cytotoxic (data not shown). Based on these results, δ-T3 at a concentration range of 10–12.5 μM was chosen for evaluating synergy with FA. Unlike δ-T3, FA did not inhibit proliferation even at concentrations as high as 50 μM (data not shown). As shown in Fig. 1C, combined treatment of 10–12.5 μM δ-T3 with 5–20 μM

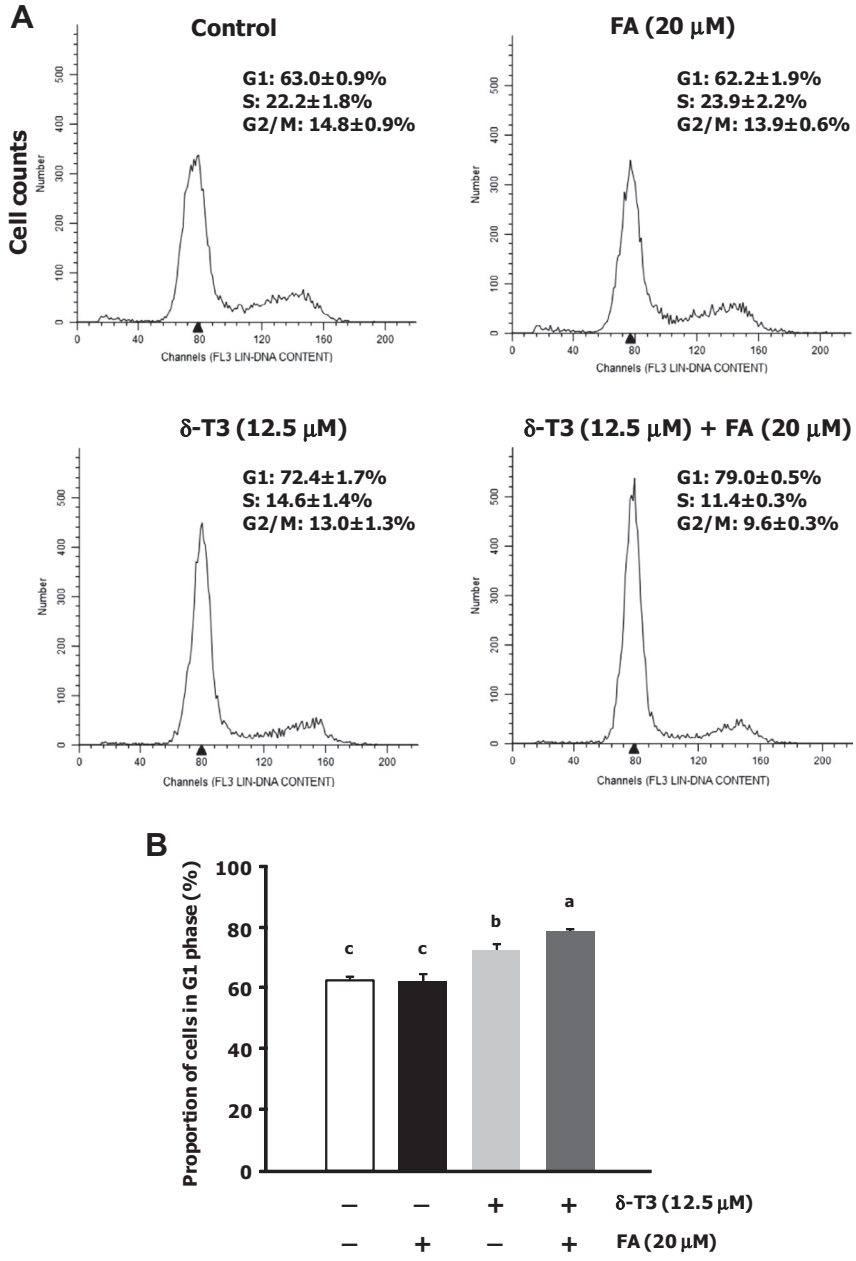

Fig. 3. Effects of δ-T3 and FA on cell cycle regulation. (A) PANC-1 cells were treated with δ-T3, FA, and δ-T3 + FA for 48 h. The cells were then stained with propidium iodide and analyzed by flow cytometry. Data were evaluated with ModFit LT 3.2 software. (B) The percentage of cells in G1 phase was calculated. Values are presented as mean ± S.D. from three independent experiments. Values not sharing a common superscript are significantly different ($P < 0.05$).

FA markedly reduced proliferation of these cells relative to δ-T3 alone, although 20 μM FA alone showed no inhibitory effect. These results clearly demonstrate the synergistic inhibition of DU-145, MCF-7, and PANC-1 cell proliferation by the combination of δ-T3 and FA. Specifically, the combination of 12.5 μM δ-T3 and 20 μM FA inhibited the proliferation of DU-145, MCF-7, and PANC-1 cells by 59%, 37%, and 32%, respectively (Fig. 1C), suggesting a sensitivity ranking to the combination of PANC-1 > MCF-7 > DU-145. In the remaining experiments described herein, PANC-1 cells were used to further investigate the synergistic effects of δ-T3 and FA.

T3 suppresses the proliferation of and induces apoptosis in a wide variety of tumor cells, including those of the breast, colon, liver, lung, stomach, skin, pancreas, and prostate [16]. Concentrations of δ-T3 used in the *in vitro* experiments (Fig. 1B and C) were comparable to those reported in other studies [16]. To determine whether our findings (Fig. 1C) generally apply to other cancer cell types, DLD-1 human colorectal adenocarcinoma, HeLa human cervical carcinoma, HepG2 human hepatoma, and A549 human lung carcinoma cell lines were treated with δ-T3 and FA. Concomitant treatment of cells with δ-T3 and FA led to synergistic growth inhibition of all cell types tested (data not shown), suggesting that the synergistic effects apply to a wide variety of cancer cells.

Many attempts have been made to enhance the efficacy of T3 by screening for molecules that synergize with T3. A number of studies have reported that T3 combined with other agents (e.g., statins [17], celecoxib [18], erlotinib/gefitinib [19], epigallocatechin gallate/resveratrol [20], sesamin [21]) significantly potentiated the anti-cancer effects of T3. Sesamin, a phytochemical found in high concentrations in sesame seed oil, improves the bioavailability of T3 *in vitro* [22] and *in vivo* [23]. Treatment of A549 cells with both T3 and sesamin substantially increased the intracellular concentration of T3 relative to when T3 was used alone [22]. Akl et al. [21] reported the synergistic inhibition of +SA mouse mammary epithelial cell proliferation by T3 and sesamin (20 μM). These reports collectively suggest that increased T3 bioavailability correlates with enhanced T3 activity.

3.2. Effect of FA on intracellular δ-T3 concentration

To examine whether FA enhances the bioavailability of T3, the intracellular concentration of δ-T3 was analyzed using HPLC. Co-treatment with δ-T3 and FA increased the intracellular concentration of δ-T3 after 48 h in PANC-1 cells, compared to when δ-T3 was used alone (Fig. 2A). This suggests that FA enhances the bioavailability of δ-T3, and that the intracellular concentration of δ-T3 correlates with the intensity of δ-T3-mediated inhibition of proliferation (Fig. 1C).

Intracellular levels of δ-T3 observed in PANC-1 cells suggest that FA either suppresses the intracellular metabolism of δ-T3 or facilitates its cellular uptake. To address the latter possibility, we evaluated the concentration of δ-T3 in culture medium using HPLC, and found that FA treatment did not influence the amount of δ-T3 in culture medium (Fig. 2B). This result suggests that FA is unlikely to affect the cellular uptake of δ-T3.

All vitamin E isoforms are catabolized to water soluble metabolites known as carboxyethyl hydroxychromans (CEHCs) [24]. Cytochrome P450 4F2 (CYP4F2), a human vitamin E-ω-hydroxylase, catalyzes the hydroxylation of one of the terminal methyl groups on the hydrophobic side chain. This ω-hydroxylation can be followed by oxidation to the corresponding carboxyl form and a series of side-chain shortening steps, ultimately leading to the formation of CEHCs, which are excreted in urine. Sesamin potently inhibits CYP4F2 activity, leading to elevated vitamin E concentrations in rat and human liver microsomes [25]. This suggests that CYP4F2 is a physiologically important determinant of vitamin E status. Since both sesamin and FA structurally belong to the family of phenylpropanoids [26,27], FA may also inhibit CYP4F2 in a similar manner.

3.3. Induction of G1 arrest by δ-T3 and FA

To further elucidate the mechanism underlying the anti-proliferative effects of δ-T3 and FA, cell cycle analysis was performed after treating cells with the combination for 48 h. While FA had no effect on cell cycle progression, δ-T3 significantly increased the proportion of PANC-1 cells in G1 phase (Fig. 3A and B) and decreased the proportion of cells in S phase. As shown in Fig. 3A and B, an even higher proportion of cells were arrested in G1 phase when treated with the combination of δ-T3 and FA.

p21 (waf-1/cip-1) plays a key role in G1 arrest. Specifically, p21 inhibits different complexes of cyclin/cyclin-dependent kinases (Cdks) (Cyclin D-Cdk4/6 and Cyclin A, E-Cdk2) that sequentially phosphorylate the retinoblastoma (Rb) protein to release the S phase-promoting E2F-1 transcription factor [28]. To examine whether treatment with δ-T3 and FA affects p21 expression, we conducted real-time RT-PCR and Western blot analysis. Treatment of PANC-1 cells with FA had little effect on both p21 mRNA (Fig. 4A) and protein (Fig. 4B) levels. Although δ-T3 alone induced

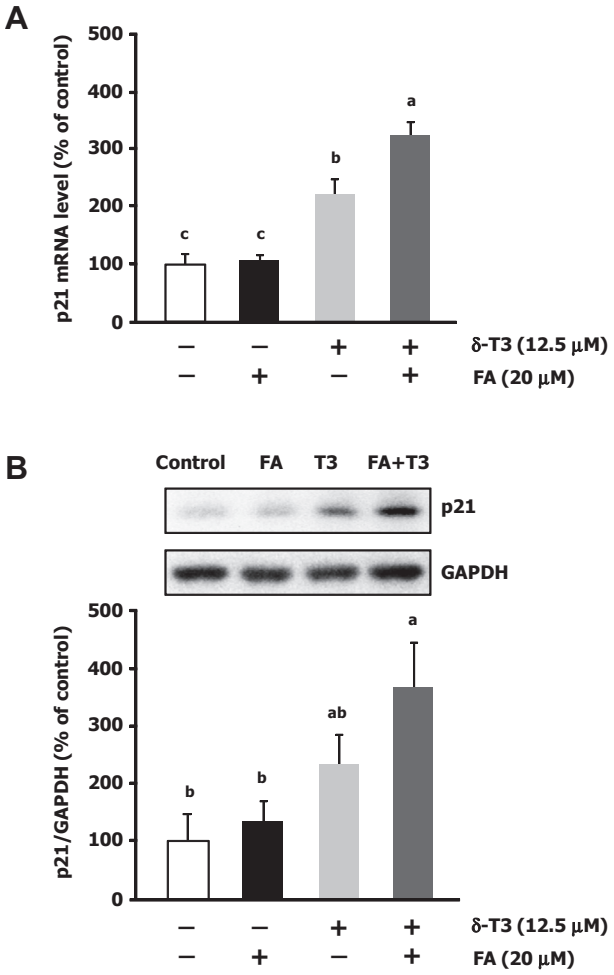

Fig. 4. Effects of δ-T3, FA, and δ-T3 + FA on p21 expression. PANC-1 cells were treated with δ-T3, FA, or δ-T3 + FA for 48 h at the indicated concentrations. mRNA (A) and protein (B) levels of p21 were measured by real-time RT-PCR and Western blot, respectively, and are expressed as percentages of the untreated control (without sample). Values are presented as mean ± S.D. from three independent experiments. Values not sharing a common superscript are significantly different ($P < 0.05$).

an approximately 2-fold increase in p21 expression, the combination of δ-T3 and FA induced higher levels (3-fold) of p21 mRNA and protein (Fig. 4A and B).

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme of the mevalonate pathway. Ras, Rho, and insulin-like growth factor I receptor rely on the mevalonate pathway for posttranslational modification and maturation, and play crucial roles in cell cycle progression (G1/S transition), apoptosis, and metastasis [29]. Ras and Rho suppress the promoter activity of p21 and inhibit its transcription [30]. Recent studies have revealed that HMG-CoA reductase inhibitors such as statins suppress the growth of tumor cells by inducing G1 arrest via p21 induction, and therefore have potential as novel chemotherapeutic agents [31]. Moreover, δ-T3 has been shown to attenuate HMG-CoA reductase activity and induce cell cycle arrest and apoptosis in tumor cells [32]. Thus, it is possible that the anti-proliferative property of δ-T3 (Fig. 1) may be attributed, at least in part, to its ability to inhibit HMG-CoA reductase.

Since chemotherapeutic agents are often associated with severe toxicity and adverse side effects, use of natural and relatively non-toxic compounds is an attractive alternative. In the present study, we demonstrated for the first time that a combination of two naturally occurring rice bran components, δ-T3 and FA, synergistically inhibited the proliferation of three cancer cell lines. The combination of δ-T3 and FA induced G1 arrest by up-regulating p21 more effectively than δ-T3 alone. The synergistic effects might be attributed to an increase in the cellular concentration of δ-T3 via FA.

In summary, the present study demonstrated the beneficial synergistic effects of two rice bran components, δ-T3 and FA. Our findings should encourage further investigation into the combination, as well as its application to cancer prevention and therapy.

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