Nutrition and Cancer

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/hnuc20

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To cite this article: Zehui Li, Shuang Zhou, Xiaodong Yang, Xiyan Li, Grace Xiaolu Yang, John Chant, Michael Snyder & Xin Wang (2021): Broad Anti-Cancer Activity Produced by Targeted Nutrients Deprivation (TND) of Multiple Non-Essential Amino Acids, Nutrition and Cancer, DOI: 10.1080/01635581.2021.2013904

To link to this article: https://doi.org/10.1080/01635581.2021.2013904

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Broad Anti-Cancer Activity Produced by Targeted Nutrients Deprivation (TND) of Multiple Non-Essential Amino Acids

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ABSTRACT

It has been known for close to 100 years that the metabolism of cancer cells is altered and different than that of healthy cells in the body. On that basis, we have developed an entirely novel approach to managing cancer, termed Targeted Nutrients Deprivation (TND). TND employs a formulated diet depleted of multiple non-essential amino acids (NEAAs) that are required by tumor cells but not by normal cells. Cancer cells specifically require those NEAAs due to their heightened and rewired metabolism. We demonstrated that our first proprietary formulated TND diet—FTN203—significantly reduced the growth of multiple human tumor xenografts in mouse. In combination with chemotherapy and immunotherapy, FTN203 further enhanced therapeutic efficacy. Reliance on FTN203 as the sole nutrition source was shown to be safe without causing detrimental body-weight loss or internal organ damage. Our findings indicate that TND is a novel and safe approach to managing cancer.

ARTICLE HISTORY
Received 21 May 2021
Accepted 24 November 2021

Introduction

For close to a century, it has been known that reprogramming of metabolism is a hallmark of cancer. Tumor cells have unique nutritional requirements to sustain their abnormal metabolic demands (1, 2). As a result, cancer-specific nutrient dependencies and metabolic vulnerabilities serve as a unique and powerful entry point of novel intervention.

Amino acid metabolism in cancer has drawn strong interest for decades due to its role in tumor development (3). For example, cancer cells can use glutamine as an alternate carbon and nitrogen source, and they can become glutamine addicted (4). Recent evidence indicates that arginine plays an important role in the tumor microenvironment for modulation of the immune response (5).

Our therapy, Targeted Nutrients Deprivation (TND), is based on the differential requirement of normal and cancer cells for certain amino acids. For humans, amino acids are classified as essential and non-essential. Essential amino acids (EAA) are indispensable in diet. Non-essential amino acids (NEAAs) are normally not required in diet. Rather, the body synthesizes these eleven NEAAs through endogenous biochemical processes (6, 7). In contrast, many cancer cells cannot secure sufficient amounts of NEAAs through de novo synthesis due to their altered metabolism and enhanced proliferation. NEAAs become conditionally essential to tumor cells. They rely on extracellular supply of these nutrients. This is the basis of TND technology.

Previous studies have shown that deprivation of a single amino acid can have anti-tumor effects in animal studies. For example, blocking glutamine utilization has been suggested as a potential mechanism for treating cancer (8–10). Glutaminase inhibitors which block the conversion of glutamine to glutamate for utilization by triple-negative breast cancer cells are being developed on this basis (11). Disruption of cysteine homeostasis by inhibiting its transport into cells impairs glutathione synthesis and results in unbalanced redox status. Inhibition leads to iron-dependent ferroptotic cell death in renal cell carcinoma and pancreatic cancer both in vitro and in vivo (12–16). Dietary starvation of serine and glycine disrupts cellular homeostasis of

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Supplemental data for this article is available online at https://doi.org/10.1080/01635581.2021.2013904.

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one-carbon metabolism and anti-oxidative capacity, thereby impeding colon cancer development in mouse studies (17, 18). Accumulating evidence suggests that enzyme-mediated arginine depletion can be a potential approach for targeting multiple cancer types including pancreatic cancer and melanoma (19–22).

Already there is one FDA-approved therapeutic with a mechanism of action based on deprivation of a single amino acid, asparagine. Injectable asparaginase (Elspar®, Erwinase®, and Oncaspar®) depletes asparagine from the bloodstream via enzymatic degradation. It is approved for treatment of acute lymphoblastic leukemia (ALL) in combination with chemotherapy (23).

TND takes the approach of depriving multiple amino acids simultaneously. We adopted this strategy because we believe that its effects would prove more robust and powerful than single amino acid deprivations. We also reasoned that efficacy from deprivation of multiple amino acids would be less vulnerable to resistance or compensatory mechanisms. In addition, TND may have efficacy more broadly on different tumor types with varying dependencies on amino acids.

Our TND approach was developed with the following rationale. By screening several human cancer cell lines representing common cancer types with single dropouts of NEAA in cell culture, we developed a formula for targeted deprivation of multiple selected NEAAs. The proprietary amino acid profile is termed FTN203. Here, we present multiple studies of FTN203, both as a single agent and in combination with standard-of-care therapies in vitro and in vivo. FTN203 holds the potential to be adopted as a new foundational module in cancer management.

Materials and Methods

Cell Culture

Cell lines U87, FaDu, Detroit 562, SCC154, HCT116, CT26, H226, H460, and H1975 were obtained from the American Type Culture Collection (ATCC). Cell lines U251, KYSE70, and OE19 were purchased from Sigma-Aldrich. Human normal primary peripheral blood mononuclear cells (PBMC) was purchased from ATCC. Stock cultures of HCT116, CT26, KYSE70, OE19, H226, H460, and H1975 were maintained in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS) at 37 °C with an atmosphere of 5% CO_2 in air. U251, U87, Fadu, Detroit 562, and SCC154 cell lines were maintained in EMEM medium containing 10% (v/v) fetal bovine serum (FBS) at 37 °C with an atmosphere of 5% CO_2 in air. Cultures were passaged for less than 6 mo, and were monitored periodically for mycoplasma contamination with PlasmoTest™ - Mycoplasma Detection Kit (InVivo Gen).

Cell Viability Assay

Cells were seeded in white-walled opaque 96-well plates with four replicate wells (5,000 cells/well) for each culture condition. Medium with different NEAA deprivation was added into plates. ATP levels, as a surrogate of cell viability, was measured 72 hrs later using the CellTiter-Glo® luminescence cell viability assay kit (Promega), according to the manufacturer's protocol. The XTT assay was used to test cell proliferation after 72 hrs. Luminescence and optical density (O.D.) were read by using a Perkin Elmer EnSpire 2300 Multilabel Multimode Plate Reader.

Tumor Xenografts

All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee (Crown BioScience Inc., Washington Biotechnolgy Inc., and Molecular Medicine Research Institute). Cells were cultured in normal condition with 10% fetal bovine calf serum (FBS) and incubated at 37 °C in a humidified atmosphere of 5% CO_2. As cells became 80% confluent, cultures were subcultivated into 150cm^2 flasks and expanded further until sufficient cells were available for injection. Cells in 100 µL (FaDu: 1×10^6 cells; A-253: 1×10^6; H69: 1×10^6 cells; PANC-1: 1×10^7 cells; PC-3: 1×10^7 cells; MB-231: 1×10^6) of medium with 20% Matrigel were injected subcutaneously into the right flank of female athymic nude mice at age 5-6 weeks. 100 µL of Jurkat ALL tumor cells (1×10^7) in 0.1 ml of PBS mixed with Matrigel (1:1) were injected subcutaneously into the right flank of female NOD SICD mice. Mice were randomized in each group based upon tumor volume and assigned to either Complete Control or FTN203 chow. All special diets were iso-caloric and provided quantitatively equal amounts of carbohydrates, lipids and micronutrients. All diets provided the same amount of nitrogen, although nitrogen content was provided by different formulations of amino aicds with ratio-matched profiles, according to these presented in Supplement Table 1. All special diets were prepared at Research Diets, Inc. (New Brunswick, NJ). For the pancreatic cancer model, 100 mg of Gemcitabine HCL (GEM) was dissolved in 10 ml of 0.9% saline to prepare 10 mg/ml solution, and 0.45 ml (10 mg/ml) was mixed with 2.55 ml of saline to prepare 1.5 mg/ml solution; mice were dosed 10 ml/kg,

i.p., twice weekly, until day 42. For the prostate cancer model, 2.5 ml of paclitaxel (PTX) solution (6 mg/ml) was mixed in 7.5 ml of 0.9% saline to prepare 1.5 mg/ml solution, mice were dosed 10 mg/kg, i.v., until day 28. The tumors were measured three times per week with a digital caliper once palpable. Palpation began on day 1 after cell inoculation. The mice were euthanized when the tumors exceeded 2000mm^3 or bodyweight loss ≥ 20%. Calculate tumor volumes using formula in excel: Tumor Volume = length x width x width x ½.

Colon Cancer Syngeneic Mouse Model

Animals were prepared for injection using an approved, standard anesthesia method and shaved prior to injection. CT26 mouse colon cancer cells (2.5×10^5) in 100 µL of PBS with 20% matrigel were injected subcutaneously into the right flank female Balb/c mice (6-7 weeks old). Mice were randomized into 6 groups ($n = 15$ per group) when the average tumor volume reaches 80-125mm^3. Dosing was initiated within 24 h, of randomization. Anti-mouse PD-1 and anti-mouse PD-L1 antibodies were injected i.p. into mice (10 mg/kg) on day 1, 4, 8 and 11. The mice were euthanized when the tumors exceeded 2000mm^3 or bodyweight loss ≥ 20%. Calculate tumor volumes using formula in excel: Tumor Volume = length x width x width x ½. On day 20, tumor and blood were collected 4 hrs post final dose from five animals from each group, and blood was analyzed by FACS to determine CD45+, CD3+, CD4+, CD8+, FOXP3+, PD-1, PD-L1, CD335+ cell population.

Blood Chemistry Analysis

Plasma samples were analyzed to determine alkaline phosphatase (ALP), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total protein (TP), albumin (ALB), globulin (GLOB), total calcium (Ca^{2+}), phosphorus (PHOS), sodium (Na^+), and potassium (K^+) using a comprehensive diagnostic profile kit (Abaxis, Cat. No. 500003824) with VetScan VS2 Analyzer (Abaxis), following manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Specific tests applied to assess significance are noted in the figure legends. Error bars indicate standard deviation. Each tested group was compared to Complete Control by One-way ANOVA or Two-way ANOVA; statistical comparison between two groups was analyzed by unpaired t-test; for all survival plot, P values were calculated with Log-rank (Mantel-Cox) test. P values < .05 were considered to be significant, and the actual P values were reported. For P values less than .001, report them as P <.001, instead of the actual exact P value.

Results

NEAA Deprivation Selectively Inhibited Human Cancer Cell Growth In Vitro

We first tested NEAA single dropouts on multiple cancer cell lines in vitro and found that certain dropouts inhibited cancer cell growth. We initially tested using three diverse cell lines: U251 (glioblastoma, GBM), FaDu (head and neck cancer, pharynx squamous carcinoma, HNC), and H1975 (non-small cell lung cancer, adenocarcinoma, NSCLC). As shown in Figure 1A–C, several single dropouts of NEAAs (Arg, Cys, Gln, and Tyr) dramatically prevented tumor cell proliferation detected by ATP-measuring CellTiter-Glo assay. The results were confirmed by XTT assay (Supplement Figure 1A-C) and in a larger panel of cancer cell lines (Supplement Figure 2). Disintegration of cancer cells cultured in single NEAA dropout media was also confirmed by microscopic examination (data not shown). These cell culture experiments demonstrated the differrent NEAA requirements for the growth of a diverse panel of cancer cell lines. For example, most cancer cell lines are sensitive to cystine dropout but esophageal cancer cell line OE19 and NSCLC H1975 are slightly less sensitive (Figure 1, Supplement Figure 1, Supplement Figure 2). Another example is that all HNC cell lines used in this study are insensitive to glutamine dropout, while two out of three NSCLC cell lines are sensitive to glutamine dropout but NSCLC cell line H226 is not sentive at all. Given different tumor tissues are heterogenous and require different NEAA for their metabolism, we conclude that deprivation of multiple NEAAs at the same time could be more beneficial for the suppression of cancer growth.

To maximize the effect of NEAA deprivation, we tested the deprivation of multiple NEAAs in different combinations (data not shown) and optimized a TND formula (FTN203) to a form that inhibited the growth of all tested cancer cell lines, including GBM, colon cancer (CC), HNC, esophageal cancer (EC), and NSCLC (Figure 1D, and Supplement Figure 1D).

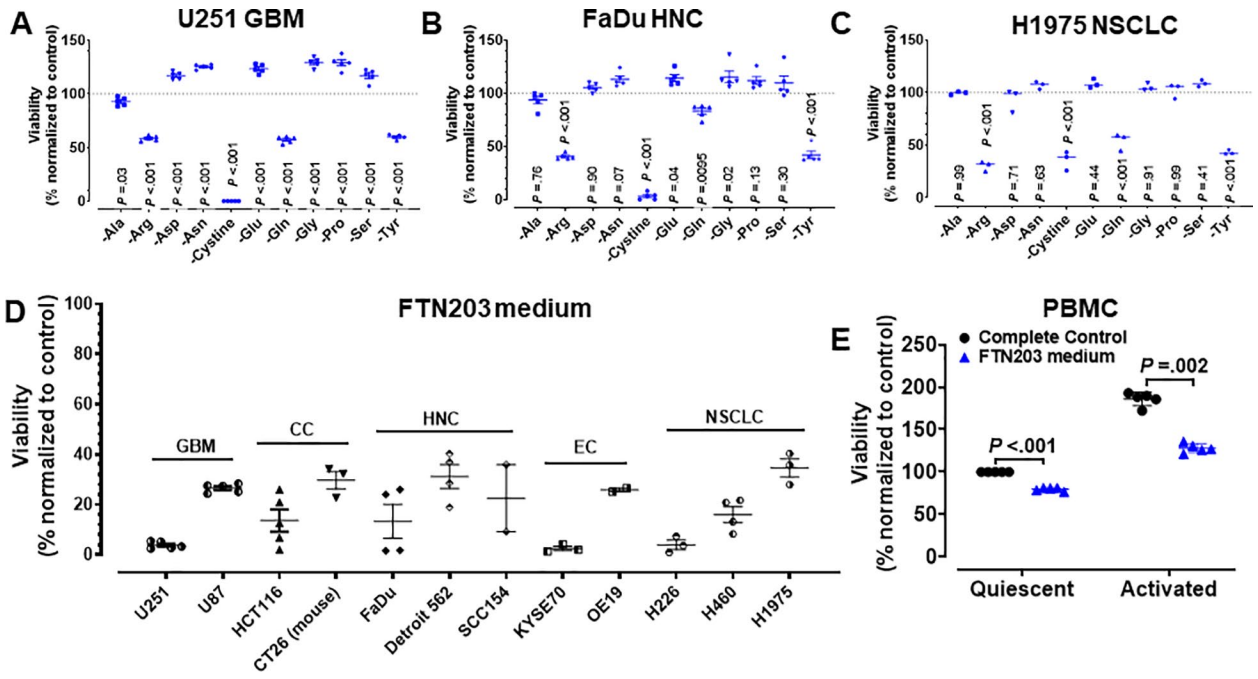

Figure 1. NEAA deprivation selectively inhibited human cancer cell growth in vitro (CellTiter-Glo® Luminescent Cell Viability Assay). Human glioblastoma U251 cells (A), head and neck cancer FaDu cells (B), non-small cell lung cancer H1975 cells (C) were seeded in 96-well plate (5,000 cells per well). Complete Control medium and single non-essential amino acid (NEAA)-dropout medium were added into plates. 12 cell lines were used to test the effect of multiple NEAA-deprived (FTN203) medium in vitro (D). Quiescent and activated human normal PBMC cells (1x10^5 cells/well) were treated with FTN203 medium (E). Cell viability was measured after 72 h by the CellTiter-Glo® luminescence cell viability assay kit and calculated as % normalized to Complete Control group. Each tested group was compared to Complete Control by One-way ANOVA (n = 5, Mean+/-SEM). Ala, Alanine; Arg, Arginine; Asp, Aspartic acid; Asn, Asparagine; Glu, Glutamic acid; Gln, Glutamine; Gly, Glycine; Pro, Proline; Ser, Serine; Tyr, Tyrosine.

We next tested the effects of FTN203 medium on the growth of non-cancerous cells. We chose human peripheral blood mononuclear cells (PBMCs) as these primary cells are commonly used to assess the potential adverse effects of interventions on the immune system. When tested on PBMCs, FTN203 medium had a small effect on the viability of quiescent PBMCs (21% in Figure 1E as measured by CellTiter-Glo; not significant in Supplement Figure 1E as measured by XTT assay). We next analyzed FTN203 medium on activated PBMCs upon stimulation by IL-2 and phytohemagglutinin (PHA), which induces proliferation and expansion of cell numbers. Stimulation led to a comparable level of PBMC expansion in both Complete Control and FTN203 medium (1.8-fold and 1.6-fold respectively). Viability was slightly inhibited by 30% when the cells were cultured in FTN203 medium. The mild effect on normal cells is consistent with our safety findings in mice described further below. Based on the strong effects of FTN203 on cancer cell lines, minor effects on PBMC viability, and a modest effect on PBMC expansion, we moved forward to examine FTN203 in mouse xenograft cancer models.

FTN203 is Efficacious Broadly across Tumor Types

To test anticancer effects in vivo, we formulated a completely synthetic mouse chow based on the NEAA depletion results described above: FTN203 chow. Amino acids were supplied as monomers as complete depletion of multiple amino acids is not possible using a complex protein source.

Before testing the FTN203 chow for anti-tumor effects, we wanted to rule out the possibility that replacement of intact protein source with free monomer amino acids, which are necessary to produce the TND diet, might have anticancer activity – an important control. We formulated a control chow (Complete Control with a complete panel of free amino acids in the same proportion as in commercially available natural chow with a complex protein source. We compared Complete Control chow to natural chow in two human HNC cancer mouse xenograft models. No significant effect was observed (Figure 2A and B), suggesting that changing intact protein to free amino acids in diet has no effect on tumor growth. The mouse body weights were similar in the Complete Control chow and natural chow groups (Supplement

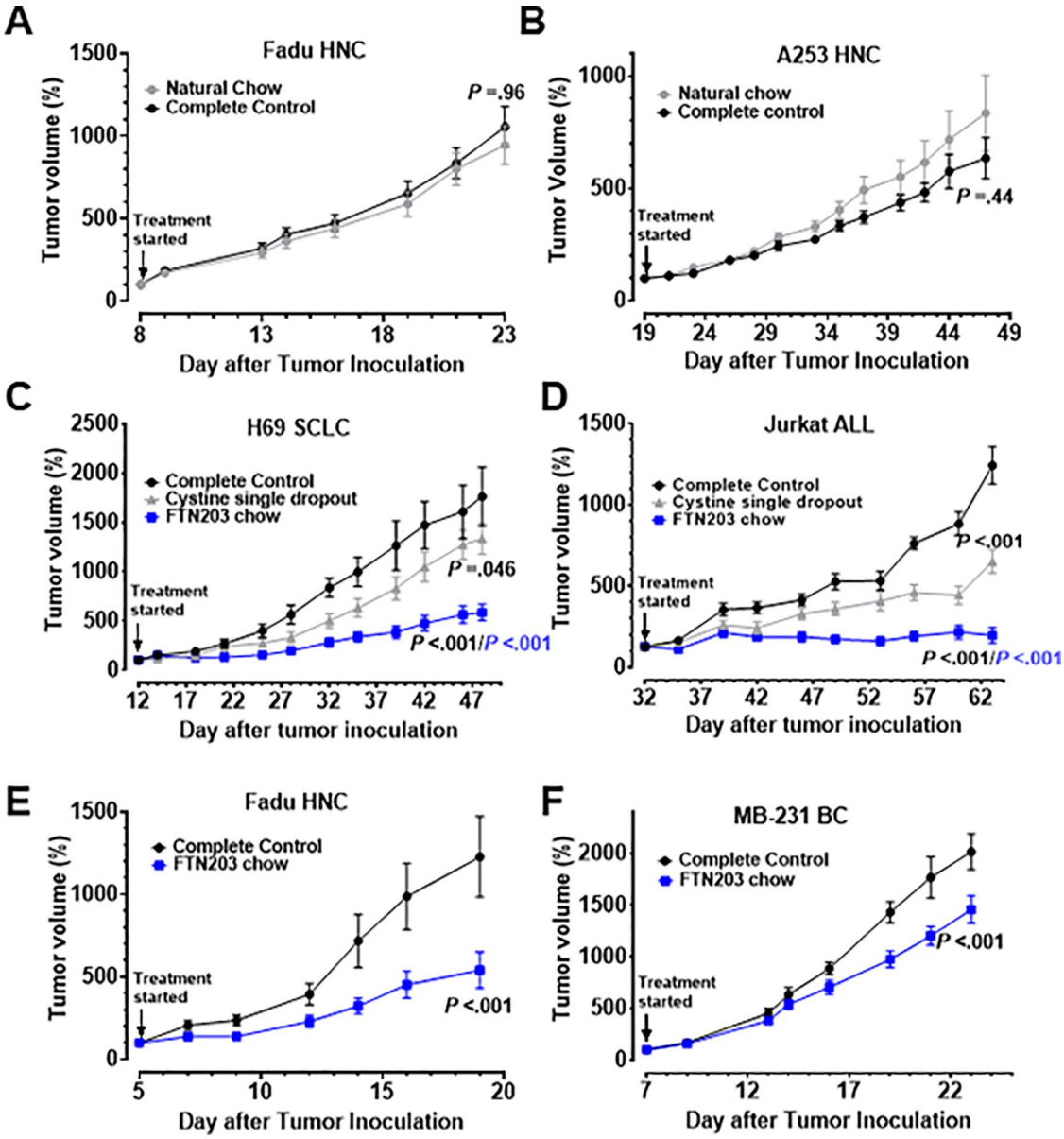

Figure 2. FTN203 chow is efficacious broadly across tumor types. Cells in 100 µL (FaDu: 1X10^6 cells (A); A-253: 1X10^6 cells (B), H69: 1X10^6 cells (C); Jurkat: 1X10^7 cells (D); FaDu: 1X10^6 cells (E); MB-231: 1x10^6 cells (F)) of medium with Matrigel were injected subcutaneously into the right flank of mice at age 5-6 weeks. Mice were randomized in each group based upon tumor volume and fed with indicated chow. The tumor volumes were measured (n = 10; Mean+/-SEM) three times weekly. Tumor Volume = L x W x W x ½. Statistical comparison between two groups was analyzed by Two-way ANOVA (n = 10, in A, B, and E; n = 12 in C and D; n = 5 in F; Mean+/-SEM; *P* value in black, compared to Complete Control, *P* value in dark blue, compared to Cystine single dropout).

Figure 3A and B). Thus, the Complete Control chow was used as the scientific control, as it allowed us to control for dietary composition variables in assessing the possible effects of FTN203 chow (Diet composition in Supplement Table 1).

We next determined whether depletion of multiple amino acids had a stronger effect than depletion of a single amino acid. In in vivo cell culture, the dropout of cystine alone had a very strong effect on tumor cell growth and survival (Figure 1A–C). In in vivo xenograft models, depletion of cystine alone had a modest effect on H69 SCLC tumor growth: 24% tumor growth inhibition (TGI) (Figure 2C; $P = 0.046$). In contrast, FTN203 chow produced TGI of 67% ($P < 0.001$). In the Jurkat ALL xenograft model, depletion of cystine alone produced TGI of 48% ($P < 0.001$, Figure 2D), while FTN203 produced TGI of 84% 63 day after tumor inoculation ($P < 0.001$). Thus, overall in vivo, FTN203 chow with multiple amino acid dropouts proved superior to the single

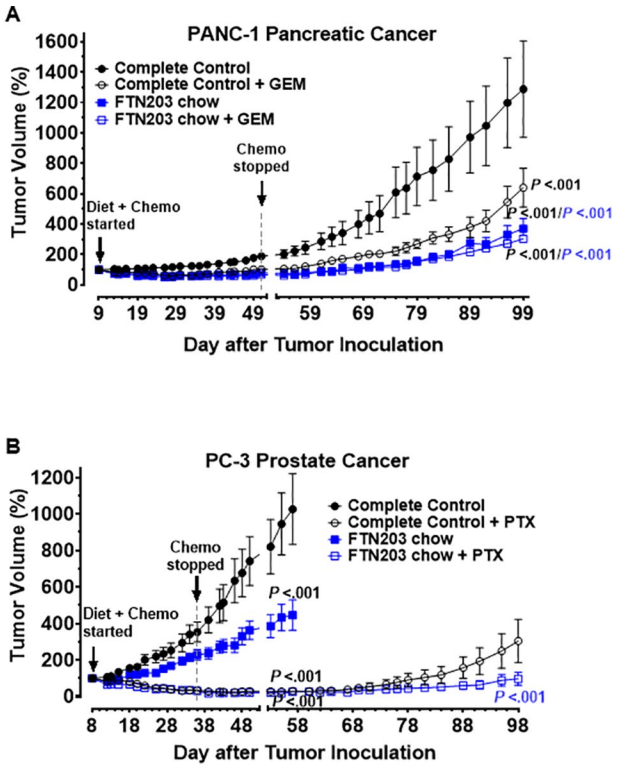

Figure 3. FTN203 inhibited tumor recurrence after chemotherapy. Cells in 100 µL (PANC-1: $1x10^7$ cells (A), PC-3: $1x10^7$ cells (B)) of medium with 20% Matrigel were injected subcutaneously into the right flank of female athymic nude mice at age 5-6 weeks. Mice were randomized in each group based upon tumor volume and fed with Complete Control or FTN203 chow. 15mg/kg of Gemcitabine (GEM) was given (i.v.) to mice bearing human PANC-1 pancreatic cancer for 42 days (twice weekly) (A). 10mg/kg of paclitaxel (PTX) were dosed (i.v.) to mice bearing human PC-3 prostate cancer for 28 days (twice weekly) (B). The tumor volumes were measured (n = 10; Mean+/-SEM) three times weekly. Tumor Volume = L x W x W x ½. Statistical comparison between two groups was analyzed by unpaired t-test (n = 10; Mean+/-SEM; *P* value in black, compared to Complete Control, *P* value in dark blue, compared to Complete Control + GEM (A) or Complete Control + PTX (B)).

dropout of cystine, which had produced the most potent effect observed earlier in cell culture.

We analyzed the FTN203 chow in several other tumor models and found that it consistently suppressed the growth of xenograft tumors: FaDu HNC (56%; Figure 2E), MB-231 breast cancer (28%; Figure 2F), PANC-1 pancreatic cancer (63%; Figure 3A) and PC-3 prostate cancer (56.6%; Figure 3B). In multiple xenograft tumor models, mice fed with FTN203 tended to lose weight ranging from 5% to 20% of their beginning body weight. Body weight loss was not observed in the mice fed Complete Control chow (Supplement Figure 3C–F). Multiple lines of evidence indicate that tumor suppression and weight loss are mechanistically unrelated, as analyzed further in the Discussion section.

Overall, we observed significant tumor inhibition by FTN203 chow in 6 human tumor types: SCLC, ALL, HNC, breast cancer, pancreatic cancer, and prostate cancer.

FTN203 Inhibited Tumor Recurrence after Chemotherapy

We next tested whether FTN203 chow would prove efficacious in combination with existing standard-of-care therapeutics. We first tested chemotherapy in models of pancreatic and prostate cancer with DNA synthesis inhibitor gemcitabine (GEM) and the mitotic inhibitor paclitaxel (PXT), respectively. In both models, FTN203 chow alone had an effect and combined well with chemotherapy. But the most significant observation was that after removal of chemotherapy, FTN203 chow reduced the rate at which tumors recurred and grew back.

In the human pancreatic cancer PANC-1 mouse xenograft model, three groups had comparable effects on tumor suppression: Complete Control + GEM, FTN203 + GEM, and FTN203 alone, in the first 42 day prior to discontinuation of GEM. In the Complete Control chow alone group, tumors grew as expected (Figure 3A). However, upon removal of chemotherapy the three curves showing efficacy gradually diverged. Following the removal of GEM, tumors of both FTN203 and FTN203 + GEM cohorts that continued using FTN203 remained suppressed and their growth slowed at later time points. Following the removal of GEM from the cohort maintained solely on Complete Control, tumors grew at a rate of growth quite similar to that of the Complete Control alone control group. Thus, FTN203 chow and GEM (on normal Complete Control chow or with FTN203 chow) were effective in suppressing tumor growth. But upon removal of GEM, FTN203 chow but not Complete Control chow effectively prevented tumor rebound and recurrence.

For the combination of FTN203 chow and PTX in the human prostate cancer PC3 model, the results were similar: FTN203 chow suppressed tumor recurrence following removal of PTX (Figure 3B). In this model, the FTN203 chow alone was significantly more efficacious than Complete Control chow but not nearly as efficacious as either of the PTX arms (Complete Control + PTX, FTN203 + PTX). However, similar results were observed in terms of tumor suppression

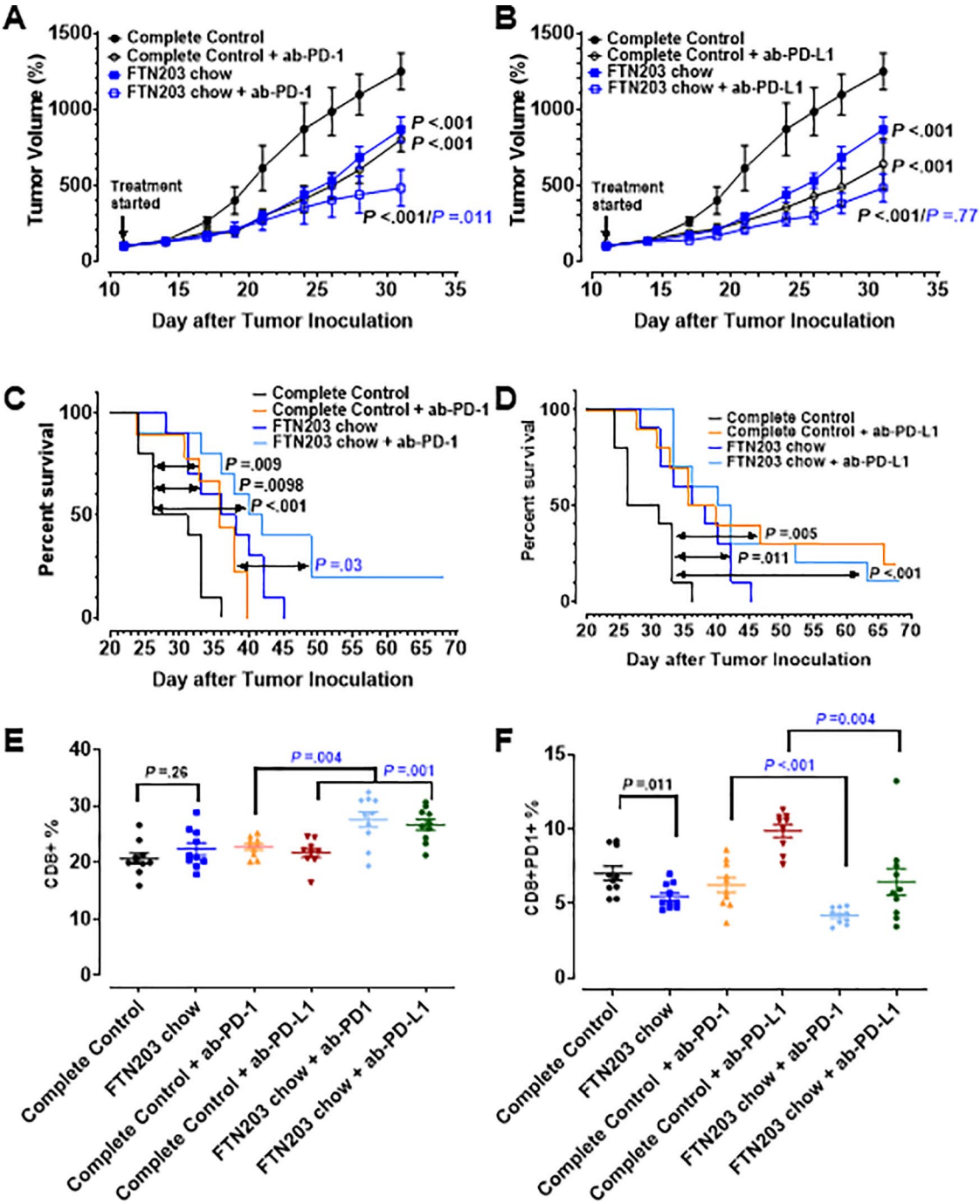

Figure 4. FTN203 enhanced the effects of anti-PD-1 and anti-PD-L1 immune checkpoint inhibitors. Mouse CT26 colon cancer cells were inoculated into female BALB/c mice. Mice were fed with Complete Control and FTN203 chow +/- anti-PD-1 and anti-PD-L1 treatment (i.p. 10mg/kg, twice per week for 2 weeks). The tumor volumes were measured three times per week (A, B). Tumor Volume = L x W x W x ½. Survival was analyzed (C, D). Mouse blood was collected on day 20, and the percentages of CD8+ and CD8 + PD-1+ cells were analyzed (E, F). Statistical comparison between two groups was analyzed by Two-way ANOVA (A, B), Log-rank (Mantel-Cox) test (C, D), and unpaired t-test (E, F) (n = 10; Mean+/-SEM; *P* value in black, compared to Complete Control, *P* value in dark blue, compared to Complete Control + ab-PD-1 (A, C, E, F) or Complete Control + ab-PD-L1 (B, D, E, F)).

after PTX removal. In the Complete Control + PTX group, following PTX removal, tumors eventually grew back at a rapid rate. While tumors grew back in the FTN203 + PTX arm following PTX removal, the rate was much reduced. Thus, in both models, the results were consistent that FTN203 chow could suppress tumor recurrence following the removal of chemotherapy.

There was a slightly lower mouse body weight observed in the FTN203 chow group than in the Complete Control group in these two models (Supplement Figure 4).

FTN203 Enhanced the Effects of anti-PD-1 and anti-PD-L1 Immune Checkpoint Inhibitors

Immunotherapy has proven an important new modality for the treatment of cancer. Several NEAAs have been implicated in modulating immune functions (24, 25), which prompted us to investigate the combined use of FTN203 chow and the immune checkpoint inhibitors (ICIs) anti-PD-1 antibody (ab-PD-1) and anti-PD-L1 antibody (ab-PD-L1). A syngeneic mouse model (BALB/c) bearing subcutaneous tumors of mouse colorectal cancer cell line CT26, which is microsatellite stable, was used.

Figure 4A and C show the effect of the FTN203 chow + ab-PD-1 combination on tumor growth. The FTN203 chow and ab-PD-1 treatments each suppressed tumor growth as compared to Complete Control alone (Figure 4A). Remarkably, the efficacy of FTN203 chow as a single agent is comparable to that of the ab-PD-1 regimen with regard to TGI in this study (30% and 36%, Figure 4A). In the combination cohort, the effects of FTN203 chow and ab-PD-1 were observed to be additive. Combination of ab-PD-1 and FTN203 chow further reduced tumor growth (TGI: 61%; Figure 4A) and increased median survival significantly ($P < 0.05$, vs. ab-PD-1; Figure 4C). In addition, the FTN203 + ab-PD-1 combination enabled some survivors to live long-term up to 70 days. Animals in all other arms were deceased by 45 days.

Figure 4B and D show the results of the FTN203 + ab-PD-L1 combination. Each single treatment produced a significant effect compared with Complete Control (TGI: 31% and 48%). The combined effect was additive. FTN203 chow boosted the TGI of ab-PD-L1 alone from 48% to 61% (Figure 4B, P = 0.81), though survival extension was not observed in this combination (Figure 4D).

To look for potential mechanisms, we analyzed the immune cell profile in the blood on day 20 of tumor growth. We found that FTN203 chow, when combined with ab-PD-1 and ab-PD-L1, significantly increased the percentage of cytotoxic CD8+ cells (Figure 4E) and decreased the percentage of exhausted CD8 + PD1+ cells in the blood (Figure 4F), when compared with respective immunotherapy controls. Interestingly, FTN203 chow alone caused a modest yet significant reduction in CD8 + PD1+ cells, while it showed a trend to increase CD8+ cells (not significant). Thus, FTN203 chow appears to modulate T cell numbers, especially in combination with ICI agents.

Taken together, our data has highlighted the exciting possibility of using TND in improving the efficacy and extending the utilization of checkpoint inhibitors in microsatellite-stable colon cancer.

FTN203 is Safe in Mice

To establish the safety profile of FTN203 chow, we analyzed multiple physiological parameters in female BALB/c mice fed with Complete Control or FTN203 chow, starting at 6-8 weeks of age. We tracked the effects of FTN203 chow for 30 day in these animals. Data is presented in Figure 5. The results are summarized and briefly discussed below.

Blood Chemistry: Standard blood chemistry analysis was performed pre- and post- treatment. No difference was observed between the two groups according to blood glucose, kidney health markers, liver health markers, plasma proteins, ion balance, and organ weights (Figure 5A–F).

Histopathology: Histological examination of organs was also performed on liver, kidney, heart, and small intestine (data not shown). None of the organs in the study showed overt signs of pathological damage.

Body Weight (Supplement Figure 5A): Body weights were monitored throughout the study in cohorts of female mice ($n = 5$). Weight change was compared between the start and the end of the study. The Complete Control chow group showed a weight gain of approximately 10%. At the end of the study, FTN203 chow group had gained less and ended up weighing 6% less than the Complete Control chow group. The changes were modest and within healthy ranges.

Behavioral Monitoring: Physiological monitoring was performed to establish the overall health and vitality of the mice with Vium Smart Housing™ system, which continuously monitored motion, breath rate, and wheel running activity (Supplement Figure 5B–D). Significant changes in these parameters are taken to provide valuable information on many aspects of mouse's physiology and pathology, including stress, anxiety, and depression-like behaviors. We did not observe any significant changes of these physiological parameters in the diet-treated animals, compared with the controls over the 30-day course of the observations, indicating that the health and wellbeing of the mice were not affected by the FTN203 chow.

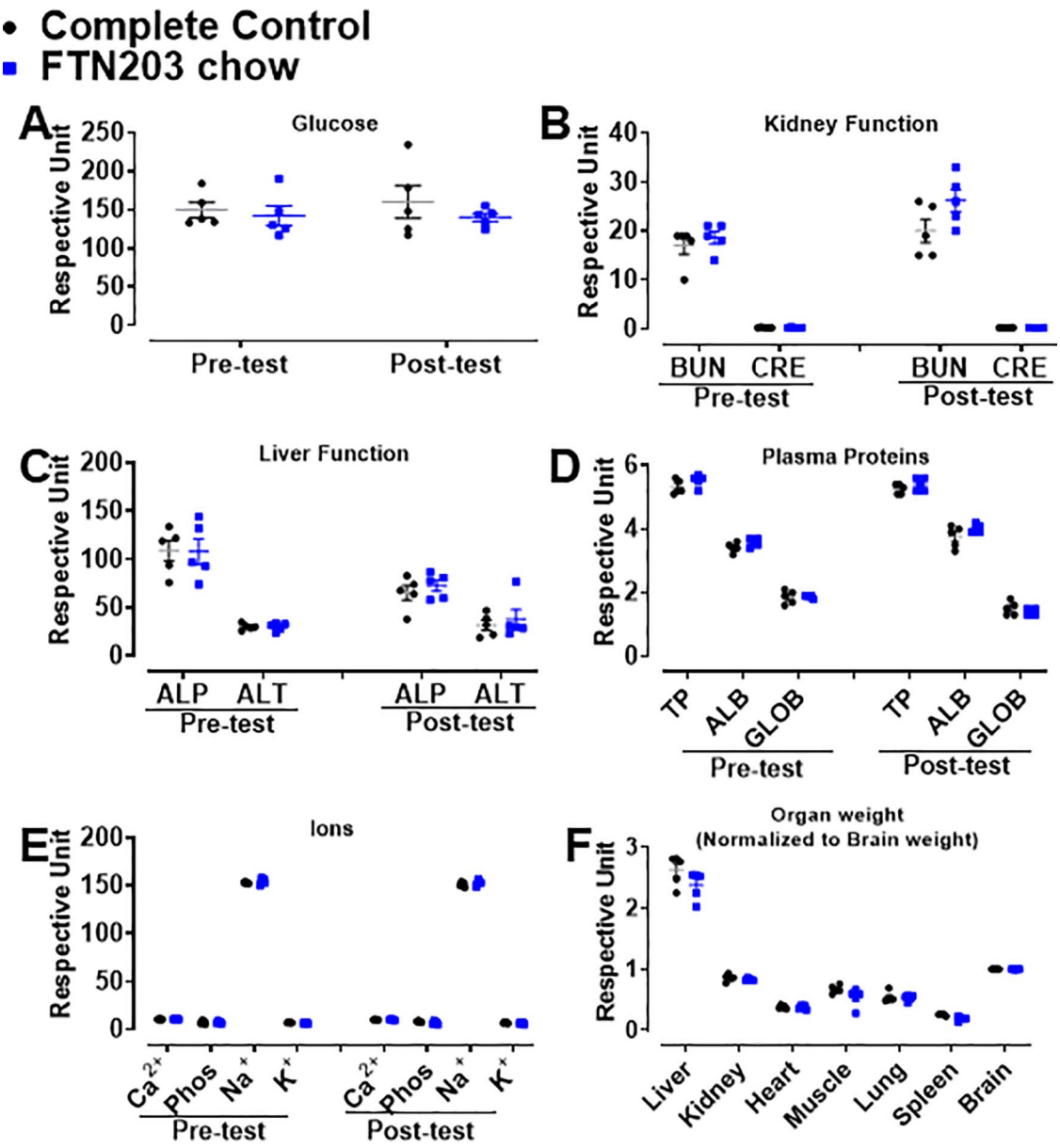

Figure 5. FTN203 therapy has a strong safety profile in mice. Female BALB/c mice were given Complete Control and FTN203 chow for 1 month. Plasma samples were collected pre- and post- treatment for analyzing levels of glucose (GLU) (A), blood urea nitrogen (BUN) and creatinine (CRE) (B), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) (C), total protein (TP), albumin (ALB), and globulin (GLOB) (D), total calcium (Ca^{2+}), phosphorus (PHOS), sodium (Na^{+}), and potassium (K^{+}) (E). Mice were euthanized after 1 month, and internal organs were collected and weighted. The organ weight was normalized to brain weight (F). Statistical comparison between two groups was analyzed by unpaired t-test (n = 5, Mean+/-SEM, compared to Complete Control).

FTN203 Inhibits Cancer Cell Growth in Part through Perturbing the Redox Balance and Inducing Ferroptosis

Finally, we looked into the mechanism(s) that might contribute to the anticancer activity of FTN203. Several NEAAs, including cysteine, glutamate, and glycine, contribute to determining the level of reduced glutathione in cells and consequently redox balance. Reduced glutathione is a major intracellular anti-oxidant that is utilized by glutathione peroxidase (GPX4) to prevent lipid peroxidation and ferroptotic cell death (26). NEAA deprivation could inhibit tumor cell growth, at least in part, by interrupting redox balance and inducing ferroptosis in tumor cells. We tested this hypothesis by measuring the viability of cancer cells (U251 GBM and FaDu HNC) cultured in Complete Control or FTN203 medium with ferroptosis inhibitors and anti-oxidants.

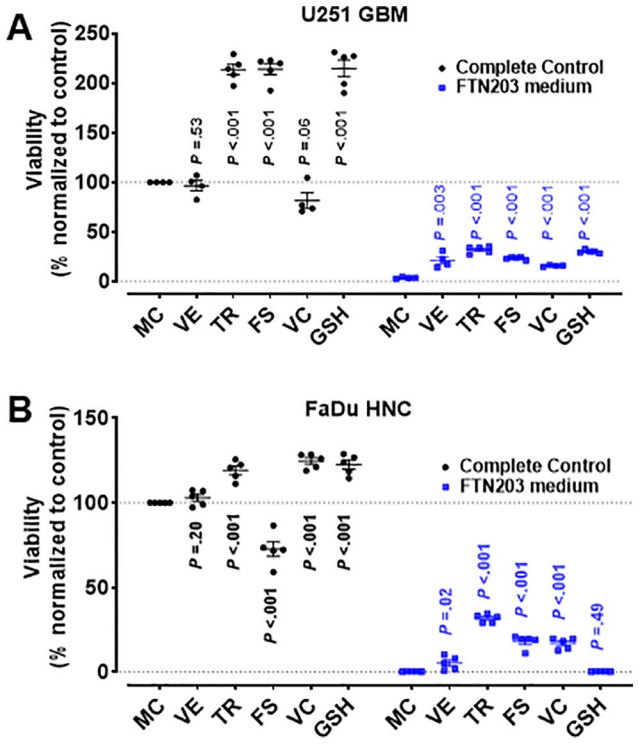

Figure 6. FTN203 inhibits cancer cell growth in part through perturbing the redox balance and inducing ferroptosis. U251 GBM (A) and FaDu HNC (B) cells were seeded in 96-well plate (5,000 cells per well). Complete Control or FTN203 medium was added into plates with Vitamin E (18uM), Trolox (1X), Ferrostatin-1(1uM), Vitamin C (57uM) and L-glutathione reduced (1X). Cell viability was assessed after 72 h by measuring cellular ATP, using the CellTiter-Glo® luminescence cell viability assay kit, and calculated as % normalized to control group. Statistical comparison between two groups was analyzed by unpaired t-test (n = 5, Mean+/-SEM; *P* value in black, compared to Complete Control MC; *P* value in dark blue, compared to FTN203 MC). MC, Medium control; VE, Vitamin E; TR, Trolox (a water-soluble analog of vitamin E); FS, Ferrostatin-1; VC, Vitamin C; GSH, L-glutathione reduced.

As expected, the FTN203 medium consistently reduced the viability of both cancer cell lines as compared to Complete Control medium (viability remaining: U251 – 3.6%; FaDu – 0.2%). As shown in Figure 6A and B, vitamin E (VE), trolox (TR, a water-soluble analog of vitamin E), and ferrostatin-1 (FR) each reversed to a significant extent the lost viability of the cancer cells on FTN203 medium (Fold reversal of lost viability: U251 - six fold to nine fold; FaDu – 26 fold to 150 fold).

We further tested two different anti-oxidants, vitamin C (VC) and L-glutathione (GSH), and found that VC could partially reverse the effect of NEAA deprivation in both cell lines, while GSH substantially increased viability of U251 cells (in both complete and FTN203 medium) but had no effects on FaDu cells (Figure 6A and B).

Taken together, the results of our in vitro study on mechanism(s) of action indicated that ferroptosis and unbalanced oxidative stress appear to contribute to the effect of FTN203 on cancer cells.

Discussion

Here, we have described a promising and completely novel approach to cancer management: targeted nutrients deprivation (TND). TND is a diet-based approach to deplete multiple NEAAs required by tumor cells but not normal cells. As shown in our studies, the TND diet (FTN203) produced strong anti-tumor activity in six xenograft tumor models and one syngeneic mouse cancer model. In combination studies, FTN203 improved the effects of chemotherapy and immunotherapy. For chemotherapy, the most marked effect was the ability of FTN203 to significantly reduce the recurrence of pancreatic and prostate cancer following the cessation of chemotherapy in long-term monitoring. For immunotherapy, FTN203 combined with ICIs produced an additive effect, and FTN203 was able to extend survival in a mouse syngeneic colon cancer model. We further observed that FTN203 affected the immune system with an increase of cytotoxic CD8+ T cells and a reduction in the exhausted CD8 + PD1+ T cells. Due to the limitation of the study design, the infiltration of CD8+ T cells or other immune cells in the tumor tissue was not assessed.

Overall our results have provided a promising entry point for targeting NEAA metabolism as a novel and safe dietary approach to cancer.

Rationale of TND and Comparison to Other Approaches

It is well known that for cancer cells to outcompete normal cells, they rewire metabolism to accommodate their high energy demand, rapid proliferation, and survival. While this helps cancer cells grow, it also provides a metabolic weakness that we sought to exploit. We focused on the role of amino acids because of their multiple roles in metabolism. We chose to deplete NEAAs because of the concern that depletion of EAAs would have a detrimental effect on normal cells and organs. Our hope was that depletion of NEAAs could manage the tumor while leaving normal

cells intact. To date, our efficacy and safety studies, presented here, support this view.

As mentioned previously, multiple studies lend support to the notion that amino acid deprivation might be a promising therapeutic approach in certain tumor types. In humans, injected asparaginase to reduce asparagine levels in the bloodstream is used for the treatment of ALL (Egler, Ahuja et al. 2016). Small molecule inhibitor-based approaches are also being taken to interrupt amino acid metabolism. CB-839, a small molecule glutaminase inhibitor, prolonged progression free survival when combined with everolimus in a Phase II clinical trial for the treatment of renal cell carcinoma (27, 28). The glutaminase inhibitor blocks the conversion of glutamine to glutamic acid, thereby preventing cells from utilizing glutamine as an alternate energy source to glucose under metabolic stress conditions.

We designed FTN203 to deplete multiple amino acids. Our hypothesis was that depletion of multiple NEAAs from the diet would prove superior to depletion of a single amino acid as a highly active approach and that it would prove safe when combined with standard cancer treatment for the reasons summarized here.

1. Broad efficacy. Amino acid dependencies of different types of cancer vary. Cancer cell responses to the dropout of a single amino acid can also vary greatly, (Figure 1). Depletion of one NEAA may significantly inhibit the growth of one cancer but may not have much effect on another. Since previous interventions only affect one amino acid, the efficacy could be limited to one type of cancer. As mentioned, asparaginase and glutaminase inhibitors are narrowly focused on treating specific cancer indications. With our TND approach, multiple NEAAs are depleted, so the nutritional vulnerabilities of many cancer types can be exploited by a single product. This approach may prove efficacious in multiple of cancer types. We have demonstrated anti-tumor efficacy of FTN203 on 11 human cancer cell lines in vitro, 6 human cancer xenograft mouse models, and 1 mouse syngeneic cancer model.
2. Preventing resistance. Often cancer treatments are successful for a period of time but then resistance emerges. Resistance can occur by a mutation that blocks the binding or activity of an inhibitor or by compensatory mechanisms. In the case of amino acid metabolism, compensation could arise via supplies produced by autophagy, proteolysis, and de novo synthesis. It is easy for resistance to occur for the depletion of one or two amino acids, but it is much harder to compensate for the depletion of multiple amino acids. FTN203 was designed to deplete multiple amino acids to prevent this form of compensation.
3. Safety. Almost invariably small molecule inhibitors or biological cancer drugs produce significant side effects, adverse events, or dose-limiting toxicity. Often these effects make frail patients ineligible for treatment or lead to dose reduction or discontinuation. The mouse diet FTN203 is optimized to the known nutritional requirements of mice. All ingredients are normal components of a healthy diet. As such we anticipated that FTN203 diet would prove entirely safe. Our safety studies reported here bear out that prediction.
4. Combinability. Treatment of cancer by combination therapies, each of which carries side effects some of which are severe, is often limited by additive or accumulative toxicities. Given that no significant side effects were observed, FTN203 diet should be easily combined with existing standards of care. We have already shown that FTN203 diet could readily be combined with chemotherapy or immunotherapy to enhance therapeutic efficacy. Given the importance of chemotherapy and immunological checkpoint inhibitors in cancer care, we anticipate extending these studies to test the combination of ICI and FTN203 diet in animal models of different cancer types.
5. Long term safety. In addition to the data presented here, we have long-term FTN203 diet safety data in mice for 9 months and one month safety data for the canine version of TND FTN203 diet in dogs (data not shown). Both mice and dogs remained healthy. No adverse events or abnormal findings were observed. We believe that this approach represents an outstanding opportunity to combat cancer with an strong safety profile. In addition to the anti-tumor effects presented here, we believe that the adequate nutritional balance of the FTN203 diet may provide important nutritional support to cancer patients, who often struggle with poor nutrition and experience cachexia.
6. Mechanism. In some of the xenograft tumor bearing mouse models, FTN203 diet did induce some level of weight loss as compared to complete control. Severe caloric restriction

associated with weight loss has been shown to be efficacious in some mouse models of cancer (29–32). We were cognizant of this concern during our studies and wondered whether anti-tumor effects could be associated with weight loss and calorie restriction. Multiple lines of evidence indicate that the effects observed are not a result of caloric restriction. First, the degree of weight loss in our studies varied between different tumor models and was not consistent with the magnitude of weight loss associated with caloric restriction. In some of the models presented, little or no weight loss was observed but the tumor growth was suppressed. Second, the genetic profiles of some of the tumors rule out entirely the possibility that caloric restriction is the underlying mechanism. Kalaany and Sabatini have demonstrated that tumor types carrying phosphoinositide 3-kinases (PI3K) activating mutations are resistant to caloric restriction, resulting in no effect on tumor growth (33). In our animal studies, PC3 and H69 cell lines, which were strongly inhibited by TND, carry activating mutations of PI3K (33, 34). Likewise, Jurkat cell lines lack of expression in PTEN which results in activated PI3K (35, 36). These three cancer cell lines showed a robust response to TND, thereby demonstrating that the mechanism of TND is not based on caloric restriction and that the specific formulation of the diet is the acting mechanism. In addition, mouse body MRI scan measurement could be very useful for evaluating the actual change of fat/lean mass content, which will be conducted in future studies.

Mechanism(s)

Given the broad effects of FTN203, the exact mechanism(s) by which FTN203 operates remains unestablished. In this paper, we have presented data for two: ferroptosis and immune modulation. Ferroptosis inhibitors and anti-oxidants were able to partially reverse some of the anti-proliferative effects of FTN203 in cell lines. These results suggest that one possible mechanism of FTN203 action involves the induction of ferroptotic death of cancer cells, which exhibit high levels of reactive oxygen species due to increased metabolism (37). Immune responses are found directly impacted by the cellular metabolism especially amino acid sensing pathways (38–41). Our initial findings do indicate that FTN203 may modulate T cell populations especially in combination with ICI therapies.

It has been well established that amino acid status is sensed by predestined distinct signaling pathways including GCN2-ATF4 and PI3K/AKT/mTOR to adjust cellular processes (42–46). Both pathways are related to autophagy regulation, protein synthesis, redox balance, and immune regulations (41, 47–50). ATF4, a stress-induced transcription factor that is frequently upregulated in cancer cells (51), controls the expression of a wide range of adaptive genes that allow cells to endure stresses, and can also induce apoptosis, cell-cycle arrest, and senescence (52). Autophagy-defective cancer cells have enhanced ATF4 transcriptional activity (53, 54), which increases expression of amino acid transporters (AATs) on cell membrane and promote increased demand for exogenous amino acids uptake. Therefore, under persistent amino acids deprivation, cancer cells fail to meet the higher demand for amino acids, and die of metabolic catastrophe (53). Activation of PI3K/AKT/mTOR is important for driving cancer cells' proliferation, however, it may also render them vulnerable to NEAA deprivation and death from metabolic catastrophe due to the promotion of cell growth and inhibition of autophagy (55, 56).

Amino acids also are central to gluconeogenesis, which influences multiple metabolic pathways in cancer cells (57). Except for two EAA leucine and lysine, all the other 18 amino acids have glucogenic capacity and contribute to gluconeogenesis. Given the extent of the depletions of NEAAs by FTN203, we believe the treatment might attenuate gluconeogenesis and perturb cancer cell metabolism, growth, and survival (58). Future investigations regarding the precise mechanism(s) of FTN203 action directly on cancer cells are warranted.

Summary

Results presented here establish TND as a novel and promising approach to cancer. TND is simply the withdrawal by diet of multiple NEAAs. We believe the approach will have a robust and long-lasting effect on tumors either alone or when combined with existing standards of care.

Our current results raise additional interesting questions. Could TND prove useful in suppressing the recurrence of cancer following a successful course of chemotherapy treatment? Does TND suppress the formation and establishment of metastasis? Could TND help overcome immunotherapy resistance in

cancer? Finally, could a periodic course of TND diet act as a cancer preventative? Each of these scenarios suggests an important use for TND in combating cancer. Our intention is to answer these questions in more animal models.

Disclosure Statement

All authors are employees and/or shareholders of Filtricine, Inc.

Author Contributions

Zehui Li, Shuang Zhou, Xiyan Li, and Xin Wang designed and conducted experiments for the whole project. Zehui Li wrote the first draft of the manuscript. Xiaodong Yang conducted cell screen assay and provided support for animal experiments. Grace Xiaolu Yang, Xiyan Li, John Chant, and Xin Wang contributed to discussion and reviewed the manuscript. Xiyan Li, John Chant, Michael Snyder, and Xin Wang contributed to revision of the manuscript.

Funding

This study was funded by Filtricine, Inc., Santa Clara, California, USA.

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