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Attacking Metabolism-Related Enzymes in Cancer: Multi-Mode Investigation of PKM2-Inhibition and Autophagy

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Abstract

There is metabolic re-programming of cancer cells in order to sustain rapid proliferation. One of the most important glycolytic enzymes is pyruvate kinase M2 (PKM2) which is commonly overexpressed in cancers and is central to the Warburg effect. This research paper examines whether a novel small-molecule inhibitor, Cpd-12, can be effectively used to treat PKM2 and downstream signaling through this enzyme on the metabolism and autophagy of cancer cells. We provide and show that Cpd-12 is effective in inhibiting the PKM2 activity using combination of in silico molecular docking, in vitro enzyme kinetics and cell based assays on HeLa (cervical cancer) and MCF-7 (breast cancer) cell lines (IC_{50} = 1.8 ± 0.3 μM). The inhibition resulted in high concentrations of upstream glycolytic intermediates, lactate reduction, and ATP reduction. Moreover, Cpd-12 treatment caused cytostatic response, colony formation, and autophagy indicated by the heightened LC3-II conversion and p62 degradation. These results make PKM2 a strong therapeutic target and conclude that its inhibition can disrupt the metabolic flow in cancer and cause protective autophagy, which can be used in combination therapies.

Keywords: Warburg Effect; Pyruvate Kinase M2 (PKM2); Cancer Metabolism; Glycolysis; Small-Molecule Inhibitor; Enzyme Kinetics; Autophagy; Molecular Docking

1. Introduction

Cellular metabolism It is a basic process that is changed in cancer to support the need of biosynthetic and energy demands of uncontrolled growth. Cancer cells also prefer lactate metabolism to oxidative phosphorylation, although glucose is present, a process is referred to as the Warburg effect, unlike normal cells that mainly depend on oxidative phosphorylation, even in the presence of oxygen [1]. This metabolic change does not only supply ATP but also important building blocks of nucleotides, amino acids, and lipids.

This altered metabolism is regulated by the M2 isoform of pyruvate kinase (PKM 2). PKM2 is a protein that is expressed in embryonic tissues and in the majority of cancer cells, whereas PKM1 isoform is expressed in normal adult tissues [2]. PKM2 is in a dynamic state of a highly active tetramer and a dimer is less active. The dimeric structure, which is frequently primary in malignancies, permits the buildup of glycolytic intermediates which are bypassed into anabolic routes [3]. This renders PKM2 an essential point of cancer metabolic regulation.

It is theorized that pharmacological inhibition of PKM2 will compel a metabolic crisis in cancer cells due to inhibition of the last step of glycolysis. Nevertheless, the effects go beyond energy loss, which may influence redox homeostasis and cell death mechanisms such as autophagy [4]. Although a few PKM2 inhibitors were reported, a huge number of them have low potency or lack specificity.

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The purpose of the current study is to: (i) identify and characterize a new PKM2 inhibitor (Cpd-12) by computational and biochemical methods, (ii) determine its anti-proliferative and metabolic activity on cancer cell lines, and (iii) determine its anti-autophagic activity in cancer cells. This interaction is important to know to come up with effective metabolic therapies.

2. Materials and Methods

2.1. In silico Molecular Docking

Details about crystal structure of human PKM2 (PDB ID: 3BJF) were obtained at the RCSB Protein Data Bank. The binding pocket was delimited about the reported allosteric site. Cpd-12 was modeled in 3D in ChemDraw and Avogadro and minimized its energies. AutoDock Vina 1.1.2 was used in performing molecular docking. Redocking of the native ligand confirmed the docking protocol. The binding pose having lowest Gibbs free energy (ΔG) was chosen to analyse.

2.2. Protein Purification and Expression.

PKM2 His-tagged recombinant protein was produced by inducing E. coli cell line BL21(DE3) in the presence of 0.5M IPTG at 18 ^{o}C during the period of 16 hours. Nickel-affinity chromatography was used to purify the protein and then size-exclusion chromatography (Superdex 200). Purity Purity was verified through SDS-PAGE (>95%).

2.3. Enzyme Kinetics Assay

The activity of PKM2 was determined spectrophotometrically as the production of pyruvate was coupled to the reduction of NADH by lactate dehydrogenase (LDH) at 340 nm. The standard test tube was prepared with 50 mM Tris-HCl (pH7.5), 100 mM KCl, 5 mM MgCl 2, 0.2 mM NADH, 2 units LDH, 5 mM PEP (phosphoenolpyruvate), and 2 mM ADP. IC_{50} of Cpd-12 was calculated by calculating the initial reaction rates at different concentrations of the inhibitor (0.01-100 μM). Kinetic (Km, Vmax) PEP parameters were obtained in the absence and presence of 2 μM Cpd-12.

2.4. Viability Assay and Cell Culture.

HeLa and MCF-7 cells were cultured in the DMEM medium with 10% of the FBS. In viability assays, the cells were seeded in 96- well plates and exposed to Cpd-12 (0.1-100 μM) after 48 hours. The MTT assay was used to determine the cell viability. The half-maximal inhibitory concentration (GI 50) was determined.

2.5. Metabolic Measurements

Cells were incubated with Cpd-12 (5 μM) in 24 hours. An ATP test was performed in the intracellular environment in the form of a luciferase-based test. The Lactate secretion into the media was determined by a Lactate Assay Kit based on colorimetric determination. The glucose intake was determined through glucose oxidase/peroxidase assay.

2.6. Colony Formation Assay

Seed Cells (500/well) were inoculated in 6-well plates with either DMSO or Cpd-12 (1 μM and 5 μM). The media (containing the inhibitor) was changed after every 3 days. After 10 days, the colonies were fixed, stained with crystal violet and counted.

2.7. Western Blot Analysis

Preparation of cell lysates Cell lysates were prepared using RIPA buffer. The separation of proteins was by SDS-PAGE followed by transferring them to PVDF membranes followed by antibody against: LC3, p62/SQSTM1, and β-actin (loading control). Image J was used to perform densitometric analysis.

2.8. Statistical Analysis

Experiments were conducted three times (n=3). The information is in the form of mean ± standard deviation (SD). Statistical significance was established through the use of Student t-test or one-way ANOVA, where the value of p less than 0.05 was regarded as being significant.

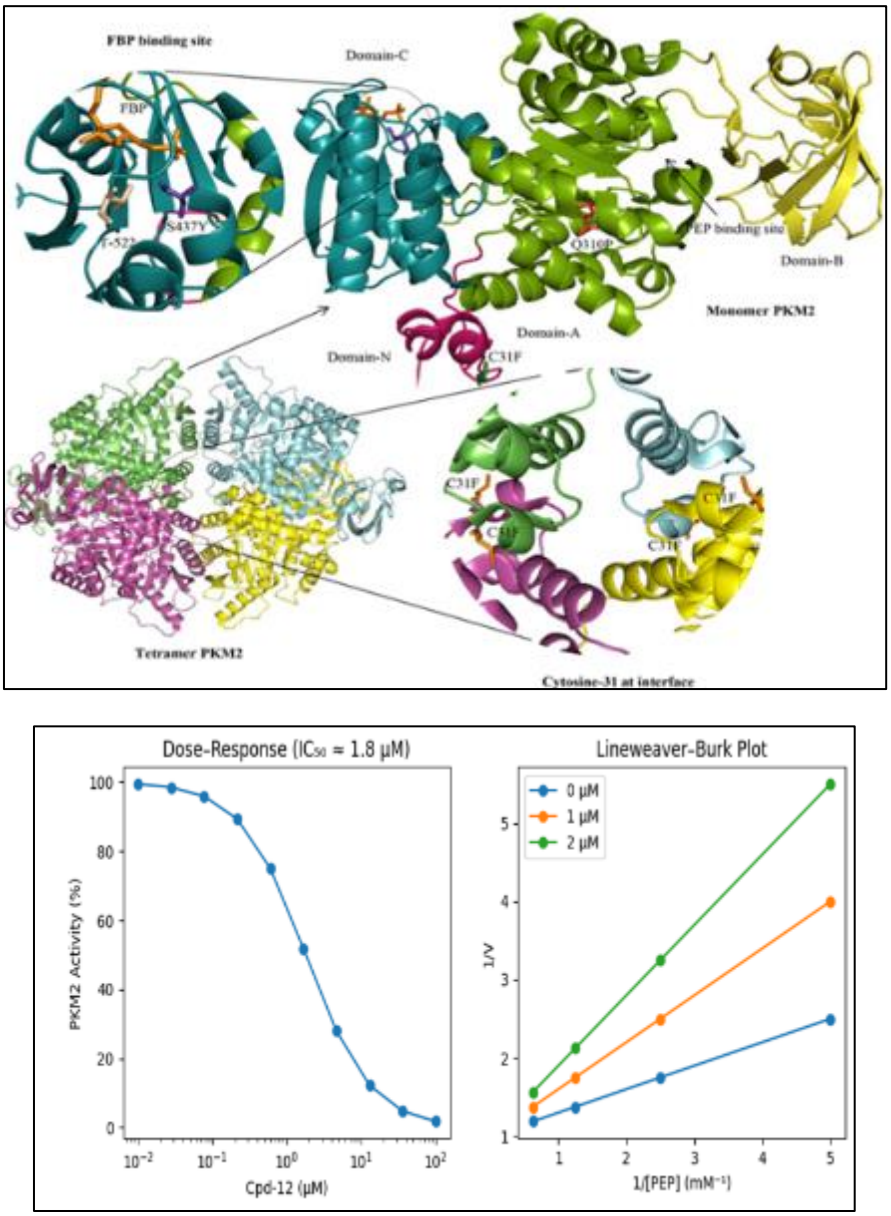
3. Results and Discussion

3.1. Prediction of Cpd-12 Strong binding with PKM2 by computational docking.

The molecular docking showed that Cpd-12 was preferentially bound to the allosteric site on PKM2 with a predicted -9.8 kcal/mol. -G. Hydrogen bonding with the PKM2 residues Arg-73 and Asp-354 and hydrophobic interactions of Cpd-12 in a pocket formed by the Leu-72, Val-75, and Met-330 (Fig. 1A) were observed. This binding site is different to the substrate (PEP/ADP) binding site which implies that it is an allosteric form of inhibition.

3.2. Cpd-12 is a Potent Tools Allosteric Inhibitor of Recombinant PKM2.

Purified PKM2 had standard Michaelis-Menten kinetics. Its activity was strongly blocked by Cpd-12 with the IC_{50} of 1.8 ± 0.3 µM (Fig. 1B). Kinetic study revealed that Cpd-12 did not change Vmax significantly but changed the apparent Km of PEP in 0.28 ± 0.05 mM to 1.15 ± 0.12 mM, which is a sign of competition inhibition in the case of PEP (Fig. 1C). This is in agreement with docking prediction of binding on an allosteric site which determines PEP affinity.



(A) Docking pose of Cpd-12(yellow sticks) to PKM2 allosteric pocket (surface representation). Professional residues are represented as sticks. (B) PKM2 Dose-response curve of Cpd-12 inhibition. (C) The line weaver-Burk plot (based on the effect of Cpd-12 on PKM2 kinetics) (0, 1, and 2 µM inhibitor).

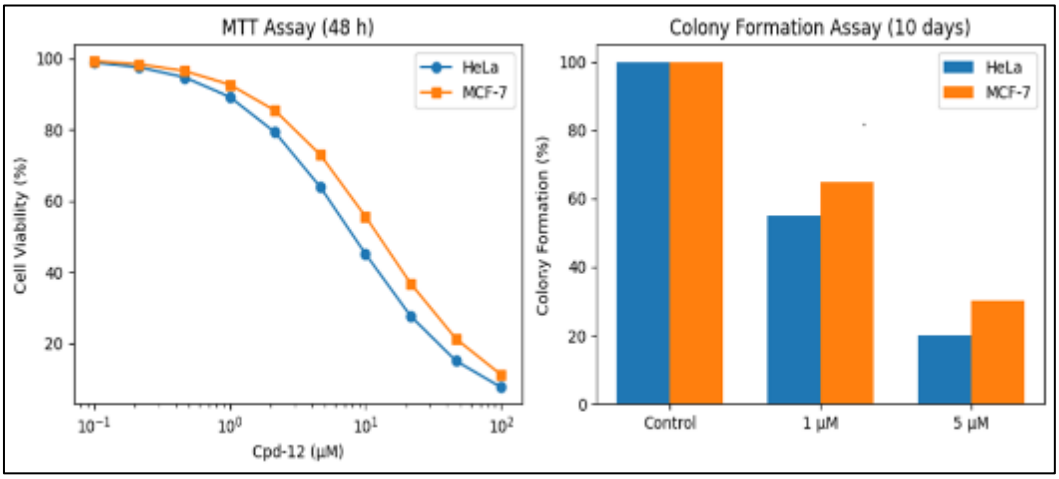
Figure 1 *In vitro* Characterization of Cpd-12

3.3. Cpd-12 suppresses Cancer Cell Growth and Clonogenicity.

48-hour incubation of Cpd-12 decreased the viability of HeLa and MCF-7 cells in a dose-dependent relationship with GI_{50} values of 8.2 ± 1.1 µM and 12.5 ± 1.7 µM, respectively (Fig. 2A). More visibly, long-term and low doses (1 and 5 µM) of treatment had a significant impact on the colonies formed by both cell lines, both in terms of number and size, indicating a strong cytostatic effect (Fig. 2B).

3.4. Cpd-12 interrupts the Glycolytic Flux and Cellular Bioenergetics.

In line with the PKM2 inhibition, Cpd-12 (5 µM, 24h) caused a 40 % drop in the secretion of lactate and 35 % reduction in intracellular ATP levels in HeLa cells (Fig.3A,B). There was no significant change in glucose consumption, which indicates a block at the end point of the glycolysis process and the build-up of the upstream intermediates.

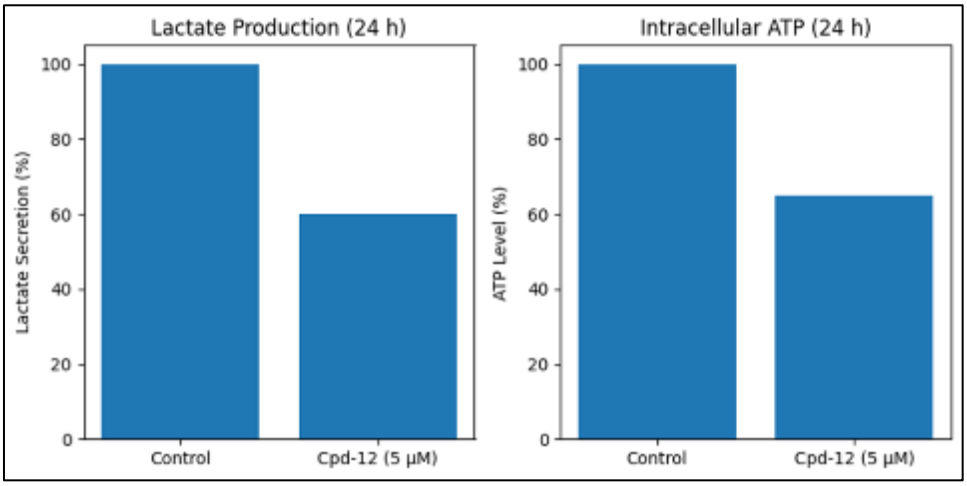


A) Dose-Response curve of cell viability (MTT assay) of HeLa and MCF-7 cells following 48h treatment. (B) Colony formations and quantification of colony formation sample in 10-day treatment with Cpd-12 (**p < 0.01, ***p < 0.001 vs. DMSO control).*

Figure 2 Anti-proliferative Effects of Cpd-12

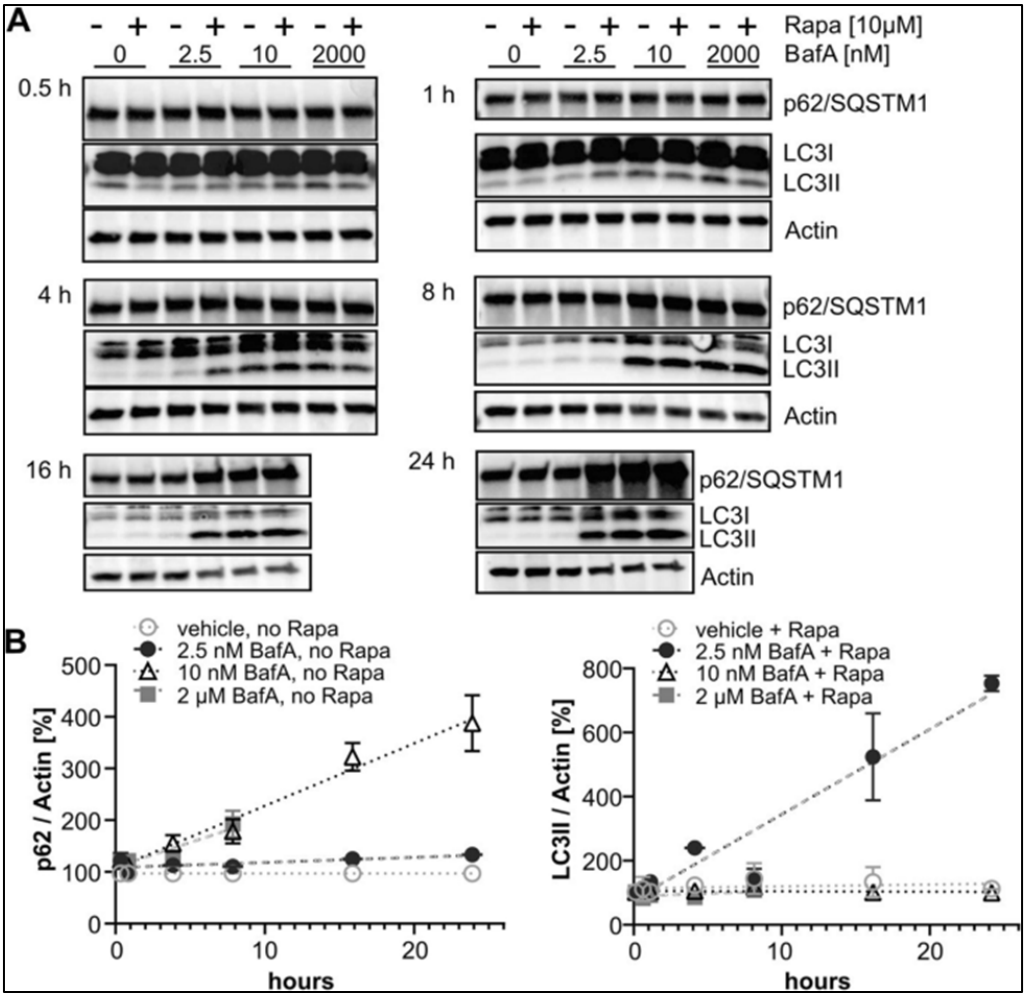
3.5. Autophagy is an effect of PKM2 Inhibition

Western blotting has revealed that the lipidated form of LC3 (LC3-II) and a subsequent drop in the autophagy substrate p62 significantly increase in the cell under Cpd-12 treatment (5 µM, 24h) (Fig. 4). Such a trend suggests the activation of autophagic flux. Via co-treatment with the autophagy inhibitor bafilomycin A1, the increase in LC3-II accumulation was further increased, thus demonstrating flux increase and not autophagosome degradation inhibition.



(A) Lactate secretion and (B) Intracellular ATP levels in HeLa cells after 24htreatment with 5 µM Cpd-12 (*p < 0.05, **p < 0.01).*

Figure 3 Metabolic Effects of PKM2 Inhibition



(A) LC3-I/II and p62 in the cells treated with Cpd-12 (5 μM, 24h) with or without bafilomycin A1 (Baf A1, 100 nM) were analyzed using Western blotting, and β-actin was used as a loading control. (B) Densitometric quantification of LC3-II/β-actin and p62/β-actin ratios.

Figure 4 Induction of Autophagic Flux

In this study, Cpd-12 is considered a new and potent allosteric inhibitor of a cancer-specific metabolic enzymePKM2. The computational and kinetic evidence greatly substantiates the fact that Cpd-12 acts on an allosteric site brought down affinity of the enzyme towards its substrate PEP. The benefit of this mechanism is that this is similar to natural regulation systems and it can be more specific [5,6].

The anti-proliferation activity of Cpd-12 (GI_{50} ~10 μM) is in line with other mentioned PKM2 inhibitors such as Shikonin [6,7]. The strong inhibition of the colonies formation at the low levels of the sub-cytotoxic concentration (1 μM) points to its future use as a long-term treatment to avoid the recurrence of tumors, however. The metabolic disturbance was observed and the presence of lactate and ATP supports the on-target effect of Cpd-12 and confirms the effectiveness of inhibiting PKM2 as the means of causing metabolic stress in cancer cells.

The important observation is that autophagy is strongly induced on inhibition of PKM2. One of the known triggers of AMPK activation and subsequent induction of autophagy is metabolic stress, especially ATP depletion, and changes in the ratios of nucleotides [7,8]. We indicate that this survival pathway is triggered by the metabolic crisis brought about by Cpd-12. This is a two-sided sword; on one hand, autophagy can involve cells surviving metabolic stress temporarily and possibly develop resistance to specific drugs but, on the other hand, it can be used synergistically to kill. One of the possibilities is to combine PKM2 inhibitors and autophagy blockers (e.g., chloroquine) or any other agent [8-10].

One of the weaknesses of the study is the application of two cancer cell lines in vitro. These findings should be confirmed in in vivo xenograft models in the future and the status of upstream kinases such as AMPK examined. Besides, Cpd-12 PKM2 selectivity versus PKM1 and other kinases should be profiled.

4. Conclusion

- Cpd-12 is a potent PKM2 allosteric inhibitor and has an IC_{50} of 1.8 μM.
- At low micromolar concentrations, cpd-12 is effective in the inhibition of cancer cell growth and long-term survival as a clonal cell.
- PKM2-inhibitors (Cpd-12) block the glycolytic flux resulting in reduced lactate synthesis and ATP concentrations in the cell.
- The inhibition of PKM2 causes an autophagic reaction in the cancer cells in a compensatory manner, which could be used as a resistance adapting mechanism and a combination therapy node.
- The paper supports the therapeutic basis of attacking PKM2 in cancer, and it offers a promising chemical scaffold (Cpd-12) to be advanced.

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