Abstract ID:2551

Pharmacodynamic Assay for Evaluation of First-In-Class Pyruvate Kinase-M2 Activators in Cancer Tissues

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Introduction

Pyruvate kinase-M2 (PKM2) is a rate-controlling enzyme of glycolysis in cancer cells. In cancer cells PKM2 exists in inactive dimeric form and diverts metabolites away from the TCA cycle to presumably promote synthesis of macromolecules (lipids and NA). A new class of compounds targets conversion of enzymatically inactive dimeric (PKM2) to an active tetramer complex (Dimitrios Anastasiou, et al., *Nature*, 2012), as a mechanism to tilt the balance between glycolysis and oxidative phosphorylation, and reverse the metabolic advantage acquired by tumor cells. In addition, PKM2 has a separate role in the nucleus, where it is present as dimer and acts as a transcription factor to regulate proliferation. We have developed a PKM2 sandwich immunoassay that measures primarily the dimeric (and monomeric) form and could indicate pharmacodynamic modulation by PKM2 activators.

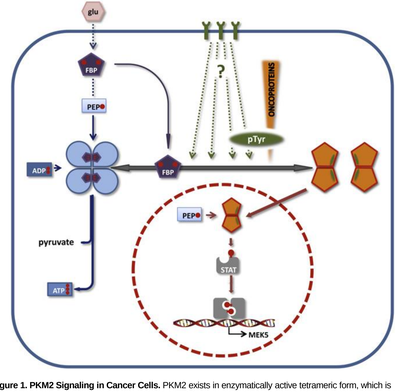

Figure 1. PKM2 Signaling in Cancer Cells. PKM2 exists in enzymatically active tetrameric form, which is allosterically regulated by fructose bisphosphate (FBP) (blue teardrops), and a less active dimeric PKM2, which causes accumulation of upstream glycolytic intermediates that can be diverted for synthesis of lipids and nucleic acids to presumably support cell proliferation in cancer cells. Binding of phosphotyrosyl peptides (green ovals) can displace FBP to promote dimerization of PKM2. The dimeric PKM2 can also translocate to nucleus to regulate transcription of a variety of proteins, signifying its nonglycolytic functions. Small molecule PKM2 activators mimic FBP to revert dimeric PKM2 to tetrameric PKM2 and restore the normal metabolism. (reprinted from Galina Semenova and Jonathan Chernoff, Mol cell 45, 583—584, 2012).

Materials and Methods

Preparation of PKM2 Calibrator. Full-length recombinant PKM2 (rPKM2) protein are produced in HEK293 cells. rPKM2 was purified using immobilized metal ion chromatography (IMAC) and diluted to 1 mg/ml concentration.

Size-Exclusion Chromatography of rPKM2: 5 ug of total protein was loaded and analyzed using size-exclusion chromatography (SEC) (Fig. 2A). Results show rPKM2 is mixture of tetrameric and dimeric forms of PKM2. The MALDI-TOF analysis confirmed the presence of dimeric PKM2 (Fig 2B). The ratio of tetramer/dimer appears to be dependent on buffer composition.

Materials and Methods (continued)

Capture and Detection of PKM2. Antibodies specific to PKM2 were coupled to Magplex Luminex beads using Mix & Go™ Coupling Reagent (Anteo Diagnostics, Brisbane, Australia). Reporter antibodies specific to an epitope distinct from the capture antibodies were labeled with EZ-link sulfo-NHS biotin kit (Thermo Scientific, Rockford, IL) and used for sandwich immunoassay development.

Tumor extracts preparation. Tumor quadrants were grinded using the Pro200 Homogenizer to generate cytosolic and mitochondrial+nuclear fractions.

Analytical Validation. Tumor lysates were used to determine reproducibility, dilution linearity, and spike recovery of the PKM2 immunoassay. Tumor samples used for validation included the fractionated extracts from A375 (melanoma), HEPG2 (hepatoma), PC3 (adenocarcinoma), and MCF-7 (adenocarcinoma) control xenografts were prepared according to tumor extraction procedures.

Preclinical Study Design. The PKM2 study was performed in human lung tumors using the H1299 cell line model. Bilateral tumors were implanted in nude mice, and once tumors reached 200 mm^3 in size, mice were given an oral dose of 60 mg/kg, 20 mg/kg, or 6 mg/kg of PKM2 activator (NSC772991, compound #9 from Kung et al., Chem Biol, 2012). Plasma and tumors were collected 2 h, 6 h, 24 h, or 48 h after oral administration of the drug and tumor quarters were flash frozen within 2 min of collection for PKM2 analysis.

Statistics: All descriptive statistics (mean, SD, and CV) were calculated with Microsoft Excel and GraphPad Prism (v6.03). The significance level for the 95% confidence interval (CI) was set at α=0.05 for a two-sided test.

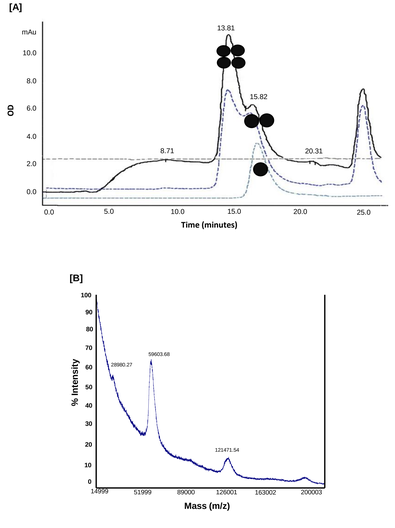

Figure 2. Size-exclusion chromatography (SEC) of recombinant PKM2. A. Analysis of recombinant PKM2 using Superdex 200 SEC showed a mixture of PKM2 dimeric and tetrameric forms in TRIS buffer (black solid line) and TRIS buffer with ß-mercaptoethanol (dark blue broken line). The ratio of tertramer/dimer appears to be dependent on buffer composition (light blue line - PBS). **B.** MALDI-TOF analysis confirmed presence of dimeric PKM2.

Results

Evidence that PKM2 immunoassay measures primarily dimeric form

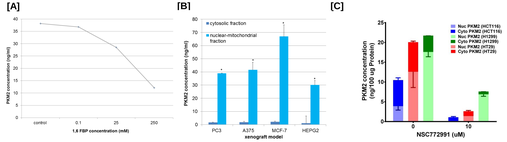

Figure 3. PKM2 immunoassay measures dimeric form. A. rPKM2 at 0.05 ug/ml concentration was incubated with 1,6 FBP for 30 min and analyzed by PKM2 assay. Results showed a 50% decrease in PKM2 levels at 250 mM, (the modest decrease in PKM2 levels appears to be related to the slow kinetics of tetramer formation). **B.** Consistent with previous findings (Gao et al., Mol Cell, 2012), >80% of PKM2 was detected in the nuclear+mitochondrial fractions. Published evidence (Gao et al.), shows that nuclear PKM2 is primarily dimeric. **C**. A known PKM2 activator (Kung et al., Chem Biol 2012) suppressed PKM2 in cytosolic fraction of cells treated for 6 h with 10 uM concentrations of drug. The PKM2 activator (Kung et al., Chem Biol 2012) is known to convert dimeric PKM2 into active PKM2 tetrameric form. The decreased levels of cytosolic PKM2 (**C**) in three different cell lines (HCT116, HT29, and H1299) suggests increase formation of tetramer. Together (A, B, and C) the above data suggests that the immunoassay is primarily detecting PKM2 dimers.

Analytical Validation of PKM2 Immunoassay

[A] Table 1. Reproducibility of the PKM2 Assay

	High Control			Medium Control			Low Control		
	n	Mean ± SD	CV (%)	n	Mean ± SD	CV (%)	n	Mean ± SD	CV (%)
Intra-assay variation	10	44 ± 3	6	10	12.3 ± 1.91	12.3	10	4 ± 0.294	7.4
Inter-assay variation	5	33.2 ± 6.4	19.3	6	11.6 ± 1.49	12.8	5	4.0 ± 0.78	19.4

[B] Table 2. Dilution Linearity Tumor Lysates

Tumor Sample (mit+nuc fraction)	Dilution Factor	Observed Concentration (ng/ml)	Recovery (%)	CVs
PC3 (50 ug/ml load)*	ND	21.2		10
	1:2	12.61	119.0	3
	1:4	5.62	106.0	4
	1:8	2.21	83.4	17
A375 (50 ug/ml load)*	ND	8.57		4
	1:2	5.27	123.0	0
	1:4	2.20	102.7	4
	1:8	1.10	102.7	6
MCF-7 (25 ug/ml load)*	ND	35.8		0
	1:2	19.4	108.2	3
	1:4	7.20	80.4	15
	1:8	4.48	100.1	2
HEPG2 (50 ug/ml load)*	ND	22.57		0
	1:2	10.28	91.1	6
	1:4	3.89	68.9	3
	1:8	1.70	60.3	8
H1299 (125 ug/ml load)*	ND	33.3		9
	1:2	20.3	121.8	4
	1:4	9.83	118.2	9
	1:8	4.27	102.7	4
Control L1 (100 ug/ml load)*	ND	35.8		1
	1:2	19.4	108.2	3
	1:4	7.20	80.4	3
	1:8	4.48	100.1	7

Tumor Sample (cytosolic fraction)	Dilution Factor	Observed Concentration (ng/ml)	Recovery# (%)	CVs
PC3 (100 ug/ml load)*	ND	7.19		21
	1:2	4.55	126.6	14
	1:4	2.73	151.9	5
	1:8	1.41	156.9	8
A375 (100 ug/ml load)*	ND	9.42		23
	1:2	4.07	86.4	10
	1:4	1.65	70.1	15
	1:8	0.88	74.7	25
MCF-7 (100 ug/ml load)*	ND	10.07		0
	1:2	4.81	95.5	8
	1:4	1.89	75.1	10
	1:8	1.08	85.8	11
HEPG2 (100 ug/ml load)*	ND	3.90		22
	1:2	1.66	85.1	20
	1:4	0.62	63.6	11
	1:8	0.27	55.4	14
H1299 (100 ug/ml load)*	ND	7.18		3
	1:2	3.61	114.3	4
	1:4	2.11	114.3	2
	1:8	1.23	114.3	7
Control (100 ug/ml load)*	ND	6.97		2
	1:2	2.79	80.1	3
	1:4	1.11	31.9	15
	1:8	0.78	22.4	4

*Values in parentheses are the total protein load for a xenograft sample. ND-no dilutions.

#Sample with PKM2 levels <20 ng/ml showed acceptable recoveries.

[C] Table 3. Spike Recovery of rPKM2 in Tumor Lysates

Tumor Sample (cytosolic fraction)	PKM2 Spike (ng/ml)	Observed Concentration (ng/ml)	Recovery (%)	CVs
PC3 (100 ug/ml load)*	0	12	-	1
	5	16	86	2
	10	19	76	0
HEPG2 (100 ug/ml load)*	0	4	-	3
	5	8	72	1
	10	12	76	0
A375 (100 ug/ml load)*	0	13	-	0
	5	17	82	5
	10	21	85	4
H1299 (100 ug/ml load)*	0	9	-	12
	5	13	80	1
	10	15	60	3

Tumor Sample (mit+nuc fraction)	PKM2 Spike (ng/ml)	Observed Concentration (ng/ml)	Recovery (%)	CVs
PC3 (25 ug/ml load)*	0	15	-	6
	5	19	81	0
	10	27	116	8
HEPG2 (25 ug/ml load)*	0	13	-	5
	5	17	80	11
	10	20	75	7
A375 (50 ug/ml load)*	0	19	-	2
	5	24	104.6	1
	10	27	83.2	1
H1299 (25 ug/ml load)*	0	3	-	2
	5	8	114.4	2
	10	12	89.8	0

*Tumor lysates at 25 ug/ml, 50 ug/ml, 100 ug/ml total protein concentrations were used for spiking rPKM2.

Summary of Analytical Validation: A. Reproducibility of PKM2 assay was sufficient to demonstrate a >50% change in response to drug. **B.** Cytosolic lysates with PKM2 concentrations above 20 ng/ml showed increased recovery upon dilution, which was probably related to kinetics of conversion between dimer and tetramer. Therefore, it was important to measure cytosolic PKM2 at two different protein loads. **C.** Spike recovery shows that rPKM2 is similar to native PKM2 in cell/tissue lysates.

Pre-clinical assessment of PKM2 activator, NSC772991, in H1299 (lung cancer) model

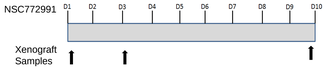

Figure 4. Study schema for preclinical evaluation of PKM2 activator (NSC772991). Mice bearing lung cancer (H1299) xenograft were given daily doses of 6, 20, and 60 mg/kg of PKM2 activator for 10 days. Tumor quadrants were collected 2 h, 6 h, 24 h, and 48 h after dosing mice.

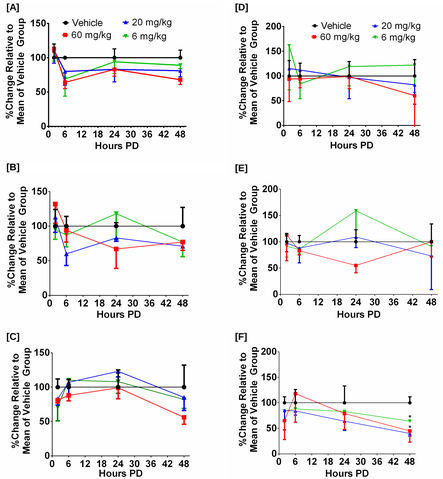

Figure 5. PD monitoring of PKM2 activator. (A-C) Measurements of PKM2 in cytosolic fractions of tumor lysates on day 1 (**A**), day 3 (**B**), and day 10 (**C**). PKM2 levels in the cytosolic fraction showed no significant change. **(D-F).** Measurements of PKM2 in nuclear-mitochondrial fractions of tumor lysates on day 1 (**D**), day 3 (**E**), and day 10 (**F**). We observed a 30-60% decrease in PKM2 levels following the 48 h sample on day 10 ($p \leq 0.05$).

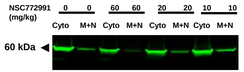

Figure 6. Western blot analysis of tumor lysates confirmed PKM2 results obtained by immunoassay. Western blot analysis of tumor lysates (25 ug protein/lane) showed a dose-dependent decrease in PKM2 levels in the nuclear mitochondrial fraction for day 10 samples. No significant changes were observable in the cytosolic fraction. These results are consistent with the changes in PKM2 levels measured by the PKM2 immunoassay.

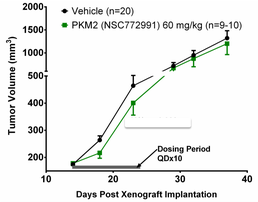

Figure 7. Effect of NSC7729991 on lung cancer H1299 xenograft tumor growth. Measurements of tumor volume over 10 days of dosing with 60 mg/kg of PKM2 activator showed a non-significant decrease (18%) in tumor volume. Tumor volume for lower doses were not shown as they are superimposable on the vehicle group.

Summary of PD Monitoring of PKM2 activator. Our preclinical evaluation of NSC772991 (at doses that were lower than MTD) did not disclose pharmacodynamic response or suppression of tumor growth in lung cancer H1299 model. We are currently testing a higher dose of PKM2 activator.

Summary and Conclusions

- The PKM2 sandwich immunoassay developed in this study appears to be reactive to the PKM2 dimer (and possibly monomer). However, the formation of a tetramer leads to loss of epitope and reduced signal by sandwich immunoassay.
- Since PKM2 activators promote tetramer formation of PKM2, we believe the assay can be used for pharmacodynamic evaluation of PKM2 activators.
- In the tumor xenograft models we tested, large amounts of PKM2 appear to be accumulated in the nuclear fractions of tissue lysates. These observations were consistent with the findings that nuclear PKM2 promotes proliferation (Gao et al., Mol Cell, 2012) in growing tumors.
- Measurement of PKM2 in the cytosolic fraction of tissue lysates was affected by the kinetics of equilibrium between dimer and tetramer. Therefore, we measured cytosolic PKM2 at two different lysate dilutions to determine PD modulation. However, treatment with NSC772991 did not show a consistent decrease in cytosolic PKM2.
- The majority of the PKM2 in tumor lysates from xenografts treated with NSC772991 was detected in nuclear fraction. Our results show that nuclear PKM2 was reduced by 30-60% between 24 h and 48 h after day 10.
- There was a non-significant reduction in tumor volume at the highest dose (60 mg/kg) of NSC772991 tested.
- The absence of PKM2 modulation was consistent with the lack of efficacy of the PKM2 activator (NSC772991) at the doses and schedule tested in this study. We are currently testing higher doses of NSC772991.

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All animals used in this research project were cared for and used humanely according to the following policies: the *U.S. Public Health Service Policy on Humane Care and Use of Animals* (2000); the *Guide for the Care and Use of Laboratory Animals* (1996); and the *U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* (1985). All Frederick National Laboratory animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Funded by NCI Contract No HHSN261200800001E.

Prepared by Scientific Publications Graphics & Media
Frederick National Laboratory for Cancer Research, Frederick, MD [204659]