This document is downloaded from DR-NTU (https://dr.ntu.edu.sg)
Nanyang Technological University, Singapore.

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

Ding, Laifeng; Zhang, Congcong; Liu, Zhigang; Huang, Qingxia; Zhang, Yinlong; Li, Suping; Nie, Guangjun; Tang, Huiru; Wang, Yulan

2020

Ding, L., Zhang, C., Liu, Z., Huang, Q., Zhang, Y., Li, S., . . . Wang, Y. (2019). Metabonomic Investigation of Biological Effects of a New Vessel Target Protein tTF-pHLIP in a Mouse Model. Journal of Proteome Research, 19(1), 238–247. doi:10.1021/acs.jproteome.9b00507

https://hdl.handle.net/10356/144048

https://doi.org/10.1021/acs.jproteome.9b00507

This document is the Accepted Manuscript version of a Published Work that appeared in final form in Journal of Proteome Research, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see https://doi.org/10.1021/acs.jproteome.9b00507

Downloaded on 02 Mar 2021 10:53:34 SGT

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

Laifeng Ding[1,2], Congcong Zhang[3], Zhigang Liu[4], Qingxia Huang[3], Yinlong Zhang[5], Suping Li[5], Guangjun Nie[5], Huiru Tang[3] and Yulan Wang[6] *

[1]CAS Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Institute of Physics and Mathematics, Wuhan 430071, P.R. China

[2]University of Chinese Academy of Sciences, Beijing 10049, P.R. China

[3]State Key Laboratory of Genetic Engineering, Zhongshan Hospital and School of Life Sciences, Laboratory of Metabonomics and Systems Biology, Human Phenome Institute, Fudan University, Shanghai, 200433, China.

[4]Division of Integrative Systems Medicine and Digestive Disease, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom

[5]CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology, Beijing100190, P.R. China

[6]Singapore Phenome Center, Lee Kong Chian School of Medicine, School of Biological Sciences, Nanyang Technological University, Singapore

*To whom the correspondences should be addressed: E-mail: yulan.wang@ntu.edu.sg; Fax: +65-65150417, Tel.: +65-69041106.

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

49 **Introduction**

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

57

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

81

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

86 Warburg effect is such an example, in which the cancer cells consumed glucose in the
87 manner of aerobic glycolysis[12]. Another feature is the shift to *de novo* synthesis of fatty
88 acids in cancer cells compared to normal cells[24]. The aberrant metabolism of cancer
89 cells drove the metabolic alteration of cancer patients at holistic levels. The cancer
90 patients usually exhibit high resting energy expenditure and breakdown of adipose
91 tissues and proteolysis in multiple viscera[25, 26]. The Cori cycle is also enhanced in
92 cancer patients and results in a waste of glucose and energy[27]. These alterations result
93 in the abnormal levels of metabolites and offer the advantages for monitoring the cancer
94 progression and prognosis of tTF-pHLIP. Metabonomics is defined as the dynamic
95 multiparametric metabolic response of living systems to pathophysiological stimuli or
96 genetic modification[28]. Metabonomics can be used both for the screen of biomarkers
97 and for drug discovery research and development. As a systems approach,
98 metabonomics can study the metabolic changes in response to drug exposure *in vivo*
99 and provide information on drug toxicity[29-33].
100
101 In this study, we aim to the potential toxicity of tTF-pHLIP fusion protein by
102 screening the metabolic effects induced by tTF-pHLIP. We employed C57BL/6J mouse
103 bearing B16F10 melanoma and then treated tumor-bearing mice with tTF-pHLIP. We
104 monitored the metabolic alteration after tTF-pHLIP treatment on the host metabolome
105 of plasma, urine and organ tissues, such as liver, spleen and kidney, using both targeted
106 and untargeted detecting techniques. Our investigation provides a holistic assessment
107 of the toxicity of tTF-pHLIP and demonstrates that metabonomics is a sensitive tool for
108 novel therapeutic based on tumor vasculature targeting.
109

110 **Materials and Methods**

111 **Chemicals**

112 Methanol, $K_2HPO_4 \cdot 3H_2O$, and $NaH_2PO_4 \cdot 2H_2O$ were purchased from Sinopharm
113 Chemical Reagent Co., Ltd (Shanghai, China), and Trimethylsilyl propanoic acid (TSP)
114 and deuterium oxide (D2O, 99.9% in D) were from Cambridge Isotope Laboratories,
115 Inc. (Miami, USA). HPLC grade methanol and acetonitrile were purchased from
116 TEDIA (Shanghai, China). The standards using for GC-MS and UPLC-MS were
117 purchased from Sigma-Aldrich (Shanghai, China).
118

119 **Synthesis and purification of tTF-pHLIP**

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

123 ACCCCGCTGCTGCTGCTGGACCTGGCTCTGCTGGTTGACGCTGACGAAGG
124 TACC, amino acid sequence:
125 AEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT) was linked to the C-
126 terminus of the extracellular domain of tissue factor (amino acids 1 to 218 of human
127 TF), which made the cDNA for tTF-pHLIP. We then cloned the cDNA into Nde I and
128 Xho I sites of the expression vector pET-30a (+). tTF-pHLIP was expressed in
129 competent *E. coli* (BL21DE3, Invitrogen, Carlsbad, CA) after vector introduction, and
130 then was purified and refolded with a His Bind Buffer Kit (BD Pharmingen, San Diego,
131 CA) according to the manufacturer's instructions.
132

133 **B16F10 melanoma cells culture**

134 B16-F10 melanoma cells were cultured in RPMI 1640 medium with 10% fetal bovine
135 serum at 37°C, 5% CO_2. B16-F10 cells were collected by dissociating the cells in
136 trypsin, washed and resuspended in PBS for tumor inoculation.
137

138 **Animal experiments and sample collection**

139 Four weeks old female C57BL/6J mice (N = 65) were purchased from Beijing Vital
140 River Laboratories and housed under specific pathogen-free conditions at Wuhan
141 Institute of Virology (Hubei, P. R. China). The experimental procedures were approved
142 by the ethics committee of Wuhan Institute of Physics and Mathematics (Hubei, P. R.
143 China, WIPM-A2017-01). Mice were randomly divided into five groups (each group
144 has 13 mice) and kept with 12 h light/dark cycles. After two weeks acclimatization,
145 three groups of mice were inoculated with tumor; one million B16F10 cells were
146 injected subcutaneously into the right flank of mice. Tumor size was measured by the
147 length and width of tumor with a vernier caliper and body weight was recorded every
148 two days. One week later, when the tumor grew to a volume of approximately 100 mm^3
149 (Volume = (length*$width^2$)/2), the three groups of mice with tumor were treated with
150 saline (Tumor 0), 2.5 μg tTF-pHLIP per mouse (Tumor 2.5) and 5 μg tTF-pHLIP per
151 mouse (Tumor 5) by tail vein injection. The two groups of mice without tumor were
152 treated with saline (Normal) and 2.5 μg tTF-pHLIP per mouse (Normal 2.5). tTF-pHLIP
153 or saline was administrated every two days for a total of 4 times (day 1, 3, 5, 7).
154

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

160 procedures. All samples were stored at -80°C following immediately frozen with liquid
161 nitrogen.
162
163 **Histopathological analysis**
164 Tissues from liver, spleen and kidney were embedded in paraffin blocks. The block was
165 sectioned into 5-μm thick sections and stained with hematoxylin and eosin (H&E) for
166 pathological examination.
167
168 **NMR sample preparation and NMR spectroscopy**
169 For plasma preparation, 30 μL of plasma was mixed with 25 μL phosphate buffer
170 (45mM) and transferred 50 μL of supernatant into a 1.7 mm NMR tube. Urine samples
171 were prepared according to the literature[34]. Briefly, 60 μL urine and 4.8 μL potassium
172 fluoride buffer (5 M) were mixed with 440 μL ultra-pure water (containing 20% D_2O,
173 v/v). The mixture was centrifuged (15,930 g, 4°C, 10 min) after vertexing and standing
174 at 25°C for 10 min. Then 450 μL supernatant was mixed with 6 μL EDTA-d12 (0.1 M)
175 and 45 μL phosphate buffer (1.5 M), and transferred to 5 mm NMR tube.
176
177 50 mg of organ tissues were extracted with 0.6 mL 66.6% cold methanol aqueous
178 solution for 3 times. Briefly, the organ tissues were homogenized with a tissue-lyser
179 (QIAGEN, Germany) and collected the supernatants after centrifugation (11,060 g, 4 °C,
180 10 min). Supernatants from the three extraction procedures were lyophilized following
181 methanol removal *in vacuo*. Then the resulted powder was dissolved in 600 μL
182 phosphate buffer (0.15 M), centrifuged and transferred 550 μL of supernatants into a 5
183 mm NMR tube for NMR analysis.
184
185 NMR spectra were collected on a Bruker AVANCE III 600 MHz NMR
186 spectrometer equipped with a cryogenic probe (Bruker Biospin, Germany) at 298 K.
187 Each spectrum was recorded with 128 transients, 32K data points and 20-ppm spectral
188 width. ^{1}H NMR spectrum for urine and tissue extracts was acquired with a standard
189 NOESYGPPR1D pulse sequence (RD-G1-90°-t1-90°-tm-G2-90°-acquisition; t1 = 4μs,
190 tm = 80 ms). The spectrum of plasma was obtained with a Carr-Purcell-Meiboom- Gill
191 sequence ((RD-90°-(τ-180°-τ) n-acquisition); τ = 350 μs, n = 100) Two-dimensional
192 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-
193 ^{13}C HSQC of selective samples were acquired for metabolites identification[35, 36].
194
195 **Amino containing metabolites extraction and detection**
196 Amino containing metabolites were quantified using an Agilent UHPLC-MS/MS 1290-

197 6460 system (Agilent Technologies, USA). Amino containing metabolites were
198 extracted before derivation with 5-aminoisoquinolyl-*N*-hydroxysuccinimidylcarbamate
199 (5-AIQC)[37, 38]. 20 μL plasma was mixing with 60 μL methanol and centrifuged to obtain
200 10 μL supernatants. 50 mg of liver tissue was frozen and thawed for three cycles in 600
201 μL 66.6% methanol aqueous solution followed by 1 min ultra-sonication and 1 min
202 pause for 8 cycles. The supernatants were collected after centrifugation and diluted with
203 equal amount of 0.1% formic acid aqueous solution. Supernatants (10 μL) from plasma
204 and liver extraction was mixed with 80 μL of 2.5 mM *N*-ethylmaleimide solution for 1
205 min. 10 μL of 1 M 4-tert-butylbenzenethiol DMSO solution was added followed by the
206 addition of 700 μL of 20 mM tris (2-carboxyethyl) phosphine hydrochloride (borate
207 buffer 0.2 M, pH 8.8). 200 μL 5-AIQC solution was then added into the extracted
208 solution after vortex-mixing and 2 min of standing, and followed by incubation at 55 °C
209 for 10 min. The mixture was added with 10 μL formic acid and centrifuged after cooling
210 down to 25 °C. The final supernatants were filtered via a 0.22 μm filter for UHPLC-
211 MS/MS analysis.
212
213 1 μL of 5-AIQC-tagged sample was injected into a column (C18, 2.1 × 100 mm,
214 1.8 μm, Agilent Technologies, USA). Mobile phase A (ultrapure water) and B
215 (methanol, containing 0.1% (v/v) formic acid) were used and 0.6 mL/min flow rate and
216 50°C column temperature was maintained. Amino containing metabolites were
217 acquired in the positive ion mode and were quantified using MassHunter Workstation
218 software by external standards method.
219
220 **Fatty acids composition analysis**
221 Fatty acids composition of liver and plasma were determined using an improved
222 method based on a previous report[39]. 10 mg of liver tissues was homogenized with 500
223 μL methanol. 20 μL internal standards (1 mg/mL methyl heptadecanoate, 0.5 mg/mL
224 methyl tricosanoate and 2 mg/mL butylated hydroxytoluene) was mixed with 100 μL
225 liver homogenate or 30 μL plasma and then mixed with 1 mL methanol-hexane solution
226 (80% methanol). The mixture reacted with 100 μL of precooled acetyl chloride at 25 °C
227 in the dark. 24 h later, the mixture was neutralized with K_2CO_3 solution and extracted
228 three times with 200 μL hexane. The supernatants were volatilized to dryness followed
229 and dissolved in hexane (100 μL for liver extracts and 50 μL for plasma extracts). The
230 fatty acids detection was performed on a Shimadzu GC 2010 Plus GC-MS spectrometer
231 (Shimadzu, Japan).
232
233 **Quantitative PCR analysis**

234 Total mRNA in the liver tissue was extracted using RNAiso Plus kit (Takara, Japan)
235 and cDNA were obtained using a reverse transcription kit (Catalog number: AT341-02,
236 TransGen, China). mRNA levels were quantified with PowerUp™ SYBR™ Green
237 Master Mix (Applied Biosystems) on a 7900HT Fast Real-time PCR System (Applied
238 Biosystems). Gapdh was applied as an internal control. The primers sequences were
239 listed in **Table S1**.
240

241 **Data processing and analysis**

242 NMR data were processed with Topspin (V3.6.0, Bruker Biospin, Germany) and
243 Mestrenova (V9.0, Mestrelab Research, Spain). In short, the free induction decay was
244 Fourier transformed with an exponential window function by multiplying a 0.5 Hz line-
245 broadening factor. All the spectra were calibrated to TSP except spectra of plasma,
246 which were calibrated to the anomeric proton of glucose[40, 41]. The calibrated spectra
247 were then integrated using equidistant binning with a width of 0.002 ppm after phase
248 and baseline correction. Water signals from δ4.7 to δ5.1 and urea signals from δ5.3 to
249 δ6.2 were excluded. The data were normalized according to the types of sample[42]:
250 urinary data were normalized to the total area, organ tissue data to the wet weight and
251 plasma without normalization.
252

253 Multivariate data analysis was performed with SIMCA-P+ (V13.0, Umetrics AB,
254 Umea, Sweden). Principal component analysis (PCA) was applied with unit variance
255 scale to detect potential outliers[43]. Orthogonal Projection to Latent Discriminant
256 Analysis (OPLS-DA) models were constructed with unit variance scale. All OPLS-DA
257 models were validated by both cross-validation[44] and CV-ANOVA method.
258

259 The levels of amino metabolites and fatty acids were quantified based on peak
260 integrations from raw UHPLC-MS/MS and GC data using calibration curves from
261 respective standards and further were analyzed using univariate data analysis after
262 normalization to wet tissue weight (organ tissue) and volume (plasma). Student's t-test
263 and non-parameters tests were applied, metabolites with $p < 0.05$ were regarded as
264 significant different metabolites. Pathway analysis was performed with Metaboanalyst
265 4.0 (http://www.metaboanalyst.ca)[45].
266

267 **Results**

268 **Histopathological analysis of major organs after tTF-pHLIP treatment**

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

271 the normal mice (**Figure S1**).
272
273 **^{1}H NMR spectroscopy of biofluids and tissue extracts**
274 Assignment of typical ^{1}H NMR spectra of the liver, plasma, tumor, kidney and urine
275 from tumor bearing mice are illustrated in Table S2. The resonance peaks were assigned
276 to metabolites according to the literature[29, 32, 36, 46], the Human Metabolome Data Base[47]
277 and further confirmed by two-dimensional NMR experiments. The plasma spectra are
278 mainly constituted of lipid, glucose, amino acids and choline metabolites (choline, O-
279 PC, GPC) (**Table S2**). The spectra of liver, kidney and spleen extracts included glucose,
280 amino acids, carbohydrates, choline metabolites and several nucleotides (**Table S2**).
281 While the spectra of urine mainly included TCA intermediates (succinate, citrate,
282 fumarate, α-ketoglutarate, cis-aconitate and malate), amino acids, organic acids, urea
283 and allantoin.
284
285 **Metabolic abnormality induced by tumor burden**
286 NMR metabolic profiles of urine, plasma, organ tissue extracts obtained from tumor
287 bearing mice were compared to relevant tissue extracts from untreated mice by OPLS-
288 DA models. The models were subsequently validated and the results suggested that the
289 metabolome of urine, plasma, liver, kidney and spleen from mice bearing with tumor
290 differed significantly from those of normal mice (**Figure 1** and **Figure S2**).
291
292 Tumor-bearing induced metabolic alterations in liver, kidney and spleen (**Figure**
293 **1A-B**), such as, the decrease in the levels of most amino acids, acetate, GABA, choline,
294 ethanolamine, hypoxanthine, xanthine and uracil and increase of inosine and UMP.
295 However, the tumor induced metabolic alterations also varied between different organ
296 tissues. The levels of succinate and sn-glycero-3-phosphocholine were upregulated in
297 both liver and kidney of tumor bearing mice. Increased levels of glutathione and
298 decreased levels of uridine and formate were observed in both liver and spleen. The
299 levels of glucose, mannose, malonate, taurine, creatine, glycerol and nicotinurate were
300 decreased in both kidney and spleen. In addition, tumor exhibited a specific influence
301 on liver metabolism such as increased levels of glutamine, AMP, β-alanine, UDP-
302 glucose and lactate and decreased levels of glycogen and fumarate. Tumor also caused
303 decreased levels of lactate and increased levels of fumarate and uridine in the kidney.
304 In the spleen, cytidine was downregulated in tumor bearing mice. Significant increase
305 of lipids, triglyceride, lactate, glycerol, alanine and GPC and decrease of tyrosine and
306 glucose were noticed in the plasma of tumor bearing mice compared to those of normal
307 mice (**Figure 1C**). Tumor also induced changes in urinary metabolic profiles and these

308 changes included increased levels of lactate, 3-indoxylsulfate and TCA cycle
309 intermediates (2-oxoglutarate, succinate, citrate) and decreased levels of betaine,
310 taurine, hippurate and N, N-dimethylglycine (**Figure 1C**).
311
312 **Therapeutic effects of tTF-pHLIP**
313 Tumor size and body weight were monitored every other day for tumor bearing mice
314 treated with and without tTF-pHLIP (**Figure 2A** and **Figure S3A-B**). Clearly, tTF-
315 pHLIP treatment resulted in significant reductions in tumor growth from four days post-
316 treatment onward for both low (2.5 μg) and high (5.0 μg) dose levels. In addition, no
317 significant effect on tumor growth was observed between low and high dosage level
318 treatments. Consistent with this observation, the tumor weight obtained at the end point
319 of the experiment showed a significant reduction in tTF-pHLIP treated groups with
320 again no distinction between low and high dose levels observed in tumor weight
321 (**Figure S3A**). No significant change in body weight was found between tumor bearing
322 group and those treated with a high or low dose of tTF-pHLIP (**Figure S3B**).
323 tTF-pHLIP administration demonstrated the capability of counteracting the
324 metabolic disruptions induced by tumor bearing. In urine metabolome, energy-related
325 metabolites in tumor bearing mice treated with tTF-pHLIP recovered to the levels of
326 the normal mice after treatment (**Figure 2B-D**), for example, the urinary levels of
327 fumarate, 2-oxoglutarate and citrate. However, the levels of 3-indoxyl sufate, TMAO
328 and lactate were not recovered to the levels of the normal control mice (**Figure S3C-**
329 **E**). The circulation fatty acids in tumor bearing mice treated with tTF-pHLIP showed a
330 trend of recovery, for example, the levels of C16:1, C18:0 and C20:4n6 of low dose
331 treated mice. However, not all the levels of fatty acids returned to the levels of normal
332 mice (**Figure 2E-F**). We also examined key enzymes of fatty acid metabolism in the
333 liver. We have shown that the mRNA levels of *Fasn* and *Acly* involved in *de novo* fatty
334 acids synthesis in the liver were increased after tumor bearing while reduced to normal
335 levels after tTF-pHLIP treatment. However, the lipid catabolism enzymes were
336 decreased in the liver of tumor bearing mice regardless of tTF-pHLIP treatment when
337 compared to normal mice (**Figure 2G**). The activity of desaturases was inhibited in
338 tumor group after tTF-pHLIP administration (**Figure 2H**). No counteractive effect of
339 tTF-pHLIP treatment on the metabolomes of liver, spleen and kidney was observed
340 (**Figure 3**, **Figure S4** and **Figure S5**) However, compared to tumor bearing group,
341 treatment with tTF-pHLIP induced decreases in the levels of glucose, alanine,
342 glutamine, succinate, fumarate, malate and GSSG and increases in the levels of
343 phosphocholine, choline, betaine, inosine, sarcosine and hypoxanthine in liver (**Figure**
344 **3A-D**). The mRNA levels involved in steatolysis, GSH metabolism, pentose phosphate

345 pathway, glutamine metabolism, TCA cycle and choline metabolism were all decreased
346 in tumor bearing mice (**Figure 3E-G**). However, compared to tumor bearing mice, tTF-
347 pHLIP induced an increase in the expression levels of *Gss* and *Pcyt1a* (**Figure 3F-G**).
348 The significant changed metabolites and enzymes induced by tumor or after tTF-pHLIP
349 treatment were mapped in the detailed pathways involved in Warburg effect, TCA
350 cycles, lipids metabolism, choline metabolism, GSH metabolism and nucleotide
351 metabolism (**Figure 4**).
352

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

355 The metabolic differences between normal mice treated with and without tTF-pHLIP
356 were compared. Levels of glucose, fumarate, malate, betaine, xanthine, uracil, and
357 phosphocholine were increased whereas the levels of 3-hydroxybutyrate, asparagine,
358 tyrosine, serine, hypotaurine and 2-aminoadipic acid were decreased in the liver of tTF-
359 pHLIP treated mice comparing to those of untreated mice (**Figure 5A**). The decrease
360 of glutamate and *myo*-inositol were found in the spleen of tTF-pHLIP treated mice
361 (**Figure 5B**). The levels of leucine, isoleucine, valine, 3-hydroxybutyrate, alanine,
362 acetate, lysine, creatine, carnitine, *myo*-inositol, mannose and phenylalanine were
363 reduced while the levels of betaine were elevated in the kidney of normal mice treated
364 with tTF-pHLIP when comparing to the kidney of untreated mice (**Figure 5C**). Plasma
365 levels of C16:0, C16:1, C18:0 and C18:2n6c were decreased in tTF-pHLIP treated mice
366 (**Figure 5D**).
367

368 **Discussion**

369 Tumor vessel targeted therapies become promising strategies because of high selectivity,
370 low toxicity and good efficacy. One of the examples is the pH-sensitive fusion protein
371 tTF-pHLIP. This targeting protein selectively induced thrombus at tumor sites and
372 blocked the nutrients supply for tumor, leading to tumor shrinkage[12]. In our previous
373 study, we have demonstrated that tTF-pHLIP can successfully induce tumor to shrink
374 in size[18]. Currently, we showed that injection of 2.5 μg tTF-pHLIP per mouse for four
375 times in total was capable of substantially inhibiting tumor growth, while a higher dose
376 (5.0 μg) had no further improvement in tumor inhibition (**Figure 2A**). In addition, no
377 difference in body weight was observed between the tTF-pHLIP exposed groups and
378 their negative tumor bearing controls (**Figure S3B**). Furthermore, no histological
379 abnormality was observed in the organs of liver, kidney and spleen (**Figure S1**), neither
380 in normal or tumor bearing mice treated with tTF-pHLIP, implying low or no toxicity
381 observed. These results suggested again that tTF-pHLIP is effective in reducing tumor

382 growth and has great potential for further development for cancer treatment. In the
383 current study, we holistically evaluated the biological effects of tTF-pHLIP in
384 rebalancing metabolic abnormality induced by cancer and the potential toxicity
385 associated with tTF-pHLIP at the molecular level by employing metabolomics
386 techniques.
387
388 The most prominent metabolic derailment induced by tumor burden was the
389 increased output of urinary TCA cycle intermediates (**Figure 1C**) and we have
390 demonstrated that tTF-pHLIP treatment re-normalized this derailment (**Figure 2B-D**
391 and **Figure 4**). Energy metabolic disorder is common phenomena associated with tumor
392 progression and this is attributed to the increased energy demands for tumor growth.
393 The re-balancing of the levels of TCA cycle intermediates and enzymes in the post-
394 treatment mice is therefore a metabolic reflection of the reduced tumor size observed
395 in mice treated with tTF-pHLIP (**Figure 2A** and **Figure 3E**). Energy disruption
396 associated with tumor growth also presents with rewired glycolysis, also known as
397 Warburg effect, and is the most important metabolic alteration which rapidly consumes
398 glucose and secret abundant lactate[48]. Our current study observed an elevated level of
399 lactate in plasma, urine and liver in tumor bearing mice, which were consistent with the
400 Warburg effect of tumors. However, tTF-pHLIP treatment was not able to revert the
401 Warburg effect associated with the tumor. We observed that tTF-pHLIP treatment can
402 re-normalize the tumor growth induced increases in the levels of C16:0, C18:0 and
403 C20:4n6 in plasma to the levels of control mice (**Figure 2E**). A previous study has
404 demonstrated alteration of the lipid metabolism associated with tumors. Specifically,
405 tumor growth induced downregulation of lipogenesis and upregulation of lipolysis
406 while the fatty acids *de novo* synthesis in tumors was enhanced for the membrane
407 synthesis[49]. We further calculated the levels of *Scd16* and *Scd18* that reflected the
408 activities of desaturases[50] and found that the desaturases were decreased in plasma of
409 mice exposed to tTF-pHLIP (**Figure 2H**). The mRNA results showed that the *de novo*
410 synthesis of fatty acids was increased in the liver of tumor bearing mice, which supports
411 the tumor fast growth. Surprisingly, the *Fasn* and *Acly* expression reduced to normal
412 levels after tTF-pHLIP treatment, suggesting that the treatment of tTF-pHLIP is able to
413 inhibit *de novo* synthesis of lipids associated with cancer growth. The fact that tTF-
414 pHLIP could counteract alterations of lipid metabolism indicated the mechanism of
415 tTF-pHLIP in inhibiting cancer growth may be via reprogramming lipid metabolism.
416 Further validation is necessary to confirm this notion.
417
418 We failed to note the re-normalization capability of tTF-pHLIP for the amelioration

419 of tumor-induced metabolic alterations in the organ tissues (**Figure 3, Figure S4 and**
420 **Figure S5**). Having said that, we noticed that tTF-pHLIP exposure to tumor bearing
421 mice induced decreased levels of glucose, accompanied by the reduced levels of TCA
422 cycle intermediates (**Figure 3A**) in the liver compared to the tumor bearing mice
423 without tTF-pHLIP treatment, implying high energy demands associated with tTF-
424 pHLIP treatment. The high energy consumption could contribute to the blood clotting
425 activity as tTF-pHLIP initiates the coagulation cascades and the clearance of formed
426 thrombus.
427
428 Another important impact of tTF-pHLIP exposure is initiation of oxidative stress,
429 which was manifested by the depleted levels of GSH and increased ratios of
430 GSSG/GSH (**Figure 3B**) and further confirmed by the expression of GSH synthetase:
431 *Gss* was increased after tTF-pHLIP exposure, implying the increased demands for GSH
432 (**Figure 3G** and **Figure 4B**). This notion is consistent with the previous finding showing
433 that the coagulation factors caused inflammation[51, 52] and the oxidation of GSH could
434 counteract this process. Furthermore, the increased betaine may also be responsible for
435 the antioxidant activity. Betaine can exert its antioxidant activity in two ways: one is
436 improving the ROS-scavenging ability of the methionine sulfoxide reductase
437 antioxidant system, and the other is to keep the free radicals away from the membrane
438 by forming a protective membrane with an electronegative outer surface around cells[53].
439 In addition, phosphatidylethanolamine (PE) methylation has been shown to be critical
440 for protecting cells against oxidative stress by generating GSH[54]. PC can be synthesized
441 from PE through methylation[55]. In the current investigation, we observed significant
442 decreases in the levels of ethanolamine and the concurrent increase in the levels of
443 phosphocholine, choline and inosine (**Figure 3A** and **Figure 3C**). *Pcyt1a*, which is the
444 key enzyme for phosphocholine synthesis, was increased in tumor bearing mice after
445 tTF-pHLIP treatment compared to untreated tumor bearing mice (**Figure 3F**). Taking
446 these together, we speculate that tTF-pHLIP induces the enhanced PE methylation,
447 which is in concordance with oxidative stress associated with tTF-pHLIP exposure.
448
449 Finally, we investigated the effects of tTF-pHLIP on normal C57 mice. tTF-pHLIP
450 induced slight perturbations on kidney and liver metabolism, but not spleen, urine or
451 plasma (**Figure 5**). A previous study showed that pHLIP accumulated in kidney, which
452 could cause the observed changes in the levels of several amino acids. While in the liver,
453 the glucose, nucleotides and energy metabolism were altered. However, compared to
454 the alteration induced by tumor or tumor treated by tTF-pHLIP, these changes were
455 negligible.

456

457 **Conclusions**

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

473

474 **Associated content**

475 **Supporting information**

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

481

482 **Author information**

483 **Corresponding Author**

484 * E-mail: yulan.wang@ntu.edu.sg; Fax: +65-65150417, Tel.: +65-69041106.

485 **Notes**

486 The authors declare no competing financial interest.

487

488 **Acknowledgements**

489 We acknowledge financial support from the National Natural Science Foundation of
490 China (21675169). We acknowledge the help of Professor Johan Trygg for MVA data
491 analysis.

492

493 **References**

494 (1) Hanahan, D.; Weinberg, R. A., The hallmarks of cancer. *Cell* **2000,** *100* (1), 57-70.
495 (2) Ferrara, N.; Kerbel, R. S., Angiogenesis as a therapeutic target. *Nature* **2005,** *438*
496 (7070), 967-74.
497 (3) Jahanban-Esfahlan, R.; Seidi, K.; Zarghami, N., Tumor vascular infarction:
498 prospects and challenges. *Int. J. Hematol.* **2017,** *105* (3), 244-256.
499 (4) Fujita, K.; Sano, D.; Kimura, M.; Yamashita, Y.; Kawakami, M.; Ishiguro, Y.;
500 Nishimura, G.; Matsuda, H.; Tsukuda, M., Anti-tumor effects of bevacizumab in
501 combination with paclitaxel on head and neck squamous cell carcinoma. *Oncol Rep.*
502 **2007,** *18* (1), 47-51.
503 (5) Ran, S.; Gao, B.; Duffy, S.; Watkins, L.; Rote, N.; Thorpe, P. E., Infarction of solid
504 Hodgkin's tumors in mice by antibody-directed targeting of tissue factor to tumor
505 vasculature. *Cancer Res.* **1998,** *58* (20), 4646-53.
506 (6) Kessler, T.; Bieker, R.; Padro, T.; Schwoppe, C.; Persigehl, T.; Bremer, C.; Kreuter,
507 M.; Berdel, W. E.; Mesters, R. M., Inhibition of tumor growth by RGD peptide-directed
508 delivery of truncated tissue factor to the tumor vasculature. *Clin. Cancer Res.* **2005,** *11*
509 (17), 6317-24.
510 (7) Bieker, R.; Kessler, T.; Schwoppe, C.; Padro, T.; Persigehl, T.; Bremer, C.;
511 Dreischaluck, J.; Kolkmeyer, A.; Heindel, W.; Mesters, R. M.; Berdel, W. E., Infarction
512 of tumor vessels by NGR-peptide-directed targeting of tissue factor: experimental
513 results and first-in-man experience. *Blood* **2009,** *113* (20), 5019-27.
514 (8) Shi, Q.; Zhang, Y.; Liu, S.; Liu, G.; Xu, J.; Zhao, X.; Anderson, G. J.; Nie, G.; Li,
515 S., Specific tissue factor delivery using a tumor-homing peptide for inducing tumor
516 infarction. *Biochem pharmacol* **2018,** *156*, 501-510.
517 (9) Huang, X.; Molema, G.; King, S.; Watkins, L.; Edgington, T. S.; Thorpe, P. E.,
518 Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor
519 vasculature. *Science* **1997,** *275* (5299), 547-50.
520 (10) Hu, P.; Yan, J.; Sharifi, J.; Bai, T.; Khawli, L. A.; Epstein, A. L., Comparison of
521 three different targeted tissue factor fusion proteins for inducing tumor vessel
522 thrombosis. *Cancer Res.* **2003,** *63* (16), 5046-53.
523 (11) Wickstrom, M.; Larsson, R.; Nygren, P.; Gullbo, J., Aminopeptidase N (CD13) as
524 a target for cancer chemotherapy. *Cancer Sci.* **2011,** *102* (3), 501-8.
525 (12) Heiden, M. G. V.; Cantley, L. C.; Thompson, C. B., Understanding the Warburg
526 effect: The metabolic requirements of cell proliferation. *Science* **2009,** *324* (5930),
527 1029-1033.
528 (13) Huber, V.; Camisaschi, C.; Berzi, A.; Ferro, S.; Lugini, L.; Triulzi, T.; Tuccitto, A.;
529 Tagliabue, E.; Castelli, C.; Rivoltini, L., Cancer acidity: An ultimate frontier of tumor
530 immune escape and a novel target of immunomodulation. *Semin. Cancer Biol.* **2017,**
531 *43*, 74-89.
532 (14) Hunt, J. F.; Earnest, T. N.; Bousche, O.; Kalghatgi, K.; Reilly, K.; Horvath, C.;
533 Rothschild, K. J.; Engelman, D. M., A biophysical study of integral membrane protein
534 folding. *Biochemistry* **1997,** *36* (49), 15156-76.

535 (15) Andreev, O. A.; Engelman, D. M.; Reshetnyak, Y. K., pH-sensitive membrane
536 peptides (pHLIPs) as a novel class of delivery agents. *Mol. Membr. Biol.* **2010,** *27* (7),
537 341-52.
538 (16) Andreev, O. A.; Engelman, D. M.; Reshetnyak, Y. K., Targeting diseased tissues
539 by pHLIP insertion at low cell surface pH. *Frontiers in physiology* **2014,** *5*, 97.
540 (17) Adochite, R. C.; Moshnikova, A.; Golijanin, J.; Andreev, O. A.; Katenka, N. V.;
541 Reshetnyak, Y. K., Comparative study of tumor targeting and biodistribution of pH
542 (Low) Insertion Peptides (pHLIP((R)) Peptides) conjugated with different fluorescent
543 dyes. *Mol. Imaging Bio.* **2016,** *18* (5), 686-96.
544 (18) Li, S. P.; Tian, Y. H.; Zhao, Y.; Zhang, Y. L.; Su, S. S.; Wang, J.; Wu, M. Y.; Shi,
545 Q. W.; Anderson, G. J.; Thomsen, J.; Zhao, R. F.; Ji, T. J.; Wang, J.; Nie, G. J., pHLIP-
546 mediated targeting of truncated tissue factor to tumor vessels causes vascular occlusion
547 and impairs tumor growth. *Oncotarget* **2015,** *6* (27), 23523-23532.
548 (19) Andreev, O. A.; Dupuy, A. D.; Segala, M.; Sandugu, S.; Serra, D. A.; Chichester,
549 C. O.; Engelman, D. M.; Reshetnyak, Y. K., Mechanism and uses of a membrane
550 peptide that targets tumors and other acidic tissues in vivo. *Proc. Natl. Acad. Sci. U. S.*
551 *A.* **2007,** *104* (19), 7893-8.
552 (20) Davalos, D.; Akassoglou, K., Fibrinogen as a key regulator of inflammation in
553 disease. *Semin. Immunopathol.* **2012,** *34* (1), 43-62.
554 (21) Witkowski, M.; Landmesser, U.; Rauch, U., Tissue factor as a link between
555 inflammation and coagulation. *Trends Cardiovasc. Med.* **2016,** *26* (4), 297-303.
556 (22) Wishart, D. S., Is cancer a genetic disease or a metabolic disease? *EBioMedicine*
557 **2015,** *2* (6), 478-9.
558 (23) Hanahan, D.; Weinberg, R. A., Hallmarks of cancer: the next generation. *Cell* **2011,**
559 *144* (5), 646-74.
560 (24) Currie, E.; Schulze, A.; Zechner, R.; Walther, T. C.; Farese, R. V., Jr., Cellular fatty
561 acid metabolism and cancer. *Cell Metab.* **2013,** *18* (2), 153-61.
562 (25) Das, S. K.; Eder, S.; Schauer, S.; Diwoky, C.; Temmel, H.; Guertl, B.; Gorkiewicz,
563 G.; Tamilarasan, K. P.; Kumari, P.; Trauner, M.; Zimmermann, R.; Vesely, P.;
564 Haemmerle, G.; Zechner, R.; Hoefler, G., Adipose triglyceride lipase contributes to
565 cancer-associated cachexia. *Science* **2011,** *333* (6039), 233-8.
566 (26) Dalal, S., Lipid metabolism in cancer cachexia. *Annals of Palliative Medicine* **2019,**
567 *8* (1), 13-23.
568 (27) Waterhouse, C., Lactate metabolism in patients with cancer. *Cancer* **1974,** *33* (1),
569 66-71.
570 (28) Nicholson, J. K.; Lindon, J. C.; Holmes, E., 'Metabonomics': understanding the
571 metabolic responses of living systems to pathophysiological stimuli via multivariate
572 statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999,** *29* (11),
573 1181-1189.
574 (29) Nicholson, J. K.; J., C.; Lindon, J. C.; Holmes, E., Metabonomics: a platform for
575 studying drug toxicity and gene function. *Nature* **2002,** *1*, 9.
576 (30) Zhao, X. J.; Huang, C.; Lei, H.; Nie, X.; Tang, H.; Wang, Y., Dynamic metabolic

577 response of mice to acute mequindox exposure. *J. Proteome Res.* **2011,** *10* (11), 5183-
578 90.
579 (31) Jiang, L.; Huang, J.; Wang, Y.; Tang, H., Metabonomic analysis reveals the CCl4-
580 induced systems alterations for multiple rat organs. *J. Proteome Res.* **2012,** *11* (7), 3848-
581 59.
582 (32) Dong, F.; Zhang, L.; Hao, F.; Tang, H.; Wang, Y., Systemic responses of mice to
583 dextran sulfate sodium-induced acute ulcerative colitis using 1H NMR spectroscopy. *J.*
584 *Proteome Res.* **2013,** *12* (6), 2958-66.
585 (33) Zhang, L.; Wang, L.; Hu, Y.; Liu, Z.; Tian, Y.; Wu, X.; Zhao, Y.; Tang, H.; Chen,
586 C.; Wang, Y., Selective metabolic effects of gold nanorods on normal and cancer cells
587 and their application in anticancer drug screening. *Biomaterials* **2013,** *34* (29), 7117-
588 26.
589 (34) Jiang, L.; Huang, J.; Wang, Y.; Tang, H., Eliminating the dication-induced
590 intersample chemical-shift variations for NMR-based biofluid metabonomic analysis.
591 *Analyst* **2012,** *137* (18), 4209-19.
592 (35) Claus, S. P.; Tsang, T. M.; Wang, Y.; Cloarec, O.; Skordi, E.; Martin, F. P.; Rezzi,
593 S.; Ross, A.; Kochhar, S.; Holmes, E.; Nicholson, J. K., Systemic multicompartmental
594 effects of the gut microbiome on mouse metabolic phenotypes. *Mol. Syst. Biol.* **2008,**
595 *4*, 219.
596 (36) Dai, H.; Xiao, C.; Liu, H.; Hao, F.; Tang, H., Combined NMR and LC-DAD-MS
597 analysis reveals comprehensive metabonomic variations for three phenotypic cultivars
598 of Salvia Miltiorrhiza Bunge. *J. Proteome Res.* **2010,** *9* (3), 1565-78.
599 (37) Boughton, B. A.; Callahan, D. L.; Silva, C.; Bowne, J.; Nahid, A.; Rupasinghe, T.;
600 Tull, D. L.; McConville, M. J.; Bacic, A.; Roessner, U., Comprehensive profiling and
601 quantitation of amine group containing metabolites. *Anal. Chem.* **2011,** *83* (19), 7523-
602 7530.
603 (38) Wang, J.; Zhou, L.; Lei, H.; Hao, F.; Liu, X.; Wang, Y.; Tang, H., Simultaneous
604 quantification of amino metabolites in multiple metabolic pathways using ultra-high
605 performance liquid chromatography with tandem-mass spectrometry. *Sci. Rep.* **2017,** *7*
606 (1), 1423.
607 (39) Xu, Z. D.; Harvey, K.; Pavlina, T.; Dutot, G.; Zaloga, G.; Siddiqui, R., An
608 improved method for determining medium- and long-chain FAMEs using gas
609 chromatography. *Lipids* **2010,** *45* (2), 199-208.
610 (40) Farrant, R. D.; Lindon, J. C.; Nicholson, J. K., Internal Temperature calibration for
611 H-1-nmr spectroscopy studies of blood-plasma and other biofluids. *Nmr Biomed* **1994,**
612 *7* (5), 243-247.
613 (41) Nicholson, J. K.; Foxall, P. J.; Spraul, M.; Farrant, R. D.; Lindon, J. C., 750 MHz
614 1H and 1H-13C NMR spectroscopy of human blood plasma. *Anal. Chem.* **1995,** *67* (5),
615 793-811.
616 (42) Craig, A.; Cloarec, O.; Holmes, E.; Nicholson, J. K.; Lindon, J. C., Scaling and
617 normalization effects in NMR spectroscopic metabonomic data sets. *Anal. Chem.* **2006,**
618 *78* (7), 2262-7.

619 (43) Trygg, J.; Holmes, E.; Lundstedt, T., Chemometrics in metabonomics. *J. Proteome*
620 *Res.* **2007,** *6* (2), 469-79.
621 (44) Wold, S., Cross-validatory estimation of the number of components in factor and
622 principal components models. *Technometrics* **1978,** *20* (4), 397-405.
623 (45) Xia, J.; Wishart, D. S., Web-based inference of biological patterns, functions and
624 pathways from metabolomic data using MetaboAnalyst. *Nat. Protoc.* **2011,** *6* (6), 743-
625 60.
626 (46) Fan, W. M. T., Metabolite profiling by one- and two-dimensional NMR analysis
627 of complex mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996,** *28*, 161-219.
628 (47) Wishart, D. S.; Feunang, Y. D.; Marcu, A.; Guo, A. C.; Liang, K.; Vazquez-Fresno,
629 R.; Sajed, T.; Johnson, D.; Li, C. R.; Karu, N.; Sayeeda, Z.; Lo, E.; Assempour, N.;
630 Berjanskii, M.; Singhal, S.; Arndt, D.; Liang, Y. J.; Badran, H.; Grant, J.; Serra-Cayuela,
631 A.; Liu, Y. F.; Mandal, R.; Neveu, V.; Pon, A.; Knox, C.; Wilson, M.; Manach, C.;
632 Scalbert, A., HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res.*
633 **2018,** *46* (D1), D608-D617.
634 (48) Bancroft Brown, J.; Sriram, R.; VanCriekinge, M.; Delos Santos, R.; Sun, J.; Delos
635 Santos, J.; Tabatabai, Z. L.; Shinohara, K.; Nguyen, H.; Peehl, D. M.; Kurhanewicz, J.,
636 NMR quantification of lactate production and efflux and glutamate fractional
637 enrichment in living human prostate biopsies cultured with [1,6‐13C2]glucose. *Magn.*
638 *Reson. Med.* **2019,** *82* (2), 566-576.
639 (49) Beloribi-Djefaflia, S.; Vasseur, S.; Guillaumond, F., Lipid metabolic
640 reprogramming in cancer cells. *Oncogenesis* **2016,** *5*, e189.
641 (50) Attie, A. D.; Krauss, R. M.; Gray-Keller, M. P.; Brownlie, A.; Miyazaki, M.;
642 Kastelein, J. J.; Lusis, A. J.; Stalenhoef, A. F. H.; Stoehr, J. P.; Hayden, M. R.; Ntambi,
643 J. M., Relationship between stearoyl-CoA desaturase activity and plasma triglycerides
644 in human and mouse hypertriglyceridemia. *J. Lipid Res.* **2002,** *43* (11), 1899-1907.
645 (51) Busso, N.; Morard, C.; Salvi, R.; Peclat, V.; So, A., Role of the tissue factor
646 pathway in synovial inflammation. *Arthritis Rheum.* **2003,** *48* (3), 651-659.
647 (52) Tobaldini, L. Q.; Arantes, F. T.; Saraiva, S. D. S.; Mazetto, B. M.; Colella, M. P.;
648 de Paula, E. V.; Annichino-Bizzachi, J.; Orsi, F. A., Circulating levels of tissue factor
649 and the risk of thrombosis associated with antiphospholipid syndrome. *Thromb. Res.*
650 **2018,** *171*, 114-120.
651 (53) Zhang, M.; Zhang, H.; Li, H.; Lai, F.; Li, X.; Tang, Y.; Min, T.; Wu, H., Antioxidant
652 mechanism of betaine without free radical scavenging ability. *J. Agric. Food Chem.*
653 **2016***, 64* (42), 7921-7930.
654 (54) Ye, C.; Sutter, B. M.; Wang, Y.; Kuang, Z.; Tu, B. P., A metabolic function for
655 phospholipid and histone methylation. *Mol. Cell* **2017,** *66* (2), 180-193.
656 (55) Bremer, J.; Greenberg, D. M., Methyl transfering enzyme system of microsomes
657 in biosynthesis of lecithin(phosphatidylcholine). *Biochim. Biophys. Acta* **1961,** *46* (2),
658 205-216.
659
660

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.

697 **Figure 4 Pathway analysis of tumor effect and therapeutic effect of tTF-pHLIP.** A,
698 metabolic alterations induced by the tumor, normal vs. B16F10 tumor bearing mice. B,
699 therapeutic effect of tTF-pHLIP, tumor vs. tumor treatment (tumor 2.5 or tumor 5). Red
700 means increased levels of metabolites or mRNA while blue decreased. "+" means the
701 levels of metabolites or mRNA recovered to normal levels after tTF-pHLIP treatment.
702 Keys: Gn, glycogen, Glc, glucose, Lac, lactate, Suc, succinate, Hxan, Hypoxanthine,
703 TG, triglyceride.
704
705 **Figure 5 Metabolic disturbance induced by tTF-pHLIP on normal mice.**
706 Significant metabolites obtained from liver(A), spleen (B), kidney (C) and plasma (D).
707 $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ indicate levels of significance when normal
708 group treated with tTF-pHLIP compared with normal group.
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733

734 For TOC Only

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

737

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

744