MedChemComm

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: X. Ning, Y. Li, H. Qi, R. Li, Y. Jin, J. Liu and Y. Yin, *Med. Chem. Commun.*, 2018, DOI: 10.1039/C8MD00062J.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/medchemcomm

View Article Online
DOI: 10.1039/C8MD00062J

Anti-cancer effect of a novel 2, 3-didithiocarbamate substituted naphthoquinone as a tumor metabolic suppressor in vitro and vivo

Xianling Ning[a], Yunqiao Li[a, c], Hailong Qi[a, b], Ridong Li[a], Yan Jin[a], Junyi Liu[d*] and Yuxin Yin[a, b, c *]

[a]*Institute of Systems Biomedicine, School of Basic Medical Sciences, Beijing Key Laboratory of Tumor Systems Biology, Peking University Health Science Center, Beijing, China.*

[b]*Peking-Tsinghua Center for Life Sciences, Peking University Health Science Center, Beijing, China.*

[c]*Department of Pathology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China.*

[d]*State Key Laboratory of Natural and Biomimetic Drugs, Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing, China.*

ABSTRACT

Tumor cells reprogram their cellular metabolism by switching from oxidative phosphorylation to aerobic glycolysis to support cell aberrant proliferation. Suppressing tumor cell metabolism has become an attractive strategy for treating cancer patients. In this study, we identified a 2, 3-didithiocarbamate substituted naphthoquinone **3i** that inhibited tumor cells proliferation by disturbing their metabolism. Compound **3i** reduced cancer cell viability with IC_{50} values from 50 nM to 150 nM against HCT116, MCF7, MDA-MB231, Hela, H1299 and B16 cells. Further, compound **3i** was found to suppress ATP production in cultured cancer cells, and inhibit M2 isoform of pyruvate kinase (PKM2) that is a rate-limiting enzyme in glycolytic pathway and block the subsequent transcription of the downstream gene GLUT1, LDH and CCND1. In addition, exposure to compound **3i** significantly suppressed tumor growth in a B16 melanoma transplantation mouse model and a spontaneous breast carcinoma mouse model in vivo. The identification of compound **3i** as a tumor metabolic suppressor not only offers a candidate compound for cancer therapy, but also provides a tool for an in depth study of tumor metabolism.

Key words

Metabolic suppressor, Compound **3i**, Antiproliferative activity, Suppressing tumor growth

1. Introduction

Cancer cells differ from most normal cells in metabolism. The normal cells rely generally on mitochondrial oxidative phosphorylation to generate energy from

* Corresponding author
Email address: jyliu@bjmu.edu.cn
* Corresponding author
Email address: yinyuxin@hsc.pku.edu.cn

View Article Online
DOI: 10.1039/C8MD00062J

glucose, whereas cancer cells instead rely on glycolysis. [1-4]. This difference suggests that targeting tumor metabolism could be a selective approach to suppress tumor growth.[5-7] Glucose goes through a series of biochemical transformations with production of ATP in the process of glycolysis, and each step is catalyzed by specific enzymes.[5] Influencing these enzymes to reduce or reverse the abnormal reprogrammed metabolism of cancer cells is an attractive therapeutic strategy for cancer patients. The pyruvate kinase (PK) is a rate-limiting enzyme that regulates the final step in glycolysis and catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to yield pyruvate and adenosine triphosphate (ATP)[8, 9]. There are four isoforms of PK (M1, M2, L and R) in mammalian cells: the M1 isoform (PKM1) is expressed in many differentiated tissues, PKM2 is expressed during embryonic development and over expressed in tumor tissues, PKL and PKR are expressed in liver and erythrocytes, respectively [10-12]. Many studies have shown that tumorigenesis is connected with the re-expression of PKM2 together with a down-regulation of the expression of PKM1 and other isozymes.[9, 13] In addition, PKM2 activates β-catenin to induce CCDN1 and c-Myc expression and upregulate GLUT1 and lactate dehydrogenase A (LDHA)[14-17]. Upregulation of these glycolysis genes increases glucose intake, consumption and lactate production to promote tumorigenesis [1].

We previously synthesized a series of dithiocarbamate substituted naphthoquinone derivatives and evaluated their anti-proliferative effects in the cellular level and PKM2 inhibition activity in enzyme level.[18, 19] To further improve the activity, we continue to optimize the structure. In this study, we synthesized a new 2, 3-didithiocarbamate substituted naphthoquinone compound **3i** (Fig. **1A**), which exhibited more potent anti-proliferative effect than previously synthesized compounds. It reduced cancer cell viability with IC_{50} values from 50 nM to 100 nM against HCT116, MCF7, MDA-MB231, Hela, H1299 and B16 cells. We also found that compound **3i** suppressed tumor cells metabolism by reducing ATP production in cancer cells, and inhibiting PKM2 activity and blocking the subsequent transcription of the downstream gene GLUT1, LDH and CCND1. In addition, exposure to compound **3i** significantly suppressed tumor growth in a B16 melanoma transplantation mouse model and a spontaneous breast carcinoma mouse model in vivo.

2. Materials and methods

*2.1 Synthesis of compound **3i***

2.1.1 Procedure for preparation of 2,3-bis-chloromethyl-[1,4]naphthoquinone

The 1,4-naphthaquinone (1 g, 6.3 mmol) in glacial acetic acid (20 mL) was taken in a 100 mL round-bottomed flask, and 36% aqueous formaldehyde (6 mL) was added. The reaction solution was cooled in ice-water. Dry hydrogen chloride passed in for 2 h. The solution became red, then being kept at room temperature for 48 h. The reaction mixture was poured on ice and extracted with ethyl acetate. The combined organic fractions were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification of the crude residue by column chromatography

View Article Online
DOI: 10.1039/C8MD00062J

(petroleum ether/ethyl acetate) afforded the title compound (yellow solid). The yield of this reaction was 68.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18-8.20 (m, 2H, Ar*H*), 7.81-7.83 (m, 2H, Ar*H*), 4.72 (s, 4H, 2CH_2Cl).

*2.1.2 Procedure for preparation of dipiperidine-dithiocarbamic acid 3-dipiperidinethiocarbamoylsulfanylmethyl-1,4-dioxo-1,4-dihydro-naphthalen-2-ylmethyl ester (**3i**)*

Carbon disulfide (180 μL, 3 mmol) and piperidine (297 μL, 3 mmol) were added to CH_3CN (5 mL) and the resulting solution was stired for 30 minutes. 2, 3-Bis-chloromethyl-[1,4]naphthoquinone (254 mg, 1 mmol) was added in portions at frequent intervals. Then the reaction mixture was kept at room temperature for 48 h. The reaction mixture was concentrated in vacuo, diluted with H_2O, and extracted with CH_2Cl_2. The combined organic fractions were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification of the crude residue by column chromatography (petroleum ether/ CH_2Cl_2) afforded compound **3i** (yellow solid). The yield of this reaction was 83.5%. Mp 142-143 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11-8.13 (m, 2H, Ar*H*), 7.73-7.75 (m, 2H, Ar*H*), 4.86 (s, 4H, 2CH_2S), 4.29 (q, 4H, 2NCH_2), 3.87 (q, 4H, 2NCH_2), 1.70 (m, 12H, 2CH_2CH_2CH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.21, 183.95, 143.97, 133.84, 132.05, 126.65, 34.11, 24.25. HR-MS (ESI$^+$) m/z: 505.1112 $[M+H]^+$, 527.0931$[M+Na]^+$. Found: 505.1100 $[M+H]^+$, 527.0903 $[M+Na]^+$.

2.2 Cell culture

Cell lines were grown with routine culture techinques in RPMI 1640 supplemented with 9% fetal bovine serum at 37°C in 5% CO_2.

2.3 MTS cell proliferation assay

Cells were plated in 96-well plates at a density of 5000-10000 cells per well. 12 h after seeding, cells were treated with various concentrations of test compounds for 48 h. Cell viability was assessed with the MTS assay (Promega) according to the manufacturer's instruction.

2.4 Measurement of ATP

HCT116 cells were seeded into 6-well plates at a density of 5×10^5 cells per well. 24 h after seeding, cells were treated with 10 μM or 20 μM compound **3i** for 6 h. ATP levels were measured using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega).

2.5 PKM2 activity assay

Pyruvate kinase activity was measured with a fluorescent pyruvate kinase-lactate dehydrogenase coupled assay as previously described [20]. To evaluate PKM2 activity in cell lysates, HCT116 cells were treated with various concentrations of compound **3i** for 8 h and lysed in NP40 lysis buffer immediately before measuring pyruvate kinase activity as described previously [21].

2.6 Quantitative real-time PCR (RT-qPCR)

HCT116 cells were seeded into 6-well plates at a density of 5×10^5 cells per well. 24 h after seeding, cells were treated with 20 μM compound **3i** for 8 h. Total RNA was extracted using TRIzol (Invitrogen). cDNA synthesis was carried out using a cDNA synthesis kit (Tansgene). qPCR was then carried out using SYBR Green Master Mix

View Article Online
DOI: 10.1039/C8MD00062J

(Transgene) in a bio-red Real-time PCR machine. PCR primers sequences used are listed in Supplementary Table S1.

2.7 B16 melanoma transplantation mouse model

Female C57BL/6 mice were injected with 1×10^6 B16 cells subcutaneously in the armpits. Approximately 6 days later, B16 tumor appeared, and mice were paired (N = 7) and injected with compound **3i** (25 mg/kg and 50 mg/kg) or vehicle. Compound **3i** was dissolved in 5% (v/v) DMAC (dimethylacetamide) and added to olive oil. Intraperitoneal injection was performed in the mouse. The animals were injected once every two days. Tumor volume was calculated using the following equation: $V=L(S^2)\pi/6$, where L is the longer and S is the shorter of the two tumor dimensions.

2.8 Spontaneous breast carcinoma mouse model

C57BL/6 spontaneous breast carcinoma mice [22] were paired (N = 3) and injected with compound **3i** (50 mg/kg) or vehicle, when spontaneous tumor volumes reached 0.1 cm^3. Intraperitoneal injection was performed in the mouse. Animals were injected once every two days.

All experiments were performed in compliance with the relevant laws and institutional guidelines of the Institute Research Ethics Committee of Peking University Health Science Center, and the committee had approved the experiments.

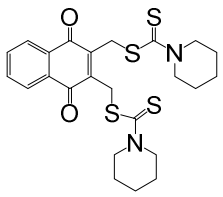
2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Data are presented as mean ± SD (n = 3).

3. Results and discussion

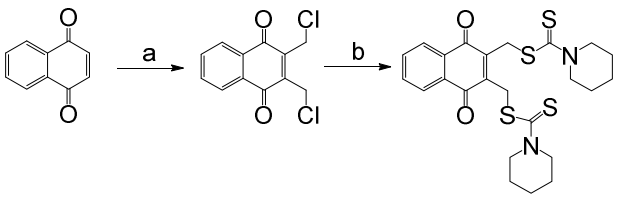
We prepared compound **3i** by the synthetic route as shown in Figure **1B**. To determine the efficiency of compound **3i** as an anti-tumor agent, we assessed the in vitro cytotoxicity of **3i** using several different tumor cell lines derived from human colon cancer (HCT116), breast cancer (MCF7), breast cancer (MDA-MB231), cervical cancer (Hela) and lung cancer (H1299), and mouse melanoma (B16). The results are presented in Table **1**. Compound **3i** reduced cancer cell viability at nanomolar concentrations with IC_{50} values against HCT116, MCF7, MDA-MB231, Hela, H1299 and B16 cells from 50 nM to 150 nM in MTS reduction assays. Specially, compound **3i** exhibited a dose-dependent cytotoxicity (Fig. **2**). To further explore the selectivity of the target compound against cancer cells, we tested its cytotoxicity in BEAS-2B cells derived from normal human bronchial epithelial cells. As seen in Table **1**, compound **3i** showed higher selectivity for cancer cells H1299 than normal cells BEAS-2B, which indicated that compound **3i** probably have low toxicity to normal cells.

A



Compound **3i**

B



View Article Online
DOI: 10.1039/C8MD00062J

Fig. 1. The structure and synthetic route of compound **3i**. (A) The structure of compound **3i**. (B) The synthetic route of compound **3i**. Reagents and conditions: (a) formaldehyde, HCl, HAc, H_2O, 0°C, 68.9%; (b) CS_2, piperidine, CH_3CN, rt, 83.5%.

Table **1**. In vitro cytotoxicity of compound **3i**

	IC_{50} ± SD (μM)
HCT116	0.077 ± 0.011
MCF7	0.061 ± 0.030
MDA-MB231	0.135 ± 0.010
Hela	0.124 ± 0.010
H1299	0.109 ± 0.015
B16	0.104 ± 0.007
BEAS-2B	32.61 ± 2.04

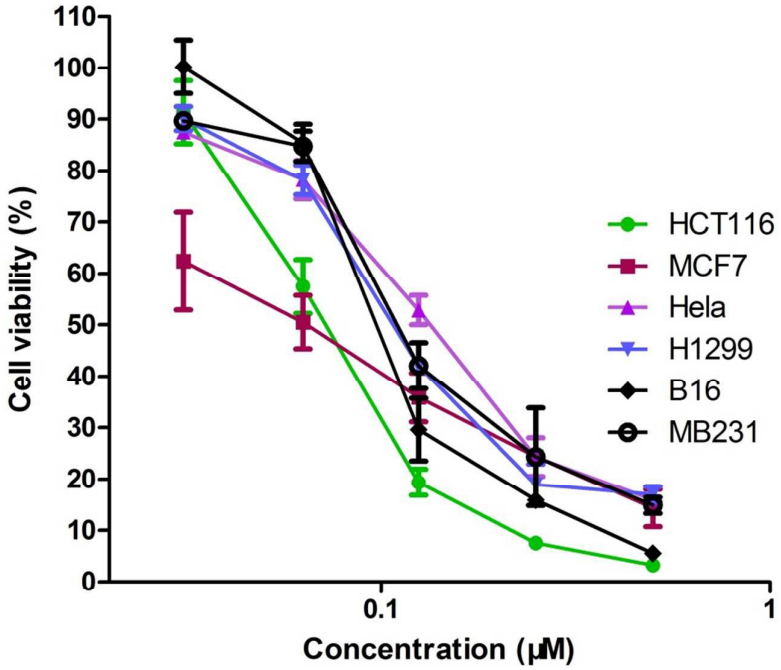


Fig. 2. Compound **3i** reduces cancer cell viability. Cells were treated with increasing concentrations of compound **3i**. Cell viability was measured using MTS.

The direct consequence of inhibiting tumor cells metabolism is the decrease of ATP production in cells. We treated HCT116 cells with 10 μM and 20 μM compound **3i** and tested ATP production with the ATP Assay Kit. The results showed that compound **3i** significantly influenced the metabolic function by impairing the cellular ATP production (Fig. **3A)**. Compound **3i** has a similar napthaquinone skeleton as in shikonin[23] that was reported to affect the metabolism of tumor cells by inhibiting PKM2 activity, therefore, we tested the influence of compound **3i** on PKM2 activity using a fluorescent PK-LDH coupled assay according to a previously reported method

View Article Online
DOI: 10.1039/C8MD00062J

[20]. Shikonin was used as the positive control. As shown in Table 2, compound **3i** (IC_{50} = 0.88±0.37) displayed the higher inhibitory activity than shikonin (IC_{50} = 8.82 ± 2.62). Moreover, compound **3i** showed inhibition of PKM2 with less inhibition of PKM1 and PKL. To investigate whether compound **3i** is able to inhibit PKM2 in cells, we treated cells in which PKM2 is highly expressed with 2.5 μM, 5 μM, 10 μM and 20 μM compound **3i** and assayed PKM2 activity in the corresponding cell lysates. We found that compound **3i** inhibited PKM2 activity in cells in a dose dependent manner (Fig. **3B**). In addition, there are extensive evidences that PKM2 coactivates β-catenin to induce its downstream gene CCND1 and c-Myc transcription, resulting in upregulation of GLUT1 and LDHA. We evaluated the transcription of these genes. Cells were treated with 20 μM compound **3i**, and RT-PCR showed significant reduction of GLUT1, LDH and CCND1 (Fig. **3C**). These data suggest that compound **3i** probably interferes with energy metabolism of cancer cells by inhibiting PKM2 activity.

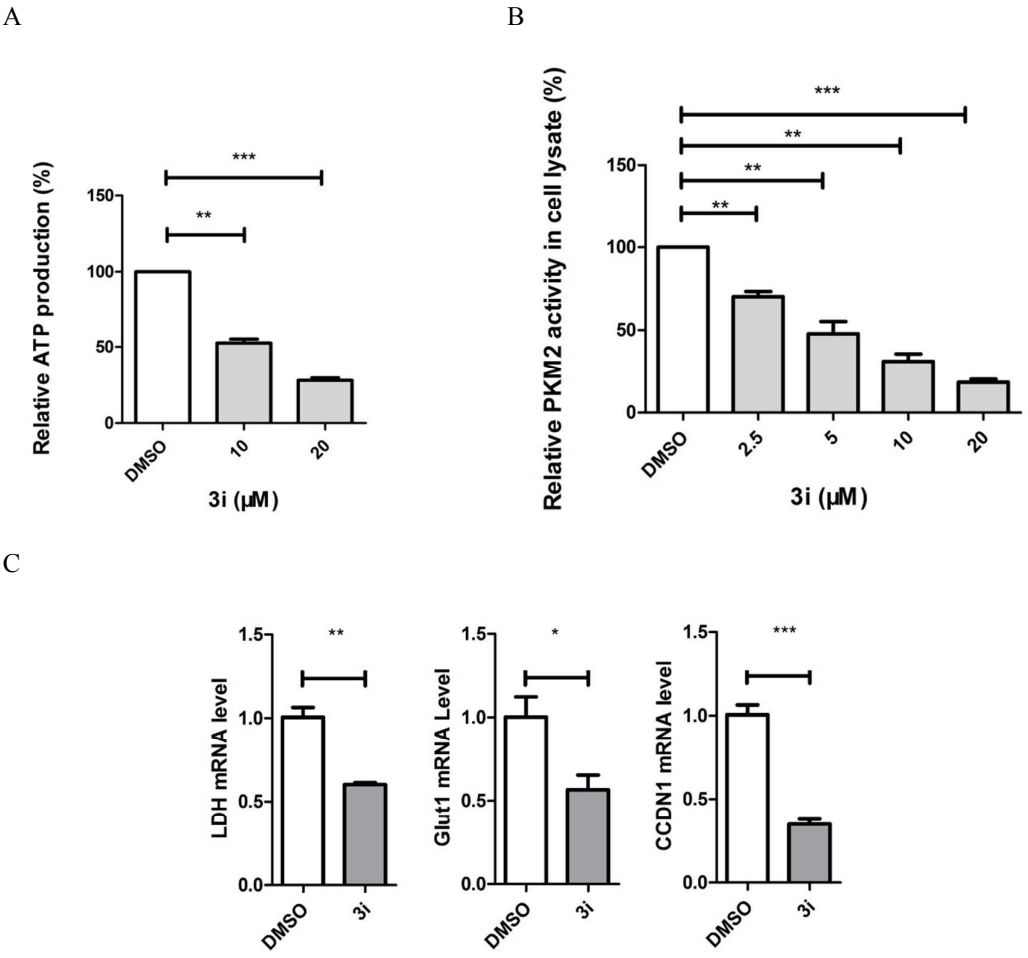


Fig. 3. Compound **3i** can regulate the metabolism of tumor cells. (A) Compound **3i** inhibited cellular ATP production. HCT116 cells were treated with 10 μM and 20 μM compound **3i** for 6 h and ATP production was tested with the ATP Assay Kit. (B) Compound **3i** inhibited PKM2 activity in cells in a dose depend manner. HCT116 cells were treated with 2.5 μM, 5 μM, 10 μM and 20 μM compound **3i** for 8 h and PKM2 activity was tested in the corresponding cell lysates using a

View Article Online
DOI: 10.1039/C8MD00062J

fluorescent PK-LDH coupled assay. (C) Compound **3i** inhibited the transcription of PKM2-regulated glycolytic genes GLUT1, LDH and CCND1. HCT116 cells were treated with 20 μM compound **3i** for 8 h and qPCR was performed.

Table **2**. In vitro inhibitory activity (IC_{50}) of **3i** and shikonin on different PKM2 isoforms

	PKM2 (μM)	PKM1 (μM)	PKL (μM)	IC_{50}(PKM1)/ IC_{50}(PKM2)	IC_{50}(PKL)/ IC_{50}(PKM2)
3i	0.88 ± 0.37	5.07 ± 0.13	2.90 ± 0.64	5.8	3.3
Shikonin	8.82 ± 2.62	12.96 ± 3.37	39.25 ± 6.53	1.5	4.5

Based on the potent inhibitory effect of compound **3i** on tumor cells in vitro, we next assessed its inhibitory efficiency in mouse models. The in vivo anti-tumor effect of compound **3i** was first evaluated in a B16 transplantation mouse model. As shown in Fig. **4A**, intraperitoneal injection of compound **3i** at 25 and 50 mg/kg every two days for a period of 12 days demonstrated significant dose-dependent inhibition of tumor growth over the course of the treatment. The mice treated with compound **3i** at 50 mg/kg showed tumors of 30% tumor weight compared to the vehicle-treated (control) group (Fig. **4B**). The T/C values (relative tumor volume growth rate) of the 50 mg/kg treatment group were close to or less than 40% at each time point, which consistent with the high efficiency of compound **3i**. (Fig. **4D**). In addition, compound **3i** had no significant effect on body weight over the course of this experiment, suggesting that it was well tolerated in vivo.

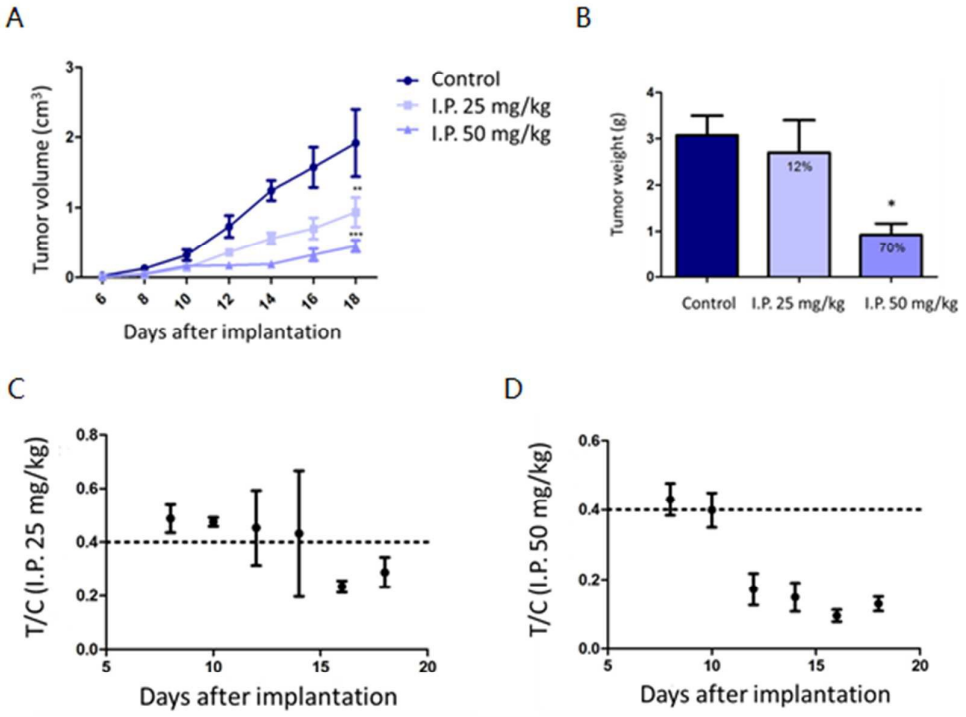


Fig. 4. Compound **3i** inhibited B16 tumor growth in a dose depend manner. (**A**) Mice were treated with 25 mg/kg or 50 mg/kg compound **3i** or vehicle. Tumor volume was measured once every two

View Article Online
DOI: 10.1039/C8MD00062J

days. (**B**) The weight of individual tumors was measured. (**C-D**) The T/C values of the 25 mg/kg and 50 mg/kg treatment group at selected time points.

To further assess the therapeutic efficiency of this compound, we used a PTEN deleted mediated mouse spontaneous breast tumor model[22]. Mice were divided into two groups when tumors reached a volume of 0.1 cm^3, and compound **3i** at 50 mg/kg or vehicle was injected intraperitoneally every two days for 36 days. As shown in Fig. **5A**, tumor growth was significantly suppressed over this course of treatment. Mouse tumor weight after treatment with compound **3i** was approximately 20% of that of the control (Fig. **5B** and **5C**).

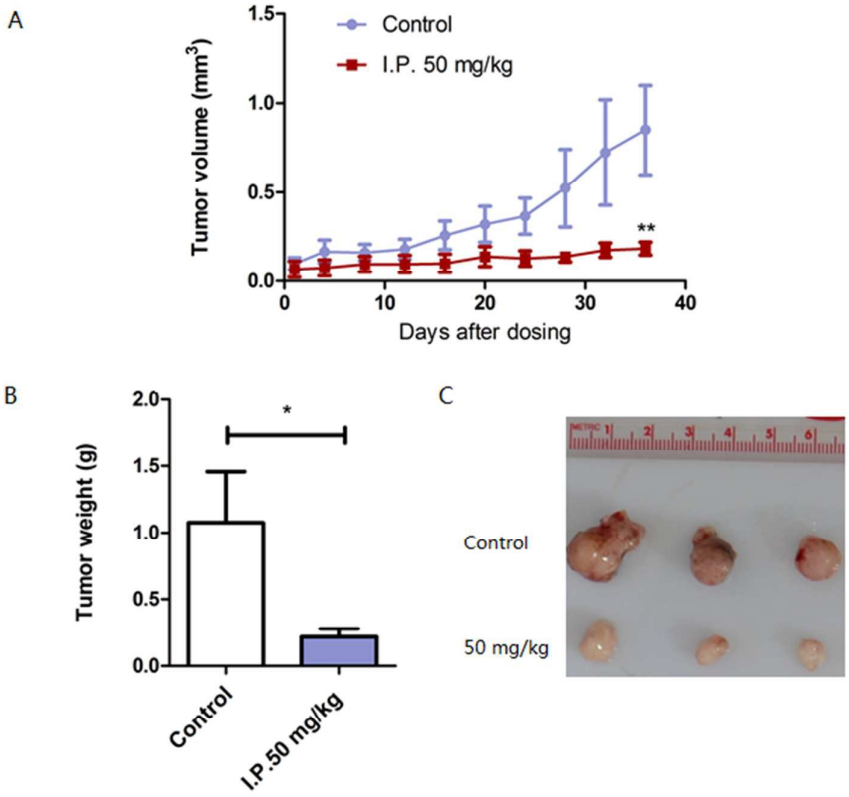


Fig. 5. Compound **3i** significantly inhibited mouse spontaneous breast tumor growth. (**A**) Mice were treated with compound **3i** or vehicle. Tumor volume was measured once every four days. (**B-C**) Tumors were removed after sacrificing mice. Weight of individual tumors was measured.

4. Conclusions

Modulation of metabolism is a key characteristic of highly proliferative cancer cells which allows both rapid ATP generation and access to metabolites needed as cellular building blocks. Here, we describe a metabolic suppressor compound **3i** that is a previously unreported compound. Compound **3i** reduced cancer cell viability in a high response with IC_{50} values in nanomolar concentrations and suppressed ATP production in cancer cells. Meanwhile, compound **3i** inhibited a rate-limiting enzyme PKM2 in glycolytic pathway. However, the cytotoxicity of compound **3i** was much

View Article Online
DOI: 10.1039/C8MD00062J

higher than PKM2 inhibitory activity, which suggested that compound **3i** had other mechanisms to influence tumor cells metabolism and suppress cell proliferation. The 1,4-naphthoquinone moiety of compound **3i** has been reported to influence other proteins, such as, DT-diaphorase[24] and P2X7 receptor[25], which may lead to the poor activity relationship in the enzyme and cell level. However, there is no doubt that compound **3i** targets PKM2 and affects tumor cells metabolism. In future study, we will focus on investigating other mechanisms of compound **3i**.

In this study, we also found compound **3i** significantly inhibited PTEN loss mediated mouse spontaneous breast tumor growth. Previous studies have indicated that PTEN-negative human hepatocellular carcinoma cell lines show up-regulation of PKM2 expression, which is advantageous for cell proliferation and anchorage-independent growth[26]. This probably accounts for the fact that the compound **3i** is very sensitive to PTEN loss in the spontaneous tumor model. The mechanism will be investigated in our further work. In addition, we will also continue optimization of the structure of 2, 3-didithiocarbamate substituted naphthoquinones and prepare suitable formulation for identifying new compounds with better physico-chemical properties and higher efficiency.

Conflict of Interest

The authors declare no competing interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Key grants #81430056, #81372491 and #81402777) and the China Postdoctoral Science Foundation (#2014M560026 and #2015T80028).

Reference

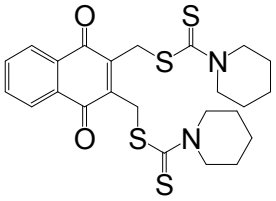
1. W. Yang and Z. Lu, *Cancer Lett*, 2013, **339**, 153-158.
2. A. T. Ooi and B. N. Gomperts, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 2015, **21**, 2440-2444.
3. J. Penkert, T. Ripperger, M. Schieck, B. Schlegelberger, D. Steinemann and T. Illig, *Oncotarget*, 2016, **7**, 67626-67649.
4. O. Warburg, *Science*, 1956, **123**, 309-314.
5. Y. Zhao, E. B. Butler and M. Tan, *Cell death & disease*, 2013, **4**, e532.
6. N. D. Amoedo, E. Obre and R. Rossignol, *Biochimica et biophysica acta*, 2017, **1858**, 674-685.
7. N. E. Scharping and G. M. Delgoffe, *Vaccines*, 2016, **4**.
8. N. Wong, J. De Melo and D. Tang, *International journal of cell biology*, 2013, **2013**, 242513.
9. V. Gupta and R. N. Bamezai, *Protein Sci*, 2010, **19**, 2031-2044.
10. W. Luo and G. L. Semenza, *Trends in endocrinology and metabolism: TEM*, 2012, **23**, 560-566.
11. T. Noguchi, H. Inoue and T. Tanaka, *The Journal of biological chemistry*, 1986, **261**, 13807-13812.

View Article Online
DOI: 10.1039/C8MD00062J

12. W. Yang and Z. Lu, *J Cell Sci*, 2015, **128**, 1655-1660.
13. H. R. Christofk, M. G. Vander Heiden, M. H. Harris, A. Ramanathan, R. E. Gerszten, R. Wei, M. D. Fleming, S. L. Schreiber and L. C. Cantley, *Nature*, 2008, **452**, 230-233.
14. W. Yang, Y. Xia, H. Ji, Y. Zheng, J. Liang, W. Huang, X. Gao, K. Aldape and Z. Lu, *Nature*, 2011, **480**, 118-122.
15. T. L. Dayton, T. Jacks and M. G. Vander Heiden, *EMBO Rep*, 2016, **17**, 1721-1730.
16. X. Gao, H. Wang, J. J. Yang, X. Liu and Z. R. Liu, *Mol Cell*, 2012, **45**, 598-609.
17. X. Gao, H. Wang, J. J. Yang, J. Chen, J. Jie, L. Li, Y. Zhang and Z. R. Liu, *The Journal of biological chemistry*, 2013, **288**, 15971-15979.
18. X. Ning, H. Qi, R. Li, Y. Li, Y. Jin, M. A. McNutt, J. Liu and Y. Yin, *Eur J Med Chem*, 2017, **138**, 343-352.
19. X. Ning, H. Qi, R. Li, Y. Jin, M. A. McNutt and Y. Yin, *J Enzyme Inhib Med Chem*, 2018, **33**, 126-129.
20. M. G. Vander Heiden, H. R. Christofk, E. Schuman, A. O. Subtelny, H. Sharfi, E. E. Harlow, J. Xian and L. C. Cantley, *Biochemical pharmacology*, 2010, **79**, 1118-1124.
21. H. R. Christofk, M. G. Vander Heiden, N. Wu, J. M. Asara and L. C. Cantley, *Nature*, 2008, **452**, 181-186.
22. A. Di Cristofano, B. Pesce, C. Cordon-Cardo and P. P. Pandolfi, *Nature genetics*, 1998, **19**, 348-355.
23. J. Chen, J. Xie, Z. Jiang, B. Wang, Y. Wang and X. Hu, *Oncogene*, 2011, **30**, 4297-4306.
24. C. Flader, J. Liu and R. F. Borch, *J Med Chem*, 2000, **43**, 3157-3167.
25. R. X. Faria, F. H. Oliveira, J. P. Salles, A. S. Oliveira, N. L. von Ranke, M. L. Bello, C. R. Rodrigues, H. C. Castro, A. R. Louvis, D. L. Martins and V. F. Ferreira, *Eur J Med Chem*, 2018, **143**, 1361-1372.
26. I. Nemazanyy, C. Espeillac, M. Pende and G. Panasyuk, *Biochemical Society transactions*, 2013, **41**, 917-922.

View Article Online
DOI: 10.1039/C8MD00062J

Graphical abstract:



Compound **3i**