Application Note

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Discovery of a New Inhibitor for PFKFB2 Kinase Activity Through Mechanistic Studies

Using complimentary metabolic analyses with an Agilent Seahorse XFe24 analyzer and an Agilent 6546 LC/Q-TOF system

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Abstract

The 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) family of proteins regulate glycolysis in insulin-sensitive tissues and cancer. In this application note, we sought to develop an assay to identify small molecules that can inhibit PFKFB kinase activities. Testing a molecule library selected by the Atomwise AtomNet AI platform, we discovered a new inhibitor, B2, which inhibits PFKFB2 and PFKFB3. Using complementary techniques, an Agilent Seahorse XFe24 analyzer showed B2 reduced glycolysis and glycolytic capacity in A-498 cells. An Agilent 6546 quadrupole time-of-flight LC/MS (LC/Q-TOF) confirmed the effect of B2 by showing decreased glycolytic intermediates and increased fructose-6-phosphate, indicating PFK-1 inhibition. These findings validate B2 as a new PFKFB kinase inhibitor and the dual metabolic analysis using a Seahorse XFe24 analyzer and a 6546 LC/Q-TOF system to facilitate drug discovery research.

Introduction

The 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) family of bifunctional enzymes plays a critical role in regulating glycolysis through their control of fructose-2,6-bisphosphate (F-2,6-BP) levels.[1–3] F-2,6-BP is a potent allosteric activator of phosphofructokinase-1 (PFK-1), the rate-limiting enzyme of glycolysis, and thus, PFKFB enzymes are essential for modulating cellular glucose metabolism. The four PFKFB isoforms (PFKFB1–4) are expressed in various tissues, each isoform playing distinct roles in metabolic processes such as glycolysis, gluconeogenesis, and cellular response to stress. Notably, PFKFB2 is the primary isoform in the heart, and its activity is regulated by phosphorylation. Dysregulation of PFKFB2 and other isoforms has been implicated in various diseases, including cancer, diabetes, and liver fibrosis.[4–9]

Given their central role in metabolism, PFKFB enzymes are promising therapeutic targets, but progress in drug discovery has been hindered by challenges in accurately measuring their activity. Specifically, the instability of F-2,6-BP, the lack of commercially available standards, and the complexity of kinase assays make it difficult to evaluate potential modulators of PFKFB activity. To address these challenges, we developed a reliable method for measuring PFKFB2 activity and used it to screen a library of small molecules selected by an AI-driven platform.[10] This screening led to the identification of a new inhibitor, B2, which effectively inhibits both PFKFB2 and PFKFB3, and blocks PFK-1 activity. This inhibitor has potential for a wide range of biological applications; the original research leading to its identification is detailed in Eyster *et al.* (2025).[11]

Experimental

Seahorse XFe24 assay method

The kidney cancer cell line A-498 (HTB-44) was obtained from ATCC. To assess the impact of PFKFB2/3 inhibitors on glycolysis, a glycolysis stress test was performed using a Seahorse XFe24 extracellular flux analyzer. The cells were cultured in DMEM media until they reached confluency, then treated with DMSO, 5 µM B2, 25 µM B2, or 5 µM PFK158 in Seahorse media. During the assay, glucose, oligomycin, and 2-deoxyglucose were added sequentially. Afterward, Hoechst 33342 was added to the wells, and live cell counts were measured using an Agilent BioTek Cytation 5 cell imaging multimode reader for normalization.

Note: A more quantitative assay for monitoring glycolysis, the XF glycolytic rate assay, is now available, which allows users to perform the assay under normal physiological conditions (no starvation needed). Additionally, data quality and precision can be improved by using the updated Agilent Seahorse XF Flex analyzer.

6546 LC/Q-TOF metabolomic analysis method

After A-498 cells reached 95% confluence in culture plates, the media was replaced with DMEM containing DMSO, 5 µM B2, or 5 µM PFK158. The cells were incubated for 60 minutes at 37 °C, then washed with PBS and snap-frozen in liquid nitrogen. The plates were stored at –80 °C and, while on dry ice, 1 mL of chilled methanol was added to each plate and swirled for 1 minute to cover the cells. The cells were scraped, transferred to Eppendorf tubes, and additional methanol was added to recover all cells. The cells were sonicated, incubated on dry ice, and then thawed. After adding 350 µL of Milli-Q water, the mixture was shaken and centrifuged. The supernatant was filtered using Agilent Captiva EMR–Lipid cartridges and dried in a vacuum centrifuge for about 4 hours. The dried samples were stored at –80 °C, then reconstituted in a 7:3 mixture of acetonitrile and water, and transferred to LC/MS vials.

LC/MS analysis was performed on an Agilent 1290 Infinity II LC with a 6546 LC/Q-TOF. In this application note, we adapted a standardized Agilent InfinityLab Poroshell 120 HILIC-Z metabolomics method documented in Yannell *et al.*[12] for use with a quaternary pump. Data analysis and peak integration of target metabolites were done using Agilent MassHunter software (v10.1). A standard mix of glycolytic and TCA cycle intermediates was analyzed along with the samples to identify metabolites. A custom Agilent Personal Compound Database and Library (PCDL) was created using METLIN to help identify target metabolites. Retention times from the standard mix analysis were added to the PCDL for easier identification. Relative abundance of metabolites was calculated by normalizing the data to total protein levels, which were measured using a Bradford protein assay on cell pellets from the methanol-water extraction.

Candidate selection using AtomNet technology

To identify small molecules that could modulate PFKFB2 kinase activity, AtomNet technology was used for virtual screening. AtomNet employs artificial intelligence (AI) and high-resolution protein crystallography data to predict potential small molecule binders to target proteins. Using the X-ray crystal structure of PFKFB2 (PDB ID: 5HTK)[13], AtomNet selected 64 compounds to be tested (at 10 µM each) for their ability to influence PFKFB2 kinase activity compared to a DMSO control (Figure 1B).

This screening identified several potential activators and inhibitors, which were further validated in subsequent experimental assays (Figure 1A). The most potent inhibitor of PFKFB2 activity was identified as B2 (5-[(2-methoxyphenyl) amino]-1,3,4-thiadiazole-2-thiol). B2 inhibits PFKFB2 with an IC_{50} of 3.289 µM (Figure 1C). It also inhibits PFKFB3 with an IC_{50} of 11.89 µM. B2 has a different chemical structure from other known PFKFB inhibitors. Compared to PFK158 (Figure 1D), a known inhibitor, B2 is slightly less potent but still effective in inhibiting PFKFB2 and PFKFB3 kinase activities. In this work, B2 will be characterized further in the enzymatic assays and cellular metabolism studies.

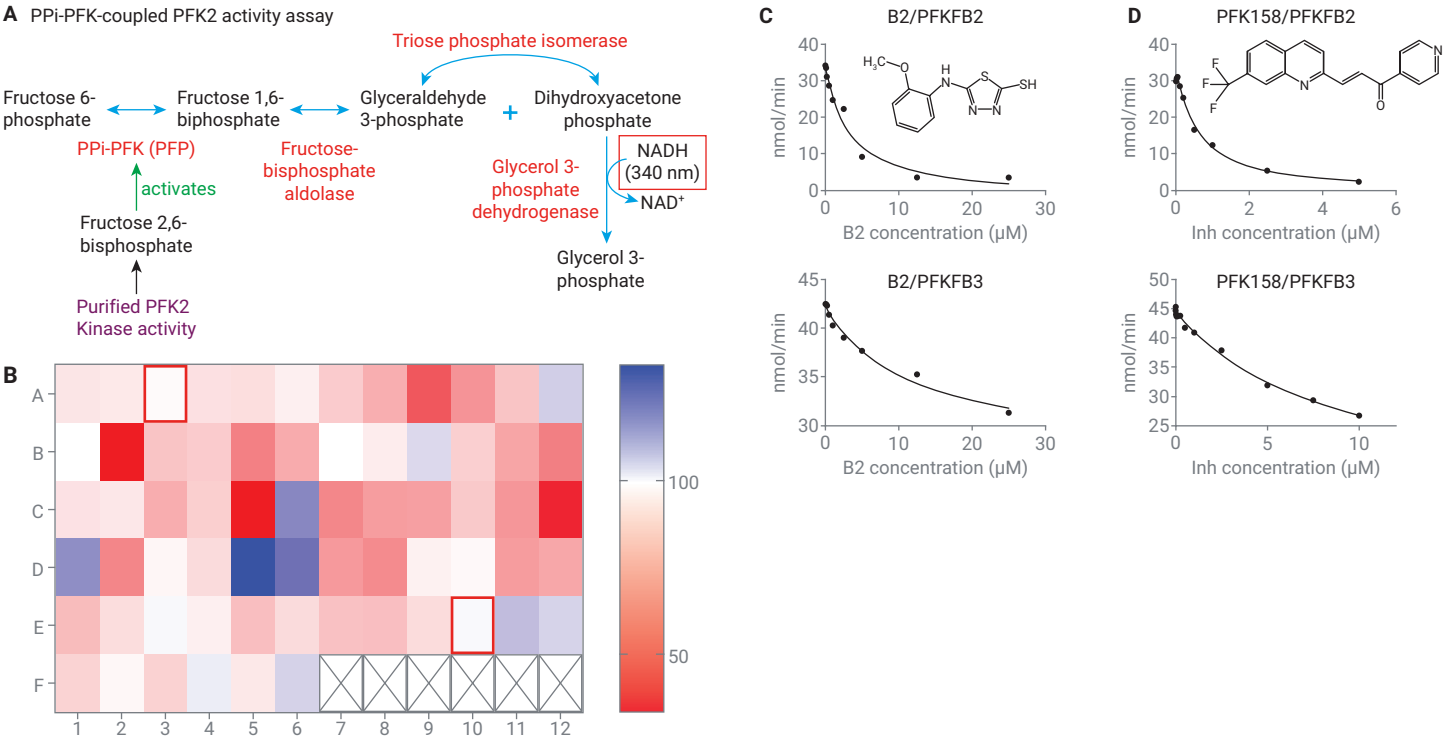

Figure 1. Development of a PFKFB kinase activity assay and screening of potential modulators. (A) Diagram of the coupled enzyme activity assay, where purple shows the reaction mix with purified PFKFB isoforms and red shows added enzymes. The product, glycerol-3-phosphate, is measured by the decrease in NADH at 340 nm. (B) Screening of candidate compounds (10 µM in DMSO), with DMSO controls in wells A3 and E10 (red boxes). (C) IC_{50} values for the candidate inhibitor B2 against PFKFB2 (top) and PFKFB3 (bottom) kinase activities (structure shown in the inset). (D) IC_{50} values for the known PFKFB3 inhibitor PFK158 against PFKFB2 (top) and PFKFB3 (bottom) kinase activities (structure shown in the inset).

Results and discussion

This paper demonstrates the benefits of using Seahorse XFe24 and 6546 LC/Q-TOF analysis together to study the impact of small molecule inhibitors on cellular metabolism, particularly glycolysis, and to characterize the enzymatic activity of PFKFB2 (phosphofructokinase-2/fructose-2,6-bisphosphatase 2). Updated versions of these instruments exist, including the Seahorse XF Flex Analyzer, with the updated Seahorse XF Glycolytic Rate Assay, and the Revident LC/Q-TOF. These instruments and reagents will be able to provide similar or enhanced results to those shown here.

Seahorse XFe24 assay results

The Seahorse extracellular flux analyzer was employed to measure the extracellular acidification rate (ECAR), which reflects cellular glycolytic activity. In the study, both B2 (at 5.0 and 25 µM) and PFK158 were shown to significantly decrease basal glycolysis in A-498 cancer cells (Figure 2A). Additionally, B2 was observed to inhibit glycolytic capacity (Figure 2B), which is the maximal glycolytic rate achievable after oligomycin treatment. These data clearly demonstrated that B2 inhibits glycolysis at a similar level to PFK158 but with a unique effect on glycolytic capacity.

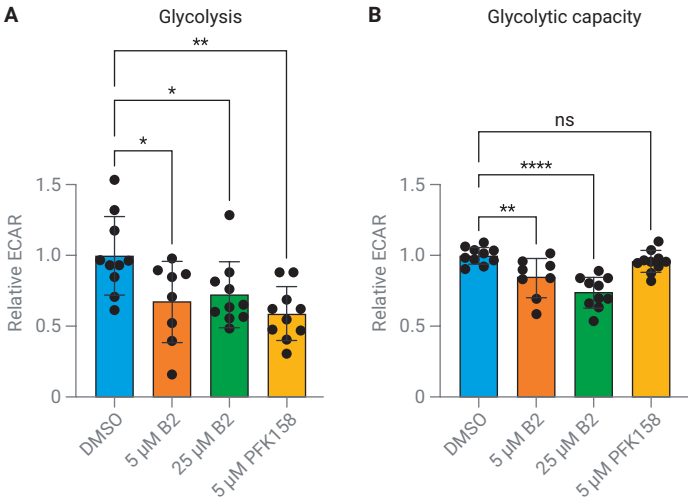

Figure 2. B2 inhibits glycolysis and glycolytic capacity. A-498 cells were treated with 5 µM B2 for 60 minutes. (A) Glycolysis rates were measured by ECAR after glucose addition, and (B) glycolytic capacity was measured by ECAR after adding oligomycin. Each data point represents a well from a Seahorse XFe24 plate, with the experiment done on two separate plates. Data are shown as mean ± SD. Statistical significance is indicated as: ns ($P > 0.05$), *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$ by one-way ANOVA with multiple comparisons to the DMSO control.

6546 LC/Q-TOF metabolomic analysis results

Targeted LC/MS analysis was conducted on glycolytic intermediates extracted from A-498 cells treated with either B2 or PFK158. This analysis revealed differential effects on specific glycolytic intermediates. B2 caused a significant decrease in fructose-1,6-bisphosphate and the ratio of fructose-6-phosphate to fructose-1,6-bisphosphate (Figure 3B). Additionally, B2 treatment led to decreased levels of downstream glycolytic intermediates such as phosphoenolpyruvate (PEP), glyceraldehyde 3-phosphate (G3P), and 3-phosphoglyceric acid (3-PGA), highlighting its specific effect on the PFK1 step in glycolysis (Figure 3C). The principal component analysis (Figure 3A) further validated that the metabolic profiles of cells treated with B2 were distinct from those treated with PFK158, reinforcing that B2 acts through a unique mechanism.

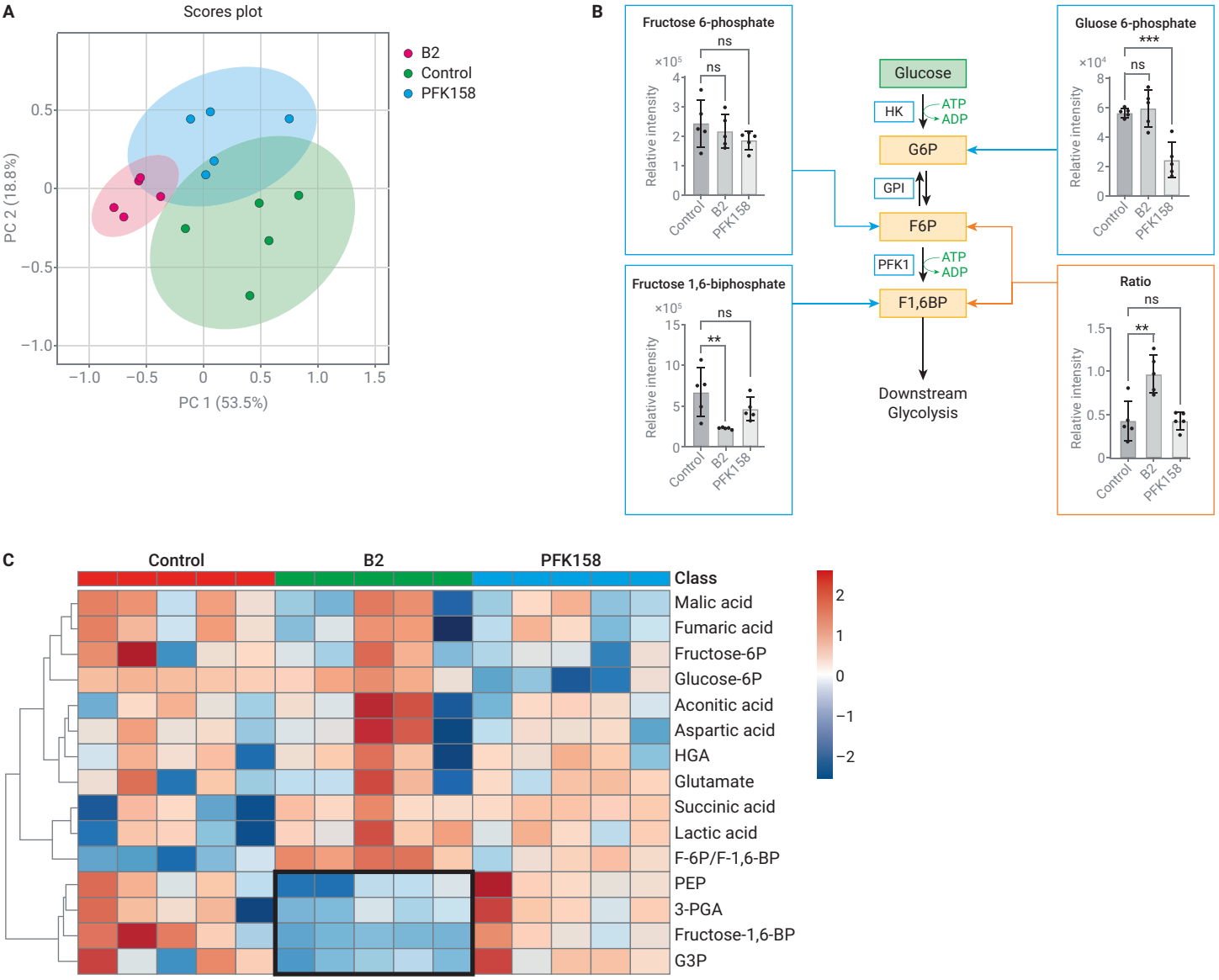

Figure 3. B2 decreases glycolytic intermediates in cells. A-498 cells were treated with B2 or PFK158 for 60 minutes, and metabolites were extracted for LC/MS analysis. (A) PCA analysis shows differences between control, B2, and PFK158 treatment groups. (B) Levels of early glycolytic intermediates and the ratio of F-6-P to F-1,6-BP are shown. (C) A heatmap of metabolomic data shows decreased downstream glycolytic intermediates in B2-treated cells, with the ratio of F-6-P to F-1,6-BP included. Other abbreviations: HGA (DL-hydroxyglutaric acid), PEP (phosphoenolpyruvic acid), 3-PGA (3-phosphoglyceric acid), G3P (glyceraldehyde 3-phosphate). Data are shown as mean ± SD (n = 5). Statistical significance: ns (not significant), **P ≤ 0.01, ****P ≤ 0.0001 by one-way ANOVA with multiple comparisons to the vehicle control. PCA and heatmap were generated with MetaboAnalyst 6.0; figure created with Biorender.

Conclusion

The Seahorse assays provided real-time, functional data on cellular glycolytic activity, while the LC/MS analysis offered a more detailed metabolomic profile of the intracellular changes. This combination of approaches enabled pinpointing the specific effects of B2 on glycolytic flux and to validate its role as a PFKFB2 inhibitor. By integrating these two techniques, the study provided a comprehensive understanding of how B2 modulates metabolic pathways, which would not have been possible with either method alone.

Acknowledgements

This work at Oklahoma Medical Research Foundation was supported in part by Mostafa Ahmed at Atomwise Inc and the Atomwise AIMS award #A18-142 to Dr. Kenneth Humphries.

References

1. Rider, M. H.; *et al.* 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase: Head-to-Head With a Bifunctional Enzyme that Controls Glycolysis. *Biochem. J.* **2004**, *381*, 561–579. DOI: 10.1042/BJ20040752
2. Bartrons, R.; *et al.* Fructose 2,6-Bisphosphate in Cancer Cell Metabolism. *Front Oncol.* **2018**, *8*, 331. DOI: 10.3389/fonc.2018.00331
3. Okar, D. A.; *et al.* PFK-2/FBPase-2: Maker and Breaker of the Essential Biofactor Fructose-2,6-Bisphosphate. *Trends Biochem. Sci.* **2001**, *26*, 30–35. DOI: 10.1016/s0968-0004(00)01699-6
4. Jones, B. C.; Pohlmann, P. R.; Clarke, R.; Sengupta, S. Treatment Against Glucosedependent Cancers Through Metabolic PFKFB3 Targeting of Glycolytic Flux. *Cancer Metastasis Rev.* **2022**, *41*, 447–458. DOI: 10.1007/s10555-022-10027-5
5. Kitamura, K.; Kangawa, K.; Matsuo, H.; Uyeda, K. Phosphorylation Of Myocardial Fructose-6-Phosphate,2-Kinase: Fructose-2,6-Bisphosphatase by Camp-Dependent Protein Kinase And Protein Kinase C. Activation by Phosphorylation and Amino Acid Sequences of the Phosphorylation Sites. *J. Biol. Chem.* **1988**, *263*, 16796–16801.
6. Xiao, M.; Liu, D.; Xu, Y.; Mao, W.; Li, W. Role of PFKFB3-Driven Glycolysis in Sepsis. *Ann. Med.* **2023**, *55*, 1278–1289. DOI: 10.1080/07853890.2023.2191217
7. Liu, Q.; *et al.* Advances in the Understanding of the Role and Mechanism of Action of PFKFB3-Mediated Glycolysis in Liver Fibrosis (Review). *Int. J. Mol. Med.* **2024**, *54*. DOI: 10.3892/ijmm.2024.5429
8. Griesel, B. A.; *et al.* PFKFB3-Dependent Glucose Metabolism Regulates 3T3-L1 Adipocyte Development. *FASEB J.* **2021**, *35*, e21728. DOI: 10.1096/fj.202100381RR
9. Yi, M.; *et al.* 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 and 4: A Pair Of Valves for Fine-Tuning of Glucose Metabolism in Human Cancer. *Mol. Metab.* **2019**, *20*, 1–13. DOI: 10.1016/j.molmet.2018.11.013
10. Atomwise, A. P. AI is a Viable Alternative to High Throughput Screening: A 318-Target Study. *Sci. Rep.* **2024**, *14*, 7526. DOI: 10.1038/s41598-024-54655-z
11. Eyster, C; *et al.* Mechanistic Studies of PFKFB2 Reveal a Novel Inhibitor of its Kinase Activity. *PLOS One* **2025**. https://doi.org/10.1371/journal.pone.0317167
12. Yannell, K.; *et al.* An End-to-End Targeted Metabolomics Workflow. *Agilent Technologies application note*, publication number 5994-5628EN, **2023**.
13. Crochet, R. B.; *et al.* Crystal Structure of Heart 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase (PFKFB2) and the Inhibitory Influence of Citrate on Substrate Binding. *Proteins* **2017**, *85*, 117–124. DOI: 10.1002/prot.25204

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DE-008623

Printed in the USA, July 29, 2025
5994-8444EN

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