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Metabonomic investigation of biological effects of a new vessel target protein tTF-pHLIP in a mouse model

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Abstract

In recent years, tumor microenvironment has been recognized as potential targets for tumor treatment and tumor vascular system is one of such targets. Fusing truncated tissue factor (tTF) with a pH Low Insertion Peptides (pHLIP), tTF-pHLIP, can target tumor vessels owing to its acidic tumor microenvironment (TME) and cause tumor vessels occlusion by blood clotting and subsequently effectively inhibit tumor growth. To evaluate its bioeffects, we exposed the tTF-pHLIP to normal mice and mice xenograft with B16F10 tumor and analyzed the metabolic profiling of various tissues and biofluids including plasma and urine from mice treated with and without tTF-pHLIP. A combination of nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) and ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) was employed in the study. We found that tTF-pHLIP treatment can effectively reduce tumor size and concurrently ameliorate tumor induced alterations in the TCA cycle metabolism and lipid metabolism. In addition, we found that toxicity of tTF-pHLIP to normal mice is minor and exposure of the tTF-pHLIP induced oxidative stress to the system. Hence, we concluded that tTF-pHLIP is of low toxicity and effective in reducing tumor size as well as rebalancing tumor induced metabolic derailment.

Keywords: vessel target protein, biological effects, metabonomics, melanoma

Introduction

Tumor vasculature is crucial to supply oxygen and nutrients for cell survival and almost all tumors share sustained angiogenesis capability[1]. Compared to traditional therapy, direct target to the tumor vasculature can be applied in a wide variety of tumors and the therapeutic effects could take place in several hours[2, 3]. In addition, the drug resistance problems may be avoided since the markers expressed on the endothelial cells are stable[4]. Selectively occluding tumor vasculature by thrombosis has proven to be effective to starve and kill tumors[5-8].

Truncated tissue factor (tTF) is capable of inducing blood clotting when combined with the membrane of endothelial cells. To exert the potential of tTF and limit the side effect, accurate delivery of tTF should be achieved. Previously, delivery of tTF with antibody or peptide ligands which recognize the tumor vessels markers[5, 6, 9, 10] has not progressed into clinical application, due to limitations of nonspecific delivery and rapid clearance by the reticuloendothelial system[11]. To achieve precise delivery of tTF, we employed a pH-sensitive delivery strategy. Due to lactate and protons accumulations induced by Warburg effect[12] associated with tumors, the tumor microenvironments (TME) is acidic in nature[13]. pH Low Insertion Peptides (pHLIP) can selectively target to low pH TME and insert across the membrane to form a stable transmembrane alpha helix[14-17]. Herein we employed the newly synthesized low pH vessel target agent by fusing pHLIP with truncated tissue factor (tTF), forming a 32 kDa protein, tTF-pHLIP (18). We previously demonstrated that systemic administration of tTF-pHLIP into tumor-bearing mice can selectively initiate the coagulation cascade and induce tumor vessels occlusion and impairing tumor growth[18]. In order for tTF-pHLIP to have clinical applications in the future, an in-depth understanding of tTF-pHLIP is required. Little is known about tTF-pHLIP except that it may accumulate in the kidney after systematic administration of tTF-pHLIP *in vivo*, since pHLIP has been found that it mainly accumulates in the tumor site and certain degree of accumulation has also been found in inflammatory foci and kidney[19]; While tissue factor induced coagulation factors or thrombin could cause inflammation[20, 21]. Till now, no investigation focused on a holistic assessment of the biological effects of the tTF-pHLIP from the metabolic view has been conducted.

To investigate the metabolic impact of tTF-pHLIP exposure, not only the ability of reducing tumor size, but also the ability of normalization of metabolic derailment associated with tumors should be considered. Cancers are increasingly considered as metabolic diseases[22] and cancer cells are capable of rewiring their metabolic pathways[23].

Warburg effect is such an example, in which the cancer cells consumed glucose in the manner of aerobic glycolysis[12]. Another feature is the shift to *de novo* synthesis of fatty acids in cancer cells compared to normal cells[24]. The aberrant metabolism of cancer cells drove the metabolic alteration of cancer patients at holistic levels. The cancer patients usually exhibit high resting energy expenditure and breakdown of adipose tissues and proteolysis in multiple viscera[25, 26]. The Cori cycle is also enhanced in cancer patients and results in a waste of glucose and energy[27]. These alterations result in the abnormal levels of metabolites and offer the advantages for monitoring the cancer progression and prognosis of tTF-pHLIP. Metabonomics is defined as the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification[28]. Metabonomics can be used both for the screen of biomarkers and for drug discovery research and development. As a systems approach, metabonomics can study the metabolic changes in response to drug exposure *in vivo* and provide information on drug toxicity[29-33].

In this study, we aim to the potential toxicity of tTF-pHLIP fusion protein by screening the metabolic effects induced by tTF-pHLIP. We employed C57BL/6J mouse bearing B16F10 melanoma and then treated tumor-bearing mice with tTF-pHLIP. We monitored the metabolic alteration after tTF-pHLIP treatment on the host metabolome of plasma, urine and organ tissues, such as liver, spleen and kidney, using both targeted and untargeted detecting techniques. Our investigation provides a holistic assessment of the toxicity of tTF-pHLIP and demonstrates that metabonomics is a sensitive tool for novel therapeutic based on tumor vasculature targeting.

Materials and Methods

Chemicals

Methanol, $K_2HPO_4 \cdot 3H_2O$, and $NaH_2PO_4 \cdot 2H_2O$ were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), and Trimethylsilyl propanoic acid (TSP) and deuterium oxide (D2O, 99.9% in D) were from Cambridge Isotope Laboratories, Inc. (Miami, USA). HPLC grade methanol and acetonitrile were purchased from TEDIA (Shanghai, China). The standards using for GC-MS and UPLC-MS were purchased from Sigma-Aldrich (Shanghai, China).

Synthesis and purification of tTF-pHLIP

tTF-pHLIP was expressed by *E. coli*[18]. In short, the pHLIP polypeptide (cDNA sequence:

GCTGAACAGAACCCGATCTACTGGGCTCGTTACGCTGACTGGCTGTTCACC

ACCCCGCTGCTGCTGCTGGACCTGGCTCTGCTGGTTGACGCTGACGAAGG TACC, amino acid sequence: AEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT) was linked to the C-terminus of the extracellular domain of tissue factor (amino acids 1 to 218 of human TF), which made the cDNA for tTF-pHLIP. We then cloned the cDNA into Nde I and Xho I sites of the expression vector pET-30a (+). tTF-pHLIP was expressed in competent *E. coli* (BL21DE3, Invitrogen, Carlsbad, CA) after vector introduction, and then was purified and refolded with a His Bind Buffer Kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions.

B16F10 melanoma cells culture

B16-F10 melanoma cells were cultured in RPMI 1640 medium with 10% fetal bovine serum at 37℃, 5% CO_2. B16-F10 cells were collected by dissociating the cells in trypsin, washed and resuspended in PBS for tumor inoculation.

Animal experiments and sample collection

Four weeks old female C57BL/6J mice (N = 65) were purchased from Beijing Vital River Laboratories and housed under specific pathogen-free conditions at Wuhan Institute of Virology (Hubei, P. R. China). The experimental procedures were approved by the ethics committee of Wuhan Institute of Physics and Mathematics (Hubei, P. R. China, WIPM-A2017-01). Mice were randomly divided into five groups (each group has 13 mice) and kept with 12 h light/dark cycles. After two weeks acclimatization, three groups of mice were inoculated with tumor; one million B16F10 cells were injected subcutaneously into the right flank of mice. Tumor size was measured by the length and width of tumor with a vernier caliper and body weight was recorded every two days. One week later, when the tumor grew to a volume of approximately 100 mm^3 (Volume = (length*$width^2$)/2), the three groups of mice with tumor were treated with saline (Tumor 0), 2.5 μg tTF-pHLIP per mouse (Tumor 2.5) and 5 μg tTF-pHLIP per mouse (Tumor 5) by tail vein injection. The two groups of mice without tumor were treated with saline (Normal) and 2.5 μg tTF-pHLIP per mouse (Normal 2.5). tTF-pHLIP or saline was administrated every two days for a total of 4 times (day 1, 3, 5, 7).

Samples of urine and feces were collected every five days from the day before tumor inoculation. Two weeks after tumor inoculation and when the largest tumor size reached about 2000 mm^3, the mice were sacrificed by decapitation after fasting for 12 h. Plasma and various organ tissues of liver, kidney, spleen and tumor were obtained. Plasma for each mouse was obtained using heparin sodium with standard centrifugation

procedures. All samples were stored at -80℃ following immediately frozen with liquid nitrogen.

Histopathological analysis

Tissues from liver, spleen and kidney were embedded in paraffin blocks. The block was sectioned into 5-μm thick sections and stained with hematoxylin and eosin (H&E) for pathological examination.

NMR sample preparation and NMR spectroscopy

For plasma preparation, 30 μL of plasma was mixed with 25 μL phosphate buffer (45mM) and transferred 50 μL of supernatant into a 1.7 mm NMR tube. Urine samples were prepared according to the literature[34]. Briefly, 60 μL urine and 4.8 μL potassium fluoride buffer (5 M) were mixed with 440 μL ultra-pure water (containing 20% D_2O, v/v). The mixture was centrifuged (15,930 g, 4℃, 10 min) after vertexing and standing at 25℃ for 10 min. Then 450 μL supernatant was mixed with 6 μL EDTA-d12 (0.1 M) and 45 μL phosphate buffer (1.5 M), and transferred to 5 mm NMR tube.

50 mg of organ tissues were extracted with 0.6 mL 66.6% cold methanol aqueous solution for 3 times. Briefly, the organ tissues were homogenized with a tissue-lyser (QIAGEN, Germany) and collected the supernatants after centrifugation (11,060 g, 4 ℃, 10 min). Supernatants from the three extraction procedures were lyophilized following methanol removal *in vacuo*. Then the resulted powder was dissolved in 600 μL phosphate buffer (0.15 M), centrifuged and transferred 550 μL of supernatants into a 5 mm NMR tube for NMR analysis.

NMR spectra were collected on a Bruker AVANCE III 600 MHz NMR spectrometer equipped with a cryogenic probe (Bruker Biospin, Germany) at 298 K. Each spectrum was recorded with 128 transients, 32K data points and 20-ppm spectral width. ^{1}H NMR spectrum for urine and tissue extracts was acquired with a standard NOESYGPPR1D pulse sequence (RD-G1-90°-t1-90°-tm-G2-90°-acquisition; t1 = 4μs, tm = 80 ms). The spectrum of plasma was obtained with a Carr-Purcell-Meiboom- Gill sequence ((RD-90°-(τ-180°-τ) n-acquisition); τ = 350 μs, n = 100) Two-dimensional NMR spectra of ^{1}H J-resolved, ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC of selective samples were acquired for metabolites identification[35, 36].

Amino containing metabolites extraction and detection

Amino containing metabolites were quantified using an Agilent UHPLC-MS/MS 1290-

6460 system (Agilent Technologies, USA). Amino containing metabolites were extracted before derivation with 5-aminoisoquinolyl-*N*-hydroxysuccinimidylcarbamate (5-AIQC)[37, 38]. 20 μL plasma was mixing with 60 μL methanol and centrifuged to obtain 10 μL supernatants. 50 mg of liver tissue was frozen and thawed for three cycles in 600 μL 66.6% methanol aqueous solution followed by 1 min ultra-sonication and 1 min pause for 8 cycles. The supernatants were collected after centrifugation and diluted with equal amount of 0.1% formic acid aqueous solution. Supernatants (10 μL) from plasma and liver extraction was mixed with 80 μL of 2.5 mM *N*-ethylmaleimide solution for 1 min. 10 μL of 1 M 4-tert-butylbenzenethiol DMSO solution was added followed by the addition of 700 μL of 20 mM tris (2-carboxyethyl) phosphine hydrochloride (borate buffer 0.2 M, pH 8.8). 200 μL 5-AIQC solution was then added into the extracted solution after vortex-mixing and 2 min of standing, and followed by incubation at 55 °C for 10 min. The mixture was added with 10 μL formic acid and centrifuged after cooling down to 25 °C. The final supernatants were filtered via a 0.22 μm filter for UHPLC-MS/MS analysis.

1 μL of 5-AIQC-tagged sample was injected into a column (C18, 2.1 × 100 mm, 1.8 μm, Agilent Technologies, USA). Mobile phase A (ultrapure water) and B (methanol, containing 0.1% (v/v) formic acid) were used and 0.6 mL/min flow rate and 50°C column temperature was maintained. Amino containing metabolites were acquired in the positive ion mode and were quantified using MassHunter Workstation software by external standards method.

Fatty acids composition analysis

Fatty acids composition of liver and plasma were determined using an improved method based on a previous report[39]. 10 mg of liver tissues was homogenized with 500 μL methanol. 20 μL internal standards (1 mg/mL methyl heptadecanoate, 0.5 mg/mL methyl tricosanoate and 2 mg/mL butylated hydroxytoluene) was mixed with 100 μL liver homogenate or 30 μL plasma and then mixed with 1 mL methanol-hexane solution (80% methanol). The mixture reacted with 100 μL of precooled acetyl chloride at 25 °C in the dark. 24 h later, the mixture was neutralized with K_2CO_3 solution and extracted three times with 200 μL hexane. The supernatants were volatilized to dryness followed and dissolved in hexane (100 μL for liver extracts and 50 μL for plasma extracts). The fatty acids detection was performed on a Shimadzu GC 2010 Plus GC-MS spectrometer (Shimadzu, Japan).

Quantitative PCR analysis

Total mRNA in the liver tissue was extracted using RNAiso Plus kit (Takara, Japan) and cDNA were obtained using a reverse transcription kit (Catalog number: AT341-02, TransGen, China). mRNA levels were quantified with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) on a 7900HT Fast Real-time PCR System (Applied Biosystems). Gapdh was applied as an internal control. The primers sequences were listed in **Table S1**.

Data processing and analysis

NMR data were processed with Topspin (V3.6.0, Bruker Biospin, Germany) and Mestrenova (V9.0, Mestrelab Research, Spain). In short, the free induction decay was Fourier transformed with an exponential window function by multiplying a 0.5 Hz line-broadening factor. All the spectra were calibrated to TSP except spectra of plasma, which were calibrated to the anomeric proton of glucose[40, 41]. The calibrated spectra were then integrated using equidistant binning with a width of 0.002 ppm after phase and baseline correction. Water signals from δ4.7 to δ5.1 and urea signals from δ5.3 to δ6.2 were excluded. The data were normalized according to the types of sample[42]: urinary data were normalized to the total area, organ tissue data to the wet weight and plasma without normalization.

Multivariate data analysis was performed with SIMCA-P+ (V13.0, Umetrics AB, Umea, Sweden). Principal component analysis (PCA) was applied with unit variance scale to detect potential outliers[43]. Orthogonal Projection to Latent Discriminant Analysis (OPLS-DA) models were constructed with unit variance scale. All OPLS-DA models were validated by both cross-validation[44] and CV-ANOVA method.

The levels of amino metabolites and fatty acids were quantified based on peak integrations from raw UHPLC-MS/MS and GC data using calibration curves from respective standards and further were analyzed using univariate data analysis after normalization to wet tissue weight (organ tissue) and volume (plasma). Student's t-test and non-parameters tests were applied, metabolites with $p < 0.05$ were regarded as significant different metabolites. Pathway analysis was performed with Metaboanalyst 4.0 (http://www.metaboanalyst.ca)[45].

Results

Histopathological analysis of major organs after tTF-pHLIP treatment

No abnormality was observed from the histopathological examination of liver, kidney and spleen obtained from tTF-pHLIP treated mice or tumor-bearing mice compared to

the normal mice (**Figure S1**).

^{1}H NMR spectroscopy of biofluids and tissue extracts

Assignment of typical ^{1}H NMR spectra of the liver, plasma, tumor, kidney and urine from tumor bearing mice are illustrated in Table S2. The resonance peaks were assigned to metabolites according to the literature[29, 32, 36, 46], the Human Metabolome Data Base[47] and further confirmed by two-dimensional NMR experiments. The plasma spectra are mainly constituted of lipid, glucose, amino acids and choline metabolites (choline, O-PC, GPC) (**Table S2**). The spectra of liver, kidney and spleen extracts included glucose, amino acids, carbohydrates, choline metabolites and several nucleotides (**Table S2**). While the spectra of urine mainly included TCA intermediates (succinate, citrate, fumarate, α-ketoglutarate, cis-aconitate and malate), amino acids, organic acids, urea and allantoin.

Metabolic abnormality induced by tumor burden

NMR metabolic profiles of urine, plasma, organ tissue extracts obtained from tumor bearing mice were compared to relevant tissue extracts from untreated mice by OPLS-DA models. The models were subsequently validated and the results suggested that the metabolome of urine, plasma, liver, kidney and spleen from mice bearing with tumor differed significantly from those of normal mice (**Figure 1** and **Figure S2**).

Tumor-bearing induced metabolic alterations in liver, kidney and spleen (**Figure 1A-B**), such as, the decrease in the levels of most amino acids, acetate, GABA, choline, ethanolamine, hypoxanthine, xanthine and uracil and increase of inosine and UMP. However, the tumor induced metabolic alterations also varied between different organ tissues. The levels of succinate and sn-glycero-3-phosphocholine were upregulated in both liver and kidney of tumor bearing mice. Increased levels of glutathione and decreased levels of uridine and formate were observed in both liver and spleen. The levels of glucose, mannose, malonate, taurine, creatine, glycerol and nicotinurate were decreased in both kidney and spleen. In addition, tumor exhibited a specific influence on liver metabolism such as increased levels of glutamine, AMP, β-alanine, UDP-glucose and lactate and decreased levels of glycogen and fumarate. Tumor also caused decreased levels of lactate and increased levels of fumarate and uridine in the kidney. In the spleen, cytidine was downregulated in tumor bearing mice. Significant increase of lipids, triglyceride, lactate, glycerol, alanine and GPC and decrease of tyrosine and glucose were noticed in the plasma of tumor bearing mice compared to those of normal mice (**Figure 1C**). Tumor also induced changes in urinary metabolic profiles and these

changes included increased levels of lactate, 3-indoxylsulfate and TCA cycle intermediates (2-oxoglutarate, succinate, citrate) and decreased levels of betaine, taurine, hippurate and N, N-dimethylglycine (**Figure 1C**).

Therapeutic effects of tTF-pHLIP

Tumor size and body weight were monitored every other day for tumor bearing mice treated with and without tTF-pHLIP (**Figure 2A** and **Figure S3A-B**). Clearly, tTF-pHLIP treatment resulted in significant reductions in tumor growth from four days post-treatment onward for both low (2.5 μg) and high (5.0 μg) dose levels. In addition, no significant effect on tumor growth was observed between low and high dosage level treatments. Consistent with this observation, the tumor weight obtained at the end point of the experiment showed a significant reduction in tTF-pHLIP treated groups with again no distinction between low and high dose levels observed in tumor weight (**Figure S3A**). No significant change in body weight was found between tumor bearing group and those treated with a high or low dose of tTF-pHLIP (**Figure S3B**).

tTF-pHLIP administration demonstrated the capability of counteracting the metabolic disruptions induced by tumor bearing. In urine metabolome, energy-related metabolites in tumor bearing mice treated with tTF-pHLIP recovered to the levels of the normal mice after treatment (**Figure 2B-D**), for example, the urinary levels of fumarate, 2-oxoglutarate and citrate. However, the levels of 3-indoxyl sufate, TMAO and lactate were not recovered to the levels of the normal control mice (**Figure S3C-E**). The circulation fatty acids in tumor bearing mice treated with tTF-pHLIP showed a trend of recovery, for example, the levels of C16:1, C18:0 and C20:4n6 of low dose treated mice. However, not all the levels of fatty acids returned to the levels of normal mice (**Figure 2E-F**). We also examined key enzymes of fatty acid metabolism in the liver. We have shown that the mRNA levels of *Fasn* and *Acly* involved in *de novo* fatty acids synthesis in the liver were increased after tumor bearing while reduced to normal levels after tTF-pHLIP treatment. However, the lipid catabolism enzymes were decreased in the liver of tumor bearing mice regardless of tTF-pHLIP treatment when compared to normal mice (**Figure 2G**). The activity of desaturases was inhibited in tumor group after tTF-pHLIP administration (**Figure 2H**). No counteractive effect of tTF-pHLIP treatment on the metabolomes of liver, spleen and kidney was observed (**Figure 3**, **Figure S4** and **Figure S5**) However, compared to tumor bearing group, treatment with tTF-pHLIP induced decreases in the levels of glucose, alanine, glutamine, succinate, fumarate, malate and GSSG and increases in the levels of phosphocholine, choline, betaine, inosine, sarcosine and hypoxanthine in liver (**Figure 3A-D**). The mRNA levels involved in steatolysis, GSH metabolism, pentose phosphate

pathway, glutamine metabolism, TCA cycle and choline metabolism were all decreased in tumor bearing mice (**Figure 3E-G**). However, compared to tumor bearing mice, tTF-pHLIP induced an increase in the expression levels of *Gss* and *Pcyt1a* (**Figure 3F-G**). The significant changed metabolites and enzymes induced by tumor or after tTF-pHLIP treatment were mapped in the detailed pathways involved in Warburg effect, TCA cycles, lipids metabolism, choline metabolism, GSH metabolism and nucleotide metabolism (**Figure 4**).

Metabonomic investigation of metabolism disturbance of tTF-pHLIP administration on normal mice

The metabolic differences between normal mice treated with and without tTF-pHLIP were compared. Levels of glucose, fumarate, malate, betaine, xanthine, uracil, and phosphocholine were increased whereas the levels of 3-hydroxybutyrate, asparagine, tyrosine, serine, hypotaurine and 2-aminoadipic acid were decreased in the liver of tTF-pHLIP treated mice comparing to those of untreated mice (**Figure 5A**). The decrease of glutamate and *myo*-inositol were found in the spleen of tTF-pHLIP treated mice (**Figure 5B**). The levels of leucine, isoleucine, valine, 3-hydroxybutyrate, alanine, acetate, lysine, creatine, carnitine, *myo*-inositol, mannose and phenylalanine were reduced while the levels of betaine were elevated in the kidney of normal mice treated with tTF-pHLIP when comparing to the kidney of untreated mice (**Figure 5C**). Plasma levels of C16:0, C16:1, C18:0 and C18:2n6c were decreased in tTF-pHLIP treated mice (**Figure 5D**).

Discussion

Tumor vessel targeted therapies become promising strategies because of high selectivity, low toxicity and good efficacy. One of the examples is the pH-sensitive fusion protein tTF-pHLIP. This targeting protein selectively induced thrombus at tumor sites and blocked the nutrients supply for tumor, leading to tumor shrinkage[12]. In our previous study, we have demonstrated that tTF-pHLIP can successfully induce tumor to shrink in size[18]. Currently, we showed that injection of 2.5 μg tTF-pHLIP per mouse for four times in total was capable of substantially inhibiting tumor growth, while a higher dose (5.0 μg) had no further improvement in tumor inhibition (**Figure 2A**). In addition, no difference in body weight was observed between the tTF-pHLIP exposed groups and their negative tumor bearing controls (**Figure S3B**). Furthermore, no histological abnormality was observed in the organs of liver, kidney and spleen (**Figure S1**), neither in normal or tumor bearing mice treated with tTF-pHLIP, implying low or no toxicity observed. These results suggested again that tTF-pHLIP is effective in reducing tumor

growth and has great potential for further development for cancer treatment. In the current study, we holistically evaluated the biological effects of tTF-pHLIP in rebalancing metabolic abnormality induced by cancer and the potential toxicity associated with tTF-pHLIP at the molecular level by employing metabolomics techniques.

The most prominent metabolic derailment induced by tumor burden was the increased output of urinary TCA cycle intermediates (**Figure 1C**) and we have demonstrated that tTF-pHLIP treatment re-normalized this derailment (**Figure 2B-D** and **Figure 4**). Energy metabolic disorder is common phenomena associated with tumor progression and this is attributed to the increased energy demands for tumor growth. The re-balancing of the levels of TCA cycle intermediates and enzymes in the post-treatment mice is therefore a metabolic reflection of the reduced tumor size observed in mice treated with tTF-pHLIP (**Figure 2A** and **Figure 3E**). Energy disruption associated with tumor growth also presents with rewired glycolysis, also known as Warburg effect, and is the most important metabolic alteration which rapidly consumes glucose and secret abundant lactate[48]. Our current study observed an elevated level of lactate in plasma, urine and liver in tumor bearing mice, which were consistent with the Warburg effect of tumors. However, tTF-pHLIP treatment was not able to revert the Warburg effect associated with the tumor. We observed that tTF-pHLIP treatment can re-normalize the tumor growth induced increases in the levels of C16:0, C18:0 and C20:4n6 in plasma to the levels of control mice (**Figure 2E**). A previous study has demonstrated alteration of the lipid metabolism associated with tumors. Specifically, tumor growth induced downregulation of lipogenesis and upregulation of lipolysis while the fatty acids *de novo* synthesis in tumors was enhanced for the membrane synthesis[49]. We further calculated the levels of *Scd16* and *Scd18* that reflected the activities of desaturases[50] and found that the desaturases were decreased in plasma of mice exposed to tTF-pHLIP (**Figure 2H**). The mRNA results showed that the *de novo* synthesis of fatty acids was increased in the liver of tumor bearing mice, which supports the tumor fast growth. Surprisingly, the *Fasn* and *Acly* expression reduced to normal levels after tTF-pHLIP treatment, suggesting that the treatment of tTF-pHLIP is able to inhibit *de novo* synthesis of lipids associated with cancer growth. The fact that tTF-pHLIP could counteract alterations of lipid metabolism indicated the mechanism of tTF-pHLIP in inhibiting cancer growth may be via reprogramming lipid metabolism. Further validation is necessary to confirm this notion.

We failed to note the re-normalization capability of tTF-pHLIP for the amelioration

of tumor-induced metabolic alterations in the organ tissues (**Figure 3, Figure S4 and Figure S5**). Having said that, we noticed that tTF-pHLIP exposure to tumor bearing mice induced decreased levels of glucose, accompanied by the reduced levels of TCA cycle intermediates (**Figure 3A**) in the liver compared to the tumor bearing mice without tTF-pHLIP treatment, implying high energy demands associated with tTF-pHLIP treatment. The high energy consumption could contribute to the blood clotting activity as tTF-pHLIP initiates the coagulation cascades and the clearance of formed thrombus.

Another important impact of tTF-pHLIP exposure is initiation of oxidative stress, which was manifested by the depleted levels of GSH and increased ratios of GSSG/GSH (**Figure 3B**) and further confirmed by the expression of GSH synthetase: *Gss* was increased after tTF-pHLIP exposure, implying the increased demands for GSH (**Figure 3G** and **Figure 4B**). This notion is consistent with the previous finding showing that the coagulation factors caused inflammation[51, 52] and the oxidation of GSH could counteract this process. Furthermore, the increased betaine may also be responsible for the antioxidant activity. Betaine can exert its antioxidant activity in two ways: one is improving the ROS-scavenging ability of the methionine sulfoxide reductase antioxidant system, and the other is to keep the free radicals away from the membrane by forming a protective membrane with an electronegative outer surface around cells[53]. In addition, phosphatidylethanolamine (PE) methylation has been shown to be critical for protecting cells against oxidative stress by generating GSH[54]. PC can be synthesized from PE through methylation[55]. In the current investigation, we observed significant decreases in the levels of ethanolamine and the concurrent increase in the levels of phosphocholine, choline and inosine (**Figure 3A** and **Figure 3C**). *Pcyt1a*, which is the key enzyme for phosphocholine synthesis, was increased in tumor bearing mice after tTF-pHLIP treatment compared to untreated tumor bearing mice (**Figure 3F**). Taking these together, we speculate that tTF-pHLIP induces the enhanced PE methylation, which is in concordance with oxidative stress associated with tTF-pHLIP exposure.

Finally, we investigated the effects of tTF-pHLIP on normal C57 mice. tTF-pHLIP induced slight perturbations on kidney and liver metabolism, but not spleen, urine or plasma (**Figure 5**). A previous study showed that pHLIP accumulated in kidney, which could cause the observed changes in the levels of several amino acids. While in the liver, the glucose, nucleotides and energy metabolism were altered. However, compared to the alteration induced by tumor or tumor treated by tTF-pHLIP, these changes were negligible.

Conclusions

In summary, we have investigated the biological effect of tTF-pHLIP *in vivo* using a holistic metabolomics approach by employing a combination of NMR and MS technologies. tTF-pHLIP has little effect on normal mice, administration of tTF-pHLIP only induced subtle metabolism perturbations in liver and kidney. We also demonstrated that tumor progression caused alteration of central carbon metabolism, TCA cycle, pentose phosphate pathway and glutamine related pathways. However, after the intervention of tTF-pHLIP, the tumor associated alterations in TCA cycle metabolism and lipid metabolism was alleviated. Furthermore, the treatment of tTF-pHLIP induced oxidative stress. Moreover, our observations were made 4 days post exposure of tTF-pHLIP, which is a relatively short time for recovery of metabolism from drug effects. The metabolic effects of tTF-pHLIP could possibly be fully recovered if given a longer time for recovery, which will be evaluated in our future study. Our investigation demonstrated that tTF-pHLIP complex is a potential tumor vessel target drugs with no severe side effects and that metabolomics is a well-suited tool for the evaluation of biological effects of complex drugs *in vivo*, such as tTF-pHLIP.

Associated content

Supporting information

Table S1. Primers used for qPCR. **Table S2.** Metabolites assigned from NMR data. **Figure S1.** Histopathological analysis of liver, kidney, spleen and tumor. **Figure S2.** Effects of tumor burden on metabolomes of the mice. **Figure S3.** Therapeutic effect of tTF-pHLIP. **Figure S4.** Comprehensive analysis of amino acids in liver, spleen and kidney. **Figure S5.** Metabolites alteration induced by tumor.

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Notes

The authors declare no competing financial interest.

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Figure caption

Figure 1 Effects of tumor burden on the metabolomes of the mice. Heatmap showing the changes metabolomes of liver, kidney and spleen (A&B) and plasma and urine (C) after xenografting of tumors. The red color means that the levels of the metabolites were higher in tumor bearing mice, while blue color means lower in tumor bearing mice. NA means not detected while 0 means no significant difference.

Figure 2 Therapeutic effect of tTF-pHLIP. A, Tumor growth curve *in vivo*. Tumor sizes scaled to the size of day -3. The mice were treated with saline or tTF-pHLIP four times on day 1, day 3, day 5 and day 7. B-D, significant changed metabolites in urine. E-F, lipids and fatty acids alteration in plasma, metabolites determined by GC (E) and NMR (F). G, Quantitative reverse transcription PCR for lipids and fatty acids metabolic pathway enzymes in the liver. H, the activity of desaturases alteration in plasma, SCD16= C16:1/C16:0;SCD18 = C18:1/C18:0. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate levels of significance when tumor groups compared with normal group, #$p <$ 0.5, ##$p < 0.01$ and ###$p < 0.001$ indicate levels of significance when tTF-pHLIP treatment groups compared with tumor group. **Keys: TG**, Triglyceride, **VLDL**, very low-density lipoprotein, ***Fasn***, Fatty acid synthase, ***Acly***, ATP citrate lyase, ***Acc***, Acetyl-CoA carboxylase, ***Scd1***, stearoyl-Coenzyme A desaturase 1, ***Atgl***, Adipose triglyceride lipase, ***Hsl***, hormone-sensitive lipase.

Figure 3 Metabolic alteration in liver after tTF-pHLIP treatment. A-D, metabolites alteration. E-G, Quantitative reverse transcription PCR for validation of enzyme expression in liver. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate levels of significance when tumor group compared with normal group, #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ indicate levels of significance when tTF-pHLIP treatment group compared with tumor group.***G6pd1***, hexose-6-phosphate dehydrogenase, ***Gls***, Glutaminase, ***Glud1***, glutamate dehydrogenase 1, ***Sdh***, Succinate dehydrogenase, ***Idh1***, isocitrate dehydrogenase 1, ***Chkb***, choline kinase beta, ***Chdh***, choline dehydrogenase, ***Bhmt***, betaine-homocysteine methyltransferase, ***Fmo3***, flavin containing monooxygenase 3, ***Pcyt1a***, phosphate cytidylyltransferase 1, choline, alpha isoform, ***Sardh***, Sarcosine dehydrogenase, ***Gss***, glutathione synthetase, ***Gsr***, glutathione reductase, ***Gclc***, glutamate-cysteine ligase catalytic subunit, ***Gstp1***, glutathione S-transferase pi 1.

Figure 4 Pathway analysis of tumor effect and therapeutic effect of tTF-pHLIP. A, metabolic alterations induced by the tumor, normal vs. B16F10 tumor bearing mice. B, therapeutic effect of tTF-pHLIP, tumor vs. tumor treatment (tumor 2.5 or tumor 5). Red means increased levels of metabolites or mRNA while blue decreased. "+" means the levels of metabolites or mRNA recovered to normal levels after tTF-pHLIP treatment. Keys: Gn, glycogen, Glc, glucose, Lac, lactate, Suc, succinate, Hxan, Hypoxanthine, TG, triglyceride.

Figure 5 Metabolic disturbance induced by tTF-pHLIP on normal mice. Significant metabolites obtained from liver(A), spleen (B), kidney (C) and plasma (D). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate levels of significance when normal group treated with tTF-pHLIP compared with normal group.

For TOC Only

Metabonomic investigation of biological effects of a new vessel target protein tTF-pHLIP in a mouse model

We assessed metabolic and anti-tumor effects of fusing truncated tissue factor (tTF) with a pH Low Insertion Peptides (pHLIP) on a mouse model. tTF-pHLIP treatment can effectively reduce tumor size and concurrently ameliorate tumor induced metabolic alterations. The toxicity of tTF-pHLIP is minor and exposure of the tTF-pHLIP induced oxidative stress. Hence, tTF-pHLIP is of low toxicity, effective in reducing tumor size and rebalancing tumor induced metabolic derailment.