Targeting pyruvate kinase M2 and hexokinase II, pachymic acid impairs glucose metabolism and induces mitochondrial apoptosis

Guopeng Miao,*a Juan Han,a Jifeng Zhang,a Yihai Wu,a and Guanhe Tong* a

a Department of Bioengineering, Huainan Normal University, Huainan, Anhui Province 232038, China

*To whom correspondence should be addressed. Guopeng Miao (xndmgp@163.com); Guanhe Tong (guanhetong2007@sina.com)
Abstract

Pachymic acid (PA), a triterpenoid from *Poria cocos*, has various pharmacological effects, including anti-inflammatory, anti-cancer, anti-aging, and insulin-like properties. PA has gained considerable research attention, but the mechanism of its anti-cancer effects remains unclear. In this study, pyruvate kinase M2 (PKM2) was discovered as a PA target via the drug affinity responsive target stability. Molecular docking and enzyme assay revealed that PA is a competing activator of PKM2, and mimics the natural activator, fructose-1,6-bisphosphate. PKM2 activation should augment the flux of glycolysis. However, decreased glucose uptake and lactate production after PA treatment was observed in SK-BR-3 breast carcinoma cells, indicating a blockage or downregulation of glycolysis. The potential of previously reported triterpenoids in blocking hexokinase II (HK2) activity inspired us to investigate the inhibition effect of PA on HK2 activity. Molecular docking and enzyme assay confirmed that PA was an inhibitor of HK2, with an IC$_{50}$ of 5.01 μM. The possible consequences of glycometabolic regulation by PA, such as dissociation of HK2 from the mitochondria, release of mitochondrial Cyt c, depletion of ATP, and generation of reactive oxygen species, were further validated. Furthermore, the details of the possible linkage of targeting PKM2 and HK2 with previously reported actions of PA were discussed. The results of our study provided valuable information on the anti-cancer mechanisms of PA.

**Key words** pachymic acid; target; pyruvate kinase; hexokinase; reactive oxygen species; apoptosis
Pachymic acid (PA) is a lanostane-type triterpenoid and major component of the alcoholic extract of *Poria cocos*. This triterpenoid possesses various pharmacological effects, including anti-inflammatory, anti-cancer, anti-aging, and insulin-like properties.¹⁻⁵)

PA inhibits phospholipase A₂, which is responsible for the hydrolysis of the sn-2 position of membrane glycerophospholipids, to release arachidonic acid, a precursor of eicosanoids, including PGs and leukotrienes.²,⁶) Several studies have revealed that PA can inhibit lipopolysaccharide- and oxidative-induced inflammation by suppressing extracellular signal-regulated kinase 1/2 (ERK 1/2) and p38 signaling pathways⁷) and nuclear factor (NF)-κB.⁸) In lung cancer cells, PA induces apoptosis by activating reactive oxygen species (ROS)-dependent c-Jun N-terminal kinase (JNK) and endoplasmic reticulum (ER) stress pathways.⁴) In the experiment that investigated the effects of PA on the invasion of breast carcinoma cells, the NF-κB signal pathway was suppressed by PA, resulting in the downregulation of matrix metalloproteinase-9, which is an extremely relevant enzyme for tumor invasion. However, PA did not alter the phosphorylation states of mitogen-activated protein kinases (MAPKs), including ERK, JNK, and p38 kinase.³) Moreover, PA suppresses the growth and invasiveness of EJ bladder cancer, osteosarcoma, and gastric cancer cells.⁹⁻¹¹) PA has gained research attention, but the mechanisms of its anti-inflammation and anti-cancer effects remain unclear.

Drug affinity responsive target stability (DARTS) is a quick and straightforward approach to identify potential protein targets for small molecules. This approach relies
on the protection against proteolysis conferred on the target protein by interacting with small molecules.\textsuperscript{12) In this study, pyruvate kinase isoform M2 (PKM2) and hexokinase II (HK2) were found potential targets of PA, through DARTS and molecular docking, respectively. \textit{In vitro} experiments showed that PA was an inhibitor of HK2 and an activator of PKM2. The decreased glucose uptake and lactate production, HK2 dissociation from mitochondria, cytochrome c (Cyt c) release, and ROS generation supported the functions of PA in glucose metabolism and illuminated the mechanism of the anti-cancer effect of PA.

**MATERIALS AND METHODS**

**Materials** The PA used in this study was purchased from Dasf-Bio, Nanjing, China, and it has a purity higher than 98\%. Unless otherwise indicated, chemicals and reagents used in this study were of analytical grade.

**DARTS with cancer cell lysates** DARTS was performed according to the method of Lomenick et al., with some modifications.\textsuperscript{13) SK-BR-3 breast carcinoma cells were lysed in PBS containing 0.5\% Triton X-100, protease inhibitor (cOmplete™, Sigma-Aldrich, USA) and phosphatase inhibitor cocktail (P8340, Sigma-Aldrich, USA). Protein concentrations were determined using a bicinchoninic acid assay kit (Beyotime, China), and bovine albumin was used as the standard. All the above steps were performed on ice. PA (final concentration of 10 \(\mu\)M) or DMSO was added into the cell lysates and incubated at 25 °C for 30 min. Samples were digested enzymatically with pronase (Sigma-Aldrich, USA) at an enzyme:lysate (w/w) ratio of 1:600 for 30 min at 25 °C. The resulting mixtures were separated via
SDS-PAGE and were stained with Coomassie blue. Gel lanes showing remarkable differences in intensity were excised manually and subjected to an in-gel digestion procedure. Peptides were analyzed via high-resolution LC–MS/MS, and raw data were analyzed through MaxQuant (version 1.2.2.5) using standard settings. Proteins with more than three peptides were considered as reliable proteins.

**Molecular docking** The crystal structures of human PKM2 (PDB code: 4IP7) and HK2 (PDB code: 2NZT) were obtained from the Protein Data Bank. Docking was conducted through SystemsDock (http://systemsdock.unit.oist.jp/iddp/home/index).

**Spectrophotometric assay for enzyme activity** HK activity was measured through a coupled reaction with glucose-6-phosphate dehydrogenase. The reaction mixture contained 100 mM Tris HCl (pH 8.0), 5 mM MgCl₂, 100 mM glucose, 0.8 mM ATP, 1 mM NADP, and 3 units of glucose-6-phosphate dehydrogenase. The NADP reduction in UV absorbance at 340 nm was monitored at the start of the reaction and after 5 min. PK activity was determined using a lactate dehydrogenase-linked assay. The reaction mixture contained 50 mM Tris HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 0.6 mM PEP, 0.9 mM ADP, 0.12 mM β-NADH, and 4.8 U/mL lactate dehydrogenase. PK activity was calculated at 25 °C by monitoring the change in absorbance at 340 nm from 0 to 20 min. PA, bromopyruvic acid (BA), or TEPP-46 [the thieno(3,2-b)pyrrole(3,2-d)pyridazinone, PubChem CID: 44246499] was dissolved in DMSO and diluted by 10-fold with cell lysis buffer, and different concentrations (5–100 μM) were used. Approximately 30 μL SK-BR-3 cell lysate (20 ng/μL) was incubated with 3 μL PA, BA, or a dissolving
vector at 37 °C for 10 min. Afterward, 300 μL reaction mixture was added.

**Glucose uptake assay** After PA treatment for 24 h, the media of SK-BR-3 cell cultures were collected via centrifugation and diluted with water to 4000-fold. Glucose uptake assay was conducted using an Amplex Red glucose assay kit (Invitrogen, USA) following the manufacturer’s instructions. Results were expressed as percentages of the vector control.

**Mitochondria isolation and protein fractionation** After treatment with 100 μM PA for 12 h at room temperature, the mitochondria and cytosol of SK-BR-3 cells were separated through conventional differential centrifugation. The pellet and resulting supernatant were eluted with the SDS sample loading buffer, separated in 10% SDS polyacrylamide gel, and immunoblotted for HK2, Cyt c, and HSP60 using appropriate antibodies.

**Measurement of lactate, ATP, ROS, and 6-phosphogluconic acid (6-PGA)**

After 24 h incubation with PA or vector solvent, the accumulation of cellular lactate was determined using a lactic acid production detection kit (KeyGen, Nanjing, China) in accordance with the manufacturer’s instructions. ROS concentrations were determined using di(acetoxyethyl ester) (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate) (Thermo Fisher Scientific, USA) according to the method of Wang and Joseph. The contents of ROS were calculated from a standard curve of H₂O₂ (50–1000 μM) and were expressed as percentages of the control. The contents of ATP and 6-PGA were measured using an ATP colorimetric/fluorometric assay kit and 6-PGA colorimetric assay kit (BioVision, USA), respectively, in accordance with the
manufacturer’s instructions. All contents measured were expressed as percentages of the vector control.

Statistics All data were reported as the mean ± SD of three replicates. Significant differences were determined through ANOVA, homogeneity of variance test, and Duncan’s multiple range test for at least three treatment groups and Student’s t-test for comparison of two groups. Statistical analysis was performed using SPSS (SPSS, IBM, USA) at a statistical significance level of 0.05. All experiments were repeated twice.

RESULTS

PK was discovered as a candidate PA target by DARTS To understand the anti-cancer mechanism of PA, DARTS was applied to find possible targets of PA besides phospholipase A2. Enzyme hydrolysis broke the complete protein (bands in the negative control, NC) into peptide fragments (Fig. 1). Addition of PA protected some peptides from digestion, and resulted in blue bands (31–45 kDa) darker than those of the untreated control. The top three possible bands were cut and analyzed via LC–MS/MS. MS data revealed that the three bands, from low to high positions, may contain PK, antitrypsin, and phosphoglycerate kinase, respectively (Table 1). Antitrypsin is involved in cancer development and is a potential target of triterpenoids. However, as mentioned above, evidence directed the dysfunction of glycolysis as a PA mechanism. Thus, we selected PK or phosphoglycerate kinase as the study subject. PK has important roles in tumor cell growth, and it contains more peptide matches than phosphoglycerate kinase (Table 1). Thus, among the potential
targets discovered through DARTS, PK was selected for following investigations.

**PA served as a competing activator of PKM2** SystemsDock is a web server for network pharmacology-based prediction and analysis. This server has an elaborately designed scoring function for molecular docking to evaluate the protein-ligand binding potential.\(^{15}\) The PKM2 isoform is the major PK in cancer cells and is overexpressed in tumor cells induced by some oncogenes and tumor suppressors.\(^{22}\) Molecular docking revealed that PA could bind PKM2 in the pocket of its natural activator, fructose-1,6-bisphosphate (FBP), and share six amino acid residues (Fig. 2a). The docking score (pK\(_d\)/pK\(_i\)) of PA (8.39) was considerably higher than that of FBP (5.29) (Fig. 2b), indicating the advantage of PA than FBP in PKM2 binding. Both of PA and the positive control (TEPP-46) stimulated the activity of PKM2 in the cell lysate, and adding FBP attenuated the increasing trend caused by PA treatment (Fig. 2c). This result corroborated the results of molecular docking. Regardless of FBP addition, the maximum PKM2 activity remained the same, which indicated that FBP could compete with PA in binding PKM2.

**PA decreased glucose uptake and lactate production** When PKM2 is activated under glucose-rich conditions, the level of glucose uptake increases, and most pyruvate is rapidly converted to lactate.\(^{23}\) Thus, we tested if PA could increase glucose uptake and lactate production. However, after PA application, glucose uptake and lactate production decreased in a dose-dependent manner (Fig. 3), indicating a blockage or downregulation of glycolysis.

**PA directly inhibited HK2 activity** A recent study has discovered that several
triterpenoids in fungi *Ganoderma sinense*, especially (22E,24R)-6β-methoxyergosta-7,9(11),22-triene-3β,5α-diol, directly inhibit HK2 activity. Similar to PA, this triterpenoid contains four cyclic rings and a side chain on the five-membered D ring. As a key enzyme in the glycolytic pathway, HK2 catalyzes the first step of glycolysis by phosphorylating glucose to glucose-6-phosphate (G6P). Thus, we examined the inhibition effect of PA on HK2. Molecular docking results showed that HK2 could bind with PA in the pocket for G6P (Fig. 4a). Furthermore, the affinity of PA to HK2 was considerably higher than that of G6P; the molecular docking scores were 8.18 and 5.31, respectively (Fig. 4b). PA substantially inhibited the HK2 activity in the cell lysate, and showed an IC50 of 5.01 μM, which was higher than the 3.07 μM of BA (Fig. 4c). HK2 activity increased after treatment with a low dose of PA (5 μM). A similar phenomenon was observed in the MDA-MB-231 cell lysate treated with metformin, which is a direct inhibitor of HK2. We speculated that this phenomenon was caused by the endogenous chemicals from the cell lysate.

**PA induced mitochondrial dysfunction, ATP depletion, and ROS generation**

After 12 h treatment with 100 μM PA, the dissociation of HK2 from the mitochondria and the release of mitochondrial Cyt c were observed through immunoblot analysis in a dose-dependent manner (Fig. 5a). Mitochondrial dysfunction may affect ATP production and ROS generation. ATP depletion of up to 0.32±0.08-fold relative to that of the control was observed after 100 μM PA treatment for 12 h (Fig. 5b). Meanwhile, the PA treatment induced ROS production to reached
up to $5.99\pm 0.50$-fold relative to that of the control (Fig. 5b). HK2 inhibition and PKM2 activation will decrease the pooling of glycolytic intermediates, which should have been shunted to the pentose phosphate pathway (PPP) for NADPH generation and ROS scavenging.\textsuperscript{36} After the PA treatment, the level of PPP intermediate, namely 6-PGA, decreased in a dose-dependent manner (Fig. 5b).

**DISCUSSION**

PA is an active component of *P. cocos*, and it possesses various pharmacological effects. However, its action mechanisms are unclear. In this study, PKM2 and HK2 were discovered as the new targets of PA.

PKM2 catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP into ATP and pyruvate, which is the final reaction in glycolysis.\textsuperscript{24} When PKM2 is mainly in its inactive dimeric form, which is the case in tumor cells, all glycolytic intermediates above the PK accumulate and are channeled into synthetic processes, such as nucleic acid, phospholipid, and amino acid syntheses, to support tumor cell proliferation.\textsuperscript{26} Furthermore, the dimeric form of PKM2 has transcriptional regulation functions that benefit cancer cells.\textsuperscript{27} Previous studies have shown that the active tetrameric form of PKM2 can be allosterically activated by activators, such as FBP.\textsuperscript{28,29} As an FBP competitor, PA may increase the ratio of tetrameric/dimeric state of PKM2, which needs in-depth investigation.

Emerging evidence has implicated PKM2 as a critical regulator of metabolism in immune cells. Moreover, PKM2 expression is substantially increased in lipopolysaccharide-activated macrophages, mainly in a monomeric/dimeric
conformation and phosphorylated state with decreased activity.\textsuperscript{30} Hence, other than inhibiting phospholipase A\textsubscript{2}, an in-depth investigation on the role of PA in inflammation is interesting.

The activation of PKM2 should have increased the uptake of glucose and production of lactate. However, opposite results were obtained in this study (Fig. 3). In malignant tumors, a high rate of aerobic glycolysis is frequently observed; this high glycolysis rate is known as the Warburg effect, which is believed to benefit tumor cell growth and survival.\textsuperscript{31} As a key enzyme in the glycolytic pathway, HK2 is overexpressed in cancer cells, and it is an important target for cancer therapy.\textsuperscript{32} The potential of triterpenoids in blocking HK2 activity inspired us to investigate the possible inhibitory effect of PA on HK2.\textsuperscript{16} \textit{In silico} and \textit{in vitro} experiments proved this hypothesis (Fig. 4).

In tumor cells, HK2 strategically binds to the transmembrane channels formed by voltage-dependent anion channel (VDAC). This interaction facilitates the growth and proliferation of tumors by reducing the sensitivity to the feedback inhibition of G6P, preferred access to ATP, and protection from proteolytic degradation.\textsuperscript{33} In contrast, the HK2 dissociation from the mitochondria will impair tumor growth and induce apoptosis, which is commonly observed in tumor cells treated with HK2 inhibitors, such as metformin, oroxylin A, and methyl jasmonate.\textsuperscript{25,34,35} Moreover, we hypothesized that PA can induce the detachment of HK2 from the mitochondrial membrane. After the PA treatment, mitochondrial HK2 dissociation in a dose-dependent manner was observed (Fig. 5a). The dissociation of HK2 and VDAC
will induce the release of mitochondrial Cyt c through either the Cyt c-conducting conduit formed by Bax and Bak on the outer mitochondrial membrane (OMM) or the direct rupture of OMM resulting from sustained VDAC closure. Thus, we parallely examined the release of Cyt c from the mitochondria after the PA treatment, and positive results were obtained (Fig. 5a).

The closure of VDAC results in a diminution of mitochondrial ADP, thereby decreasing the respiratory rate and stimulation of ROS generation due to the highly reduced state of the components of the electron transport chain. Thus, we examined the influence of PA on the intracellular ROS contents and ATP level. PA stimulated the production of ROS and depletion of ATP (Fig. 5b); this result was consistent with the work of Jeong et al. Furthermore, through the inhibition of HK2 and activation of PKM2, PA decreased the intermediate PPP level for NADPH generation and ROS scavenging, which may have contributed to the stimulation of ROS.

ROS can activate intrinsic and extrinsic apoptosis pathways and modulate the pro-apoptotic molecules, including MAPK, Bax, and Bcl2; moreover, it can regulate the expression levels of various transcription factors, including Sp1, AP1, and NF-κB, and other pro-oncogenic genes that are engaged in cancer cell proliferation, survival, and metastasis. In the study of Jeong et al., blocking of ROS by N-acetyl-L-cysteine, which is a ROS scavenger, effectively reduced the PA-induced apoptosis. Moreover, ROS-mediated ER stress is associated with apoptosis initiation, which has been observed in PA-treated lung cancer cells. In summary, ROS generation by PA may be a fundamental mechanism for the anti-cancer activity of PA.
In conclusion, two new PA targets, PKM2 and HK2, were discovered in this study. PA is an activator of PKM2 and an inhibitor of HK2. In the study of Tee et al., increased glucose consumption as a result of PKM2 activation by TEPP-46 resulted in sensitized antiproliferative effects of the toxic glucose analog, 2-deoxy-D-glucose. Combination treatment resulted in reduced viability of a range of cell lines in standard cell culture conditions. Moreover, 2-deoxy-D-glucose competitively inhibited the production of G6P from glucose, which resembles that of PA. This study supported the beneficial effects of dual targeting of PA to PKM2 and HK2. Possible consequences of this dual efficacy, such as dissociation of HK2 from the mitochondria, release of the mitochondrial Cyt c, depletion of ATP, generation of ROS, and declination of PPP intermediate, were also validated. The results of our study provide valuable information on the anti-cancer mechanisms of PA.

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**Conflict of interest** The authors declare no conflict of interest.
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**Figure Captions**

Fig. 1. DARTS results for pronase-digested SK-BR-3 breast carcinoma cell lysates with or without PA protection. The NC had no proteinase addition.

(Color figure can be accessed in the online version.)

Fig. 2. Molecular docking and enzyme assay for PA and PKM2. (a) Detailed protein-ligand interactions between PKM2 and FBP (left) or PA (right); (b) Docking scores of PKM2 with FBP and PA. (c) Enzyme activity of PKM2 in cell lysate after treatment with different concentrations of TEPP-46 and PA (with or without FBP). *, P<0.05 vs PA treatment alone.

(Color figure can be accessed in the online version.)

Fig. 3. (a) Glucose uptake and (b) lactate production in SK-BR-3 cells after 24 h treatment with 10 or 100 μM PA. *, P<0.05 vs vector control (0 μM PA).

Fig. 4. Molecular docking and enzyme assay for PA and HK2. (a) Detailed protein-ligand interactions between HK and (left) G6P or (right) PA. (b) Docking scores of HK2 with G6P and PA. (c) Enzyme activity of HK2 in cell lysate after treatment with different concentrations of PA or BA.

(Color figure can be accessed in the online version.)

Fig. 5. (a) PA-induced HK2 dissociation from the mitochondria, Cyt c release, (b) ATP depletion, generation of ROS, and declination of 6-PGA. *, P<0.05 vs vector control (0 μM PA).
Fig. 1

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Fig. 2

(a) Diagram showing interactions between various amino acids.
(b) Bar graph comparing FBP and PA.
(c) Graph showing enzyme activity as a function of PA concentration.

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Fig. 3
Fig. 5

(a) Western blot analysis showing the expression levels of HK2, Cyt c, and HSP60 in mitochondria and supernatant under different PA concentrations (Cont, 10, and 100 µM).

(b) Graphs depicting the changes in ATP, ROS, and 6-PGA levels relative to the control (0 µM PA) at various PA concentrations (0, 10, and 100 µM).
Table 1. Possible target proteins identified by LCMS*

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*: “Score” measures how well an acquired spectrum matches the theoretical fragment masses, while, “Matches” indicates the intensity of the protein in the unique band.