

Induction of Apoptotic Cell Death and *in Vivo* Growth Inhibition of Human Cancer Cells by a Saturated Branched-Chain Fatty Acid, 13-Methyltetradecanoic Acid¹

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Abstract

A saturated branched-chain fatty acid, 13-methyltetradecanoic acid (13-MTD), was purified from a soy fermentation product, which was used by many cancer patients as a treatment supplement. Our preliminary study indicated that 13-MTD could induce cell death in human cancer cell lines K-562, MCF7, DU 145, NCI-SNU-1, SNU-423, NCI-H1688, BxPC3, and HCT 116. The ID₅₀ dosage of 13-MTD for these tumor cells ranged from 10 to 25 µg/ml. Further investigation revealed that 13-MTD caused tumor cell death through rapid induction of apoptosis, which could be detected 2 h after the treatment of tumor cells with 13-MTD. Xenograft tumors of prostate carcinoma cell line DU 145 and hepatocarcinoma LCI-D35 were orthotopically implanted into nude mouse prostate and liver, respectively. 13-MTD was administered p.o. once daily to the implanted mice for ~40 days. Our results showed that 13-MTD could effectively inhibit the growth of orthotopic tumor implants of both cell lines compared with control groups. The average inhibition rate was 84.6% for DU 145 and 65.2% for LCI-D35 ($P < 0.01$). LD₅₀ test results showed that mice could well sustain the oral feeding of 5 g/kg/day without observable anomaly. Our preliminary data demonstrated that 13-MTD could effectively inhibit *in vitro* and *in vivo* growth of various cancer cell lines by inducing apoptosis without significant toxic side effects, suggesting 13-MTD as a potential candidate for chemotherapy of human cancers.

Introduction

The regulation of cell growth is a homeostatic balance between stimulatory and inhibitory signals. Negative growth control by tumor suppressor genes, differentiation factors, and programmed cell death (apoptosis) provides alternative strategies for treatment of malignancies and other diseases. Among them, apoptosis is a highly attractive and widely studied area to search for more effective agents for treatment of human cancers. Variety of *in vivo* and *in vitro* studies published in recent years suggested that many chemotherapeutic agents could induce apoptotic cell death in different cancer cells (1–6).

Although these results are important and exciting, most chemotherapeutic agents studied thus far have undesired toxic side effects to the host animals and humans. Therefore, it is very critical that efforts should be made to discover new agents with which cancers could be treated safely without serious side effects. A soy fermentation product, Yang Zhen Hua 851, manufactured by Pentagenic Pharmaceuticals, Inc. (Diamond Bar, CA) through an innovative bacterial fermentation, has been used as a nutritional and therapeutic supplement by tens of thousands of cancer patients with different clinical stages since 1985. The results from these patients indicated that the product could effectively improve patients' clinical condition as well as survival

rate, and laboratory studies showed inhibition of tumor cell growth by this product.³ Several compounds with similar anticancer effects were purified from this product and named SBAs⁴ (United States patent pending). The structural and biochemical analysis had shown that SBAs represent a group of terminally branched-chain saturated fatty acids (C_{15–21}). One of them, 13-MTD (iso-C₁₅), is the most abundant component in Yang Zhen Hua 851 and was chosen for this study. The branched-chain fatty acid occurs naturally only in trace amounts and is not synthesized *de novo* in human and other mammals (7–9). The presence of the isopropyl group gives 13-MTD a characteristic gas chromatographic behavior to serve as a structural marker for investigating adipose tissue turnover in human and other mammals (8–10).

In the present report, we studied 13-MTD as a potential anticancer agent to inhibit tumor cell growth. Our *in vitro* cell culture studies showed that 13-MTD could cause cell death through apoptosis in several human tumor cell lines tested. The cell death could be observed as early as 2 h after treatment of tumor cells with 13-MTD. The MetaMouse orthotopic model was used to evaluate the *in vivo* inhibition of tumor growth by 13-MTD. The results indicated that oral administration of 13-MTD could effectively inhibit the growth of tumors orthotopically implanted into nude mice. Altogether, our *in vitro* and *in vivo* data demonstrated that 13-MTD could effectively inhibit tumor cell growth by inducing apoptosis and is a potential agent for cancer chemotherapy.

Materials and Methods

Isolation and Purification of 13-MTD. The crude product of Yang Zhen Hua 851 was produced through fermentation of soybean by a unique strain of bacteria (United States Patent No. 4877739; ATCC No. 202105). This product was then analyzed to isolate the components with anticancer activity. Several compounds were obtained by high-performance liquid chromatography and tested for growth-inhibitory activity to cultured cancer cells. The ones with inhibitory activity to cancer cells were named SBAs. The chemical and structural analysis revealed that one of the most abundant SBAs is 13-MTD (iso-C₁₅). 13-MTD is a saturated branched-chain fatty acid and can be chemically synthesized. It was reported that the synthesized 13-MTD had the same biological property as the naturally occurring form (8, 9). Our preliminary *in vitro* and *in vivo* data also showed that the synthesized 13-MTD had the same biological activities compared with native form purified from crude fermentation solution (data not shown). Therefore, we used synthesized 13-MTD for our study.

Cell Culture. Human cancer cell lines DU 145 (prostate carcinoma), K-562 (leukemia), HCT 116 (colon carcinoma), NCI-H1688 (lung small cell carcinoma), SNU-423 (liver carcinoma), MCF7 (mammary adenocarcinoma), BxPC-3 (pancreatic adenocarcinoma), and NCI-SNU-1 (gastric carcinoma) were obtained from ATCC (Manassas, VA). All cell lines were maintained in RPMI 1640, DMEM, or McCoy's medium supplemented with 10% fetal

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³ Zhenhua Yang, unpublished data.

⁴ The abbreviations used are: SBA, small biosynthetic anticancer agent; 13-MTD, 13-methyltetradecanoic acid; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

bovine serum according to the culture conditions suggested by ATCC. K-562 and NCI-SNU-1 are suspension cells, and the subcultures were established by centrifugation of the suspension with subsequent resuspension in fresh medium. The other tumor cell lines are adherent cells, and the subcultures were achieved by dispersing the cells with 0.05% trypsin/0.01% EDTA (Irvine Scientific, CA) and by subsequently resuspending the cells in fresh medium.

Cell Cytotoxicity Assays. ID₅₀, ID₇₅, and ID₉₀ were defined as the concentration of 13-MTD killing 50, 75, and 90% of the cells, respectively. ID₅₀, ID₇₅, and ID₉₀ were determined for all eight tumor cell lines. Serial dilutions of 13-MTD were used at 0, 1.5, 3.0, 6.0, 15.0, 30.0, and 60.0 µg/ml. All tumor cell lines were seeded in 96-well microplates with 5×10^4 cells/well and divided into control and treatment groups. The control groups consisted of controls treated with same volume of solvent and controls without any treatment. After 48 h, cells were removed from each well, stained with trypan blue, and counted under a microscope for viable and dead cells. The results were then calculated by nonlinear regression analysis with CalcuSyn software (Biosoft, Cambridge, United Kingdom).

MTT assays were performed in the K-562 cell line to measure the cytotoxicity of MTD to tumor cells. The rationale of MTT assay is based on the mitochondrial metabolism of the MTT salt into formazan. Because the conversion takes place only in living cells, the amount of formazan produced correlates with the number of viable cells present.

13-MTD Treatment of Cancer Cells. Tumor cells were seeded in T75 flasks at 2×10^6 cells/flask in culture medium supplemented with 10% fetal bovine serum and incubated overnight at 37°C with 5% CO₂. 13-MTD was dissolved in PBS with 0.8% Tween 80 and sterilized by filtration. After testing different concentrations of 13-MTD in all cell lines, 35 µg/ml of 13-MTD was chosen for this study because significant tumor cell death was observed at this concentration. After incubating with either 35 µg/ml 13-MTD or solvent for 1, 2, 4, 8, 24, and 48 h, cells were collected and prepared for *in situ* cell death detection assay and DNA fragmentation analysis.

DNA Fragmentation PCR and Electrophoresis. Cells treated with either 13-MTD or solvent for 8 and 24 h were collected for DNA extraction. Cells were lysed in lysis buffer containing 0.2 M NaCl, 2% SDS, and 20 mM EDTA. Cell lysates were treated with DNase-free RNase at 37°C for 1 h and then extracted with phenol/chloroform. Fragmentation from cellular endonuclease cleavage of genomic DNA during apoptosis in tumor cells treated by either 13-MTD or solvent was examined by using the ApoAlert LM-PCR Ladder Assay kit (Clontech, Palo Alto, CA), following the procedure from the manufacturer. Briefly, 0.5 µg genomic DNA from each sample was ligated to adaptors by T4 ligase and then amplified by LM-PCR with Advantage cDNA polymerase from the manufacturer. The PCR product was electrophoresed on a 1.2% agarose/ethidium bromide gel at 100 V for about 1 h, and the ladder of fragmented DNA was visualized under UV light.

***In Situ* Apoptotic Cell Death Detection.** The cell suspensions from 13-MTD or control treatments were counted and adjusted to 0.5×10^6 cells/ml. Fifty µl of each cell suspension were dropped onto a glass slide and air dried. The apoptotic cell death was detected in the cell preparations using the *In Situ* Cell Death Detection Kit, peroxidase from Boehringer Mannheim. Briefly, the cells were fixed with freshly prepared formaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. After incubating with blocking solution (0.3% H₂O₂ in methanol) and subsequently in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate), each slide was incubated with 50 µl of terminal deoxynucleotidyl transferase-mediated nick end labeling reaction mixture in a humidified chamber for 60 min at 37°C and then incubated with 50 µl of Converter-POD for 30 min at 37°C. The slides were then incubated with 3-amino-9-ethylcarbazole substrate solution for the signal conversion, counterstained with hematoxylin, and mounted with glass coverslips. The results were analyzed under light microscope and documented on film.

***In Vivo* Determination of LD₅₀.** To determine the acute toxicological effect of 13-MTD, the LD₅₀ test was performed in ICR mice. ICR mice of both sexes at 6 weeks of age were randomly selected for each experiment group with body weight of 20.5–22.5 g. The mice were treated with 13-MTD at 20, 40, 80, 160, and 800 mg/kg and 2.5 g/kg body weight by intragastric administration twice daily for 14 days. The control mice were administered with same volume of solvent for the same period of time.

Inhibition of Tumor Growth in MetaMouse Orthotopic Model. The ability of 13-MTD to inhibit tumor growth *in vivo* was tested by the MetaMouse orthotopic model performed by AntiCancer, Inc. (San Diego, CA) for

human hepatocellular carcinoma LCI-D35 and human prostate cancer DU 145 cell lines. Human hepatocellular carcinoma cell line LCI-D35 was established by AntiCancer, Inc. from a primary liver tumor. The tumor cells were first implanted and maintained s.c. in athymic nude mice. The tumors were then harvested, and any grossly necrotic or suspected necrotic tumor tissues were removed prior to orthotopic implantation. The tumor tissues were then cut into small pieces about 1 mm³ each. Three DU 145 fragments were implanted into each mouse prostate, and animals were randomly divided into four groups with eight mice for each group. The mice were administered with solvent, 35, 70, or 105 mg/kg of 13-MTD for 43 days. Two LCI-D35 fragments were orthotopically implanted into the left lobe of liver of each BALB/c mouse, and animals were then randomly divided into two groups with eight mice each. One group of mice was given 70 mg/kg/day of 13-MTD p.o. for 40 days, and the other group was given the same volume of the solvent solution. The administration of 13-MTD and solvent was carried out through gavage once a day. At the end of the study, mice were sacrificed. Tumors grown in mouse liver or prostate were inspected and weighed. Tissue sections were prepared for H&E staining and routine pathological analysis. The primary tumor weight data were analyzed by Student *t* test with α equal to 0.05 (one-tailed).

Results

Cytotoxicity of 13-MTD to Tumor Cells. The cytotoxicity activity and proliferation inhibition of 13-MTD in tumor cells were measured by ID₅₀, ID₇₅, ID₉₀, and MTT. The ID₅₀, ID₇₅, and ID₉₀ for various tumor cell lines were shown in Table 1. Strong cytotoxic activities could be detected in all human tumor cell lines treated with 13-MTD. The ID₅₀ ranged from about 10 µg/ml for MCF7 cells to <25 µg/ml for SNU-423 cells. MTT assay was performed in K-562 cells, and the inhibition rates were measured as 85.3, 83.1, 71.6, 50.1, and 26.2% for 90, 60, 30, 15, and 7.5 µg/ml of 13-MTD, respectively. Our results indicated that 13-MTD could effectively kill the tumor cells at relatively low dosage.

The acute toxicity of 13-MTD was tested by LD₅₀ assay. After 14 days of intragastric administration, all mice were alive, and there were no major anomalies observed in the animals. The results suggested a LD₅₀ greater than 5 g/kg/day, indicating the low acute toxicity of 13-MTD in laboratory mice.

13-MTD Causes Tumor Cell Death through Induction of Apoptosis. DNA fragmentation electrophoresis was performed to illustrate the apoptotic changes in 13-MTD-treated tumor cells in this study. Our results show that apoptotic DNA fragmentation could be detected in all cancer cell lines studied after 8 h of 13-MTD treatment. The representative results from cell lines MCF7 and DU 145 were shown in Fig. 1.

In situ terminal deoxynucleotidyl transferase-mediated nick end labeling assay is a very sensitive and specific cell death detection to reveal the DNA degradation of apoptotic cells. Started within 2 h of treatment, all cancer cell lines showed positive apoptosis after treatment with 35 µg/ml 13-MTD compared with controls. The representative results from NCI-H1688, MCF7, and DU 145 were shown in Fig. 2. The number of apoptotic cells increased proportionally with the time length of 13-MTD treatment (Fig. 3).

Inhibition of *in Vivo* Tumor Growth. The *in vivo* tumor growth inhibition experiments were performed in the MetaMouse orthotopic model for two cancer cell lines. The mice implanted with DU 145 xenograft tumors were p.o.

Table 1 Cytotoxicity of 13-MTD in human tumor cells

Cell line	ID ₅₀ (µg/ml)	ID ₇₅ (µg/ml)	ID ₉₀ (µg/ml)
MCF-7	10.03 ± 0.97	15.99 ± 1.28	25.49 ± 1.68
K-562	11.45 ± 1.82	22.27 ± 4.60	43.57 ± 6.71
DU 145	13.98 ± 2.15	40.43 ± 5.72	81.87 ± 8.85
H1688	15.08 ± 1.92	35.03 ± 3.59	61.37 ± 8.06
HCT-116	18.49 ± 6.23	67.96 ± 8.25	108.65 ± 13.35
SNU-1	20.77 ± 2.47	47.43 ± 4.95	80.49 ± 10.03
SNU-423	24.26 ± 3.98	70.46 ± 9.36	120.77 ± 15.82

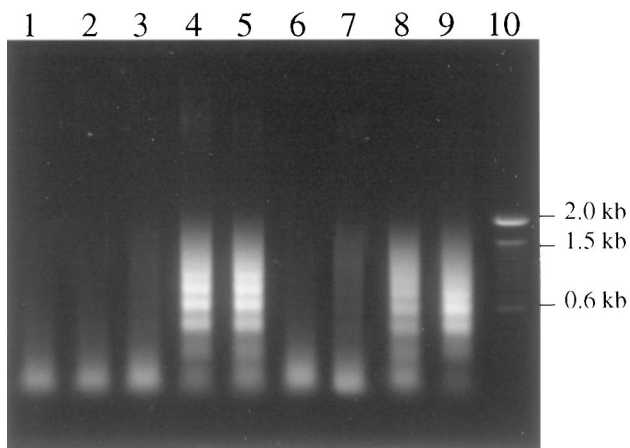


Fig. 1. Induction of DNA fragmentation by 13-MTD in DU 145 and MCF7 cells. DNA ladder formation is observed in 13-MTD-treated DU 145 (Lane 4, 8 h and Lane 5, 24 h) and MCF7 (Lane 8, 8 h and Lane 9, 24 h) cells. Lane 2 (8 h), Lane 3 (24 h), Lane 6 (8 h), and Lane 7 (24 h) are results from solvent-treated DU 145 (Lanes 2 and 3) and MCF7 (Lane 6 and Lane 7) cells and did not show DNA ladder formation. Lane 1 is no DNA control, and Lane 10 is the molecular marker.

fed with 13-MTD at doses of 35, 70, and 105 mg/kg once per day. The differences of tumor weight between 13-MTD-treated groups and control groups were summarized in Fig. 4A. Oral administration of 13-MTD inhibited DU145 tumor growth in experimental mice, and the inhibition rates of tumor growth were 54.8% ($P = 0.042$), 84.6% ($P = 0.007$), and 65.2% ($P = 0.022$) for respective 13-MTD doses. Mice implanted with human hepatocellular

carcinoma LCI-D35 xenograft tumors were only treated with 70 mg/kg of 13-MTD because this concentration gave the best inhibition in DU145. The results showed that 13-MTD could effectively inhibit LCI-D35 tumor growth in nude mice when the final tumor weights were compared with those of controls (Fig. 4B) with the inhibition rate at 64.9% ($P = 0.0086$).

Discussion

In the present study, we investigated the inhibitory effects of 13-MTD to malignant tumor cell lines including seven carcinoma cell lines from different organs and one leukemia cell line. The results from *in vitro* ID_{50} and MTT assays showed that 13-MTD could effectively cause tumor cell death at low concentrations. The results from the *in situ* cell death assay and DNA fragmentation assay suggested that 13-MTD triggers tumor cell death through induction of apoptosis in these cells. The apoptotic changes were detected within 2 h of 13-MTD treatment, as shown by the *in situ* cell death assay, and were prominent within 8 h of treatment, as shown by DNA fragmentation gel electrophoresis. Furthermore, oral administration of 75 mg/kg/day of 13-MTD to mice could effectively inhibit tumor growth in our MetaMouse orthotopic model implanted with either prostate carcinoma cell line DU 145 or hepatocarcinoma cell line LCI-D35. 13-MTD is a saturated branched-chain fatty acid purified from a soy fermentation product, Yang Zhen Hua 851. The soy fermentation product was used as a supplement for cancer treatment originally in China and now in many countries, including the United States. The effectiveness of this soy fermentation product in cancer treatment and prevention had been demonstrated by many clinical cases.³ Our *in*

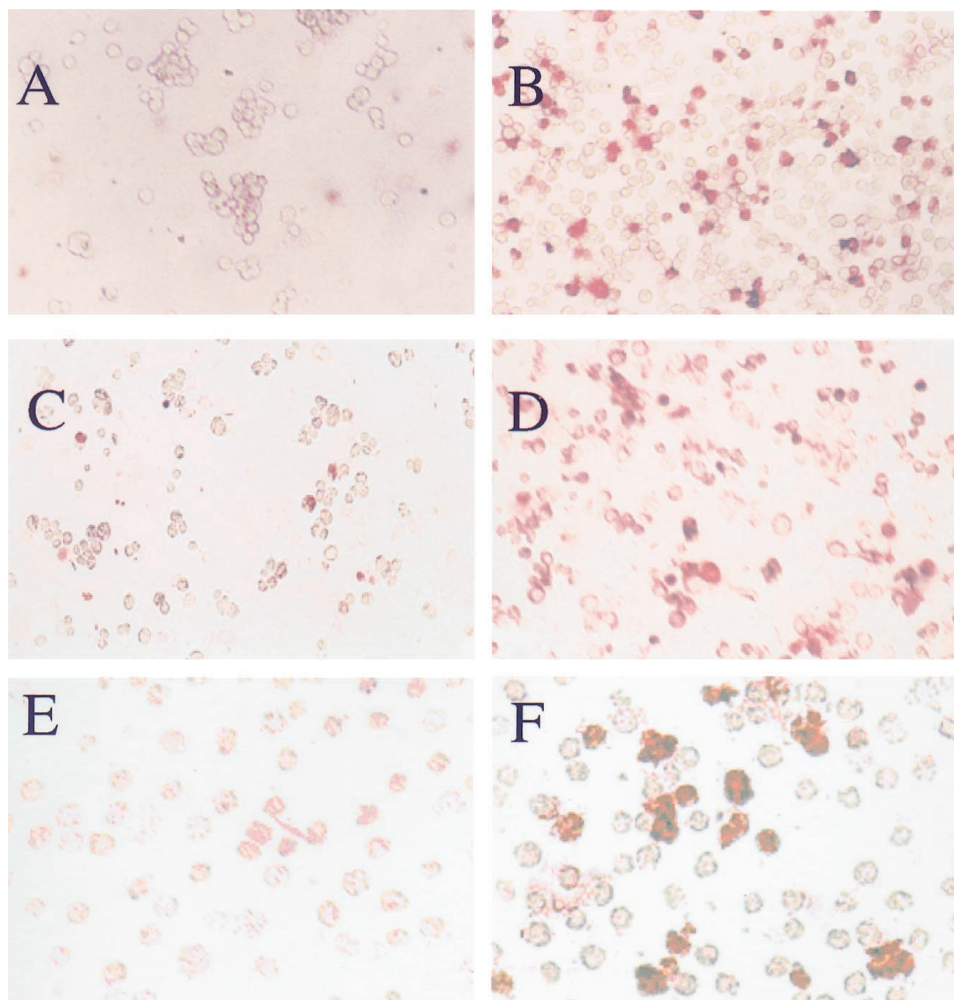


Fig. 2. *In situ* cell death detection in 13-MTD-treated cells. Apoptotic cells are intensely stained (red) in NCI-H1688 (B), MCF7 (D), and DU 145 (F) cell lines treated with 13-MTD for 4 h. A, C, and E are results of solvent control in corresponding cell lines.

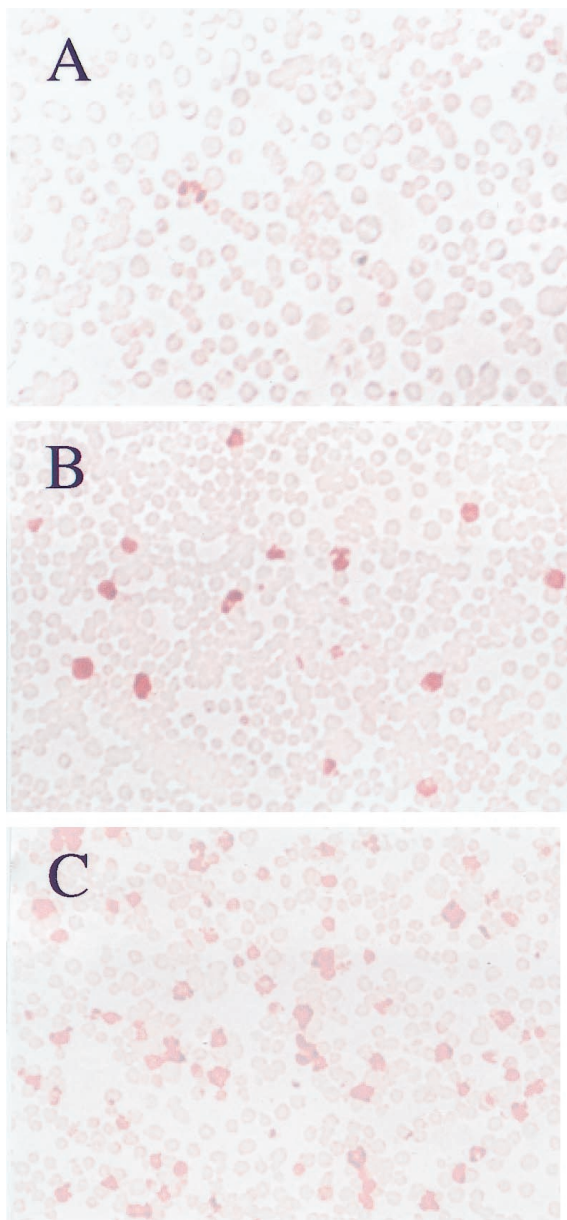


Fig. 3. Induction of cell death by 13-MTD in K-562 cells. Apoptotic cells (red) were detected at 2 h (B) and were dramatically increased at 4 h (C) by 13-MTD treatment. Solvent treatment for 4 h did not induce apoptosis in K-562 cells (A).

vitro and *in vivo* data indicated that 13-MTD is probably the major component in this soy fermentation product to account for the anti-cancer function by means of triggering apoptosis in tumor cells.

Fatty acids and unsaturated fatty acids have been reported to induce apoptosis in several cell types including cancer cells (11–16). The role of ceramide and other metabolites of sphingomyelin pathway in apoptosis has been well established (17–21). Ceramide-mediated apoptosis has been primarily described as a result of hydrolysis of a plasma membrane-derived pool of sphingomyelin (22). In comparison with other fatty acids related to apoptosis, 13-MTD has unique and distinguished characteristics and could be a better agent for cancer chemotherapy. 13-MTD occurs naturally only in trace amounts and is not synthesized *de novo* in the body of mammals (7–9), whereas cellular ceramide is normally produced either by the cleavage of sphingomyelin by sphingomyelinase or by *de novo* synthesis from palmitate and stearate (11). Because ceramide is a normal element of the sphingomyelin pathway, its apoptosis induction often depends on

cell type and stress-related, environmental factors. Ceramide can also give rise to proliferation and differentiation (22). On the other hand, 13-MTD is foreign to the body and therefore may be more effective and less affected by cell type and stress-related environment factors in triggering apoptosis in tumor cells. 13-MTD induced apoptosis in eight different cancer cell lines in our study, despite of the differences of the genetic alterations among them, and the induction was not affected by the fetal bovine serum in the culture medium, whereas ceramide and other fatty acids worked in serum free medium or with only 2% of fetal bovine serum (12, 18, 23). Furthermore, the terminally branched methyl group may give 13-MTD advantages over straight carbon chain fatty acids to perform the function of apoptotic induction.

As a 15-carbon fatty acid, 13-MTD is absorbed by the intestine when given *p.o.* and transported primarily as chylomicrons in the lymph into the circulation through the thoracic duct (8–10). The process avoids the transportation of this fatty acid by the portal circulation directly into the liver to be oxidized and therefore delivers a higher concentration of 13-MTD in the circulation to reach organs and tissues. This property might be important for 13-MTD to reach proper concentration in local tumor tissues to induce apoptosis. The results of MetaMouse orthotopic models showed that oral administration of 13-MTD at a dosage as low as 35 mg/kg of mouse body weight daily could significantly inhibit the growth of orthotopically implanted tumors, suggesting the significance of the absorption and transportation through lymph to bypass the liver metabolism of the branched-chain fatty acid. In addition, the present of the isopropyl group gives 13-MTD a characteristic gas chromatographic behavior to serve as a structural marker for investigating its tissue distributions and turnover rate to monitor the *in vivo* effectiveness of tumor treatment.

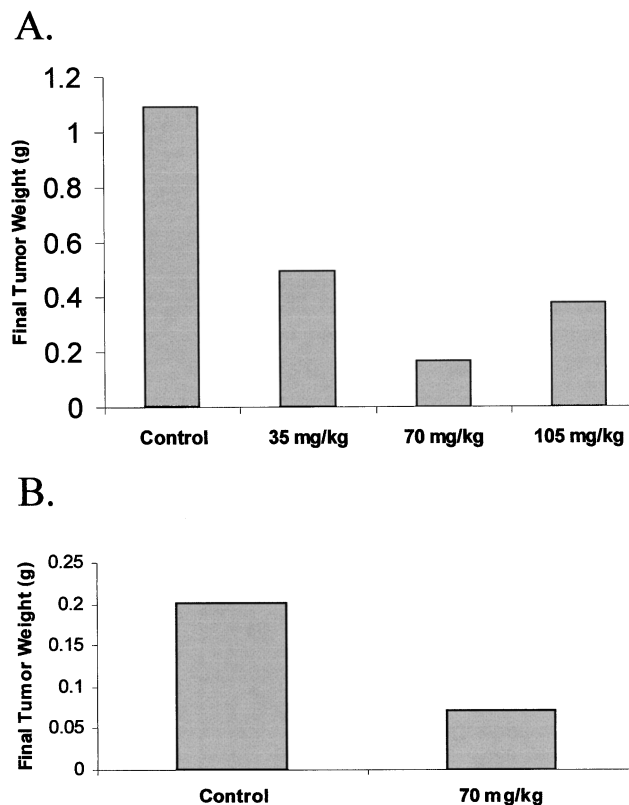


Fig. 4. Tumor growth inhibition by 13-MTD in the MetaMouse orthotopic model. Tumors from control groups and 13-MTD groups were compared by tumor weights for mice implanted with DU 145 (A) and LCI-D35 (B).

The other advantage of 13-MTD in cancer treatment is its low toxicity. LD₅₀ for intragastric administration of 13-MTD is more than 5 g/kg/day, whereas the therapeutic dosage in our orthotopic tumor implant mouse model was <75 mg/kg/day. This offers a wide safety zone between the lethal dosage and therapeutic dosage and suggests that 13-MTD may be a potent and safe agent for cancer chemotherapy. This notion was supported by the normal gross and microscopic morphology in the different mouse organs and tissues from Meta-Mouse orthotopic experiments for both DU 145 and LCI-D35 cancer cell lines after fed mice with 13-MTD for 40 days. It was also supported by the results from published studies in which 13-MTD was safely used as an indicator of adipose tissue turnover in experimental animals and human volunteers at high doses for as long as 42 days (8–10).

In summary, 13-MTD is a saturated branched-chain fatty acid purified from the soy fermentation product Yang Zhen Hua 851. Our results suggest that 13-MTD could effectively inhibit *in vitro* and *in vivo* tumor growth by inducing apoptosis of cancer cells. Although the mechanism for inducing apoptosis in tumor cells is not known and needs further investigation, 13-MTD presents a potential chemotherapeutic agent without significant undesired side effects.

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