Metabolic Response to the Mitochondrial Toxin 1-Methyl-4-phenylpyridinium (MPP+) in LDH-A/B Double-knockout LS174T Colon Cancer Cells

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Abstract. Background/Aim: Rapid glycolytic substrate-level phosphorylation (SLP) and accumulation of lactic acid are characteristics of diverse cancers. Recent advances in drug discovery have included the use of glycolytic inhibitors with mitochondrial targeting drugs to attempt to invoke an energy crisis in aggressive metabolically active chemo-resistant cancers. In this work, we examine the consequences of inhibiting mitochondrial oxidative phosphorylation (OXPHOS) with 1-methyl-4-phenylpyridinium (MPP+) in LS174T colon cancer cells containing a genetic double knockout (DKO) of lactic acid dehydrogenase (LDHA and LDHB).

Materials and Methods: Several metabolic parameters were evaluated concomitant to whole transcriptomic (WT) mRNA, microRNA, and long intergenic non-coding RNAs using Affymetrix 2.1 human ST arrays. Results: MPP+ effectively blocked OXPHOS where a compensatory shift toward anaerobic SLP was only observed in the control vector (CV), and not observed in the LDH-A/B DKO (lacking the ability to produce lactic acid). Despite this, there was an unexpected resilience to MPP+ in the latter in terms of energy, which displayed significantly higher resting baseline respiratory OXPHOS capacity relative to controls. At the transcriptome level, MPP+ invoked 1738 differential expressed genes (DEGs) out of 48,226; LDH-A/B DKO resulted in 855 DEGs while 349 DEGs were found to be overlapping in both groups versus respective controls, including loss of mitochondrial complex I (subunits 3 and 6), cell cycle transcripts and fluctuations in epigenetic chromatin remodeling systems. In terms of energy, the effects of MPP+ in the CV transcripts reflect the funnelling of carbon intermediates toward glycolysis. The LDH-A/B DKO transcripts reflect a flow of carbons away from glycolysis toward the production of acetyl-CoA. Conclusion: The findings from this study suggest a metabolic resilience to MPP+ in cancer cells devoid of LDH-A/B, explainable in part by higher baseline OXPHOS respiratory ATP production, necessitating more toxin to suppress the electron transport chain.

The "Warburg effect" describes an abnormal metabolic phenotype of cancer cells relative to healthy adjacent tissue, evidenced by a high glycolytic rate, over-expression of glycolytic enzymes, a rise in lactic acid production, and a preference for fermentative aerobic glycolysis, with diminished oxygen use by the mitochondria (1). While the original understanding of the Warburg effects deduced a probable mitochondrial defect in cancer cells (2), the current understanding has evolved, particularly with the discovery that glycolytic inhibitors or lactic acid dehydrogenase (LDH) inhibitors such as NCI-066 merely shift metabolic reliance toward greater oxidative phosphorylation (OXPHOS)/mitochondrial respiration and therefore more efficient production of ATP (3-5). In addition, high levels of oxygen and/or greater mitochondrial biogenesis drive tumorigenic processes (6-8) and appear to be prevalent in the stem cell metabolic phenotype (9, 10).

Interestingly, we have been studying the consequences of LDHA inhibitors for many years in diverse tumor cells and repeatedly failed to see any adverse effects on the production of lactic acid or tumor cell energy survival systems, with the only possible consequence being a reduction in cell proliferation (11-13). In collaboration with Dr. Jacques Pouysségurs laboratory (Nice, France), our recent work further defined transcriptomic changes in colon cancer cells which were genetically engineered to knockout both LDHA/B genes (14), and in doing so, constructed a cell line...
lacking transcripts required to synthesize numerous LDH isoforms (15). That work was crucial in demonstrating the need to simultaneously down-regulate LDH-A and LDH-B genes in order to shut off the production of lactic acid in cancer cells. However, even with this, cancer cells remain immortal, experience slower doubling rates, and give rise to increased mitochondrial OXPHOS capacity (15).

Findings such as these demonstrate a robust metabolic plasticity inherent to cancer cells that enable a continuous supply of energy produced by either or both glycolytic substrate-level phosphorylation (SLP) or mitochondrial oxidative phosphorylation (OXPHOS) to foster growth and metastasis under various micro-environments (16-18). Cancer cell metabolic rewiring is also a critical factor in driving chemo and radiation-resistant tumors (19, 20), where attempts have been made to combine chemotherapy drugs with metabolic inhibitors associated with anabolic fatty acid synthesis and catabolic oxidation [e.g., stearoyl-CoA desaturase-1 (SCD1), ATP-citrate lyase (ACLY), acyl-coenzyme A synthetase short-chain family member 2 (ACSS2), acetyl CoA carboxylase (ACC), fatty acid synthase (FASN), and Carnitine palmitoyltransferase I (CPT1)] (21-23). More recently, there has been a surge in experimentation involving the use of glycolytic or LDH inhibitors such as (IACS-010759) (4, 18) with mitochondrial based targets such as conjugated mitocan based triphenylphosphonium (TPP), BAY87-2243 with drugs such as CoA desaturase-1 (SCD1), ATP-citrate lyase (ACLY), acyl-fatty acid synthesis and catabolic oxidation [20 U/ml] and a chromogenic solution and quantified at 490 nm on a Synergy HTX multi-mode reader (Bio-Tek) as previously described (15). The data for glucose consumption was calculated as % of media blanks, housed in the first column of each 96 well.

In this study we explore the metabolic consequences of using a well-known multi-complex mitochondrial complex I, IV inhibitor,1-methyl-4-phenylpyridinium (MPP+) in LDH-A/B DKO colon cancer cells which are devoid of the metabolic apparatus to carry out anaerobic glycolytic SLP (15).

Materials and Methods

Materials. Ninety-six well plates, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin/ streptomycin, general reagents, and supplies were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and VWR International (Radnor, PA, USA). All microarray equipment, reagents, and materials were purchased from Affymetrix/ Thermo Fisher (Waltham, MA, USA).

Cell culture. Dukes’ type B, colorectal adenocarcinoma LS174T (ATCC® CL-188™) WT vector control (VC) versus LDHA/B double knockout (DKO) were established and kindly provided by Dr. Jacques Pouyssegur’s facility at the University Cote d’Azur, Lacassagne, Nice, France (15). Cells were cultured in 75 cm² flasks with high glucose DMEM (4,500mg/l) supplemented with 6% FBS and 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Cells were grown at 37°C in 5% CO₂ and sub-cultured every three to five days. The CV had a rapid doubling time, produced high lactic acid levels, and grew in multi-cellular aggregates, which required trypsin detachment [Trypsin-EDTA (0.25%), phenol red] and pressurized distribution using a 5 ml serological pipette, and subsequent monolayer re-seeding. High glucose DMEM (4,500 mg/l) was used for all studies, except for monitoring studies to monitor glucose consumption. A switch to low glucose DMEM (1000 mg/l) was required to unmask rapid glucose utilization.

Cell viability. For basic experiments, cells were plated in 96 well plates at 0.5x10⁶ cells/ml. Resazurin (Alamar Blue) indicator dye was used to measure cell viability/metabolic rate (26). Briefly, a working solution of resazurin (0.5 mg/ml) was prepared in sterile PBS, filtered through a 0.2-micron filter, added to the samples [15% (v/v) equivalent], and returned to the incubator for 2-6 hours. The data were quantified using a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT, USA) set on 530 nm (excitation) and 590 nm (emission) filters.

Lactic acid. Determination of cellular production of lactic acid produced was obtained using a colorimetric enzymatic assay with a lactate reagent according to the manufacturer (Trinity Biotech, Jamestown, NY, USA). The amount of lactic acid was measured in phenol red-free low serum media. The reagent was briefly added to the samples, incubated for 8 minutes, and quantified at 490 nm using a Synergy HTX multi-mode reader (Bio-Tek).

Glucose consumption. Briefly, cellular glucose consumption was quantified using an enzymatic assay comprised of glucose oxidase (20 U/ml) and a chromogenic solution and quantified at 490 nm on a Synergy HTX multi-mode reader (Bio-Tek) as previously published (15). The amount of glucose was measured in phenol red-free low serum media. The reagent was briefly added to the samples, incubated for 8 minutes, and quantified at 490 nm using a Synergy HTX multi-mode reader (Bio-Tek) set on luminescent measurement.

Oxygen consumption. A working solution of Janus Green (0.5 mg/ml) was prepared in sterile PBS and added to the samples [20% (v/v) equivalent]. Samples were then returned to the incubator to monitor differential profiles in the control group. Janus Green is a dye used to assess mitochondrial activity. As oxygen is consumed, the blue dye changes to a pink color and is quantified using a Synergy HTX multi-mode reader (Bio-Tek) with 530 nm (excitation)/590 nm (emission) filters. Imaging was performed using an inverted fluorescent microscope under basic settings using the TRITC (red) filter cube.

NADH/NAD+. NADH/NAD+ cellular concentrations were determined according to the manufacturer’s protocol (Sigma #MAK037 NADH/NAD determination kit), and data were quantified using the optical density on a Biotek Synergy Multi-Mode Reader. NADH concentrations were subtracted from the total NAD+ and NADH values to obtain NAD+, and data were calculated as a total ratio.
Lipids. According to the manufacturer’s guidelines, lipid isolation was carried out using several methods, including a slight modification of the Folch’s extraction procedure consisting of a biphasic solvent system of chloroform/methanol/water in a volumetric ratio of 8:4:3 (v/v/v). All lipid studies were carried out under anaerobic state with ample glucose supply.

Cholesterol esters. Briefly, the cells were scraped, washed 3x with cold PBS, and pelleted into microcentrifuge tubes. 440 μl of isopropanol, 280 μl of chloroform, and 40 μl of NP-40 solution were added to each pellet and samples were homogenized for 30 seconds, spun at 15,000 × g for 10 minutes at 4˚C and the organic phase transferred to a new tube, then dried at 50˚C. The determination of cholesterol/cholesterol esters was carried out using Abcam product #ab102515 (Cambridge, MA, USA) according to the manufacturer’s guidelines and quantified using a Synergy HTX multi-mode reader (Bio-Tek) with 530 nm (excitation)/590 nm (emission).

Triglycerides. Briefly, the cells were scraped, washed 3x with PBS, and pelleted into microcentrifuge tubes. According to the manufacturer guidelines, triglycerides were determined using Abcam product #ab65336. After adding NP-40 solution and subjecting the samples to a series of heat cycles at 100˚C, the upper layer was combined with assay buffer and lipase, placed on a rocker shaker for 20 minutes, followed by addition of a detection reagent, incubation for 1 hour and quantification using a Synergy HTX multi-mode reader (Bio-Tek) with the following settings: 530 nm (excitation)/590 nm (emission).

Neutral lipids. Briefly, the cells were scraped, washed 3x with PBS, and pelleted into microcentrifuge tubes. According to the manufacturer guidelines, neutral lipids were determined using Abcam #ab242307. After pelleting, 200 μl methanol and 400 μl chloroform was added before homogenization. 150 μls of pure water was added to the samples, followed by vortexing and centrifuging at 1000 × g for 5 min. Approximately 150 μl of the lower layer was isolated into a glass vial and left to evaporate (55˚C), then reconstituted to 150 μl with isopropanol. Subsequently, 40 μls of that sample was collected, and neutral lipids were determined using Abcam #ab242307 carried out according to the manufacturer guidelines. Neutral lipids were quantified using a Synergy HTX multi-mode reader (Bio-Tek) with the following settings: 480 nm (excitation)/580 nm (emission).

Microarray WT 2.1 human datasets. After the experimental treatment, the cells were scraped, washed three times in ice-cold HBSS, spun down, the supernatant removed, and the remaining pellet was rapidly frozen and stored at –80˚C. Total RNA was isolated and purified using the Trizol/chloroform method. The RNA quality was assessed, and concentrations were equalized to 82 ng/μl in nuclease-free water. According to the GeneChipTM WT PLUS Reagent Manual for Whole Transcript (WT) expression arrays, whole transcriptome analysis was conducted. Briefly, RNA was reverse transcribed to first strand/second strand cDNA followed by cRNA amplification and purification. After 2nd cycle ss-cDNA Synthesis and hydrolysis of RNA, ss-cDNA was assessed for yield, fragmented, labeled, and hybridized onto the arrays before being subjected to fluidics and imaging using the Gene Atlas (Affymetrix, ThermoFisher Scientific). The array data quality control and initial processing from CEL to CHP files were conducted using an expression console before data evaluation, using the Affymetrix transcriptome analysis console, String Database (String Consortium 2020), and DAVID functional annotation microarray tools (27-29). The dataset has been deposited to NIH Gene Expression Omnibus located at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149289.

Data analysis. Statistical analysis was performed for basic studies using GraphPad Prism (version 3.0; Graph Pad Software Inc., San Diego, CA, USA). Significant differences between the groups were assessed using either the student’s t-test or one-way ANOVA, followed by Tukey’s post hoc analysis.

Results

The effects of MPP+ (0.014-7.4 mM) on basic metabolic parameters in both control vectors (CV) (Figure 1A) and LDH-A/B DKO cells (Figure 1B) were determined, including viability/metabolic rate, ATP, cell respiration, glucose use, and lactic acid production. The study’s high glucose requirement (4,500 mg/l) in Figure 1 becomes a critical control issue because a high plating cell density in a micro-titer plate containing finite glucose, when employing a mitochondrial toxin, will trigger rapid glycolysis/exhaustion of glucose supplies and therefore indirect cell death. The effects of MPP+ observed in the CV are well within the bounds of what we have observed in diverse cancer cells over the years (Figure 1A) (30). This includes a compensatory reliance on SLP in glycolysis to sustain ATP levels coinciding with the rise in lactic acid and a gradual accelerated decline in glucose concentration. We anticipated that treating the LDH-A/B DKO cells with MPP+ would be catastrophic, simply because blocking both OXPHOS and SLP should evoke an energy crisis. However, on the contrary, the death curve in the LDH-A/B was relatively mild, with very little difference from the CV, as shown in Figure 1A. After repeating this work several times, we ultimately figured that the baseline differential between the CV of Figure 1 is inherently different from the LDH-A/B DKO in terms of OXPHOS resting capacity, shown as % control. In Figure 2A, we show the resting differential in OXPHOS between the CV and the LDH-A/B DKO by relative fluorescence and corresponding images evaluated with Janus Green: B (1) CV and B (4) LDHAB-DKO, where it becomes evident that the cellular respiration rate of the LDA-A/B DKO cell lines is amplified at baseline. Here we show that the CV group mitochondria are very close to being completely shut down by 116 μM of MPP+ [Figure 2B (2)] and fully suppressed at 463 μM [Figure 2B (3)]. In contrast, in the LDH-A/B DKO, even at 1.8 mM MPP+, the cells are still respiring, which coincides with the data obtained in Figure 1.

Further studies were conducted to determine the inherent metabolic differences between the CV and the LDH-A/B DKO in resting untreated cells (Figure 3). The data show that the CV uses glucose rapidly and produces abundant lactic acid (Figure
3A), which is opposite to the LDH-A/B DKOS, which show little to no glucose use, and a complete absence of lactic acid. Even though there was a dramatic metabolic divergence between how the CVs and LDH-A/B DKOs produce energy, the NADH to NAD+ ratio appears to follow a steady-state equilibrium (Figure 3B). The basic lipid profiles between the two cell lines do not vary significantly under a high glucose fed state for either neutral lipids or cholesterol esters (Figure 4B, C) with a slight increase in intracellular triglycerides (Figure 4A). As to the direct involvement of metabolic rewiring...
in the LDH-A/B DKO resting vs. CV to use fats for energy, further studies will be required to understand this aspect fully.

Next, optimal concentrations of MPP+ were established to conduct comparative microarray transcriptomic analysis, which we set at 231 μM MPP+, n=3 (Figure 5). An overall summary of differentially expressed genes (DEGs) evoked by MPP+ in CV or LDH-A/B DKO was conducted. Out of 48,226 gene transcripts tested, there were 1738 DEGs by

Figure 2. Differential cellular respiratory profile in the CV vs. LDH-A/B DKO LS174T cells cultured in high glucose media. The data are expressed as mean±SEM, n=4, for cell respiration with significant differences between the CV and the LDH-A/B DKO determined by student’s t-test, *p<0.05, n=4. (A) Fluorescent images represent higher intensity (cellular respiration). (B), where the data represent B (1): (CV), B (2): CV with 116 μM MPP+, B (3): CV with 463 μM MPP+, B (4): LDH-AB/DKO controls, B (5): LDHA/B DKO with 463μM MPP+ and B (6): LDHAB DKO with 1.8 mM MPP+.
MPP+ in the CV group and 855 DEGs by MPP+ in the LDHA/B - DKO group. Of these changes, MPP+ caused very similar changes in both groups for 79-up-regulated genes and 270 down-regulated ones. The largest DEG reduction for MPP+ in both groups was HIST1H2BM (-9.16 FC, FDR -p=0.0000621, Control Vector) and (-136.76 FC, FDR -p=0.0000011, LDH-A/B DKO).

Using the String interactive database, we looked at overlapping DEGs in the CV and the LDH-A/B DKO group evoked by MPP+ on OXPHOS related processes. MPP+ down-regulated several mitochondrial-related genes, including transcripts coding sub-units of NADH dehydrogenase (Complex I), mitochondrial outer compartment and fatty acid metabolism genes (Figure 6). More specifically, in both groups MPP+ reduced cytochrome b (CTYB), ATPase, class V, type 10A (ATP10A), NADH dehydrogenase, subunit 3 (complex I) (ND3) and NADH dehydrogenase, subunit 6 (complex I) (ND6) (Refer to NIH Gene Expression Omnibus located at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149289).
In response to MPP+, the global transcriptome differential expression for both groups ± MPP+ are shown by the volcano plot summary in Figure 7 (CV) and Figure 8 (LDH-A/B DKO). Using the DAVID comprehensive functional annotation bioinformatics array analysis tools, we provide the most significantly impacted biological systems by MPP+ in both groups (CV and LDH-A/B DKO) (Table I). It is evident that in both groups, MPP+ caused a down-regulation of transcripts which are essential for cell division, progression through the cell cycle, DNA replication, DNA strand elongation and excision repair, underscored by large changes in the epigenetic activity (Table I, Annotation cluster 8 and 14).

These changes were also observed using the Affymetrix transcriptome analysis (TAC) Wiki pathway analysis tool describing the effects of MPP+ on the CV (Figure 9) and the LDH-A/B DKO cell line (Figure 10). MPP+ caused significant changes to everything from the origin recognition complexes, minichromosomal maintenance complexes, and genes involved with each of the four phases of the cell cycle (G1, S, G2, and M). With very few up-regulatory DEGS in both groups by MPP+, DAVID analysis tools fall short of providing solid enrichment scores but rather indicate a probability of stress-related genes (Table II). Refer to the data located in NIH Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149289.

Next, we provide the unique DEG profiles elicited by MPP+ being specific to only the LDH-A/B DKO cell lines in Table III with ** denoting elements incorporated into the discussion regarding the differential transcriptiones related to glycolysis, the TCA cycle and reducing equivalents shuttled to the mitochondria. A general schematic is presented in Figure 11. These findings provide a basic platform for future analysis into understanding how cancer cells might overcome such dramatic simultaneous insult to both OXPHOS and SLP.

**Discussion**

Biological response to hypoxia, anoxia, or mitochondrial impairment in eukaryotes involves a compensatory shift toward anaerobic glycolysis, which sustain ATP through substrate level phosphorylation (SLP), resulting in accumulation of lactic acid. SLP is strictly dependent upon the function of lactic acid

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**Control Vector: Control vs. Control + MPP**
- Control: 3 samples Control + MPP: 3 samples

**Filter Criteria**
- Fold change: >2 or <2
- P-val: <0.05

**Total number of Genes: 48226**
- Genes Passed Filter Criteria: 1738 (3.6%)
- Up-Regulated: 520
- Down-Regulated: 1218

**LDH-A/B DKO vs. LDH-A/B DKO + MPP**
- LDH-A/B DKO: 3 samples LDH-A/B DKO + MPP: 3 samples

**Filter Criteria**
- Fold change: >2 or <2
- P-val: <0.05

**Total number of Genes: 48226**
- Genes Passed Filter Criteria: 855 (1.8%)
- Up-Regulated: 323
- Down-Regulated: 532

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Figure 5. Human transcriptome summary report for CV vs. CV+ MPP+ and LDH-A/B DKO vs. LDH-A/B DKO + MPP+. The summary of differentially expressed genes (DEGs) from the total number of genes evaluated; 48.226 using Affymetrix 2.1 ST Human Arrays. There were 416 overlapping DEGs of the same gene when treated by MPP+ in control vs. LDH-A/B DKO.
dehydrogenase (LDH) in order to convert pyruvate to lactic acid to generate oxidized NAD+ to drive ATP production through phosphoglycerate kinase and pyruvate kinase. However, in cancer cells, even in the presence of normoxia or hyperoxia, there is predominant glycolytic activity and lactic acid accumulation, originally believed to involve a defect in mitochondria and referred to as the Warburg effect (31, 32). Recently, there has been a paradigm shift due to advances in
cancer research. First, the Warburg effect is not necessarily a
function of faulty mitochondria, but one of its main purposes is
to sustain tumor microenvironment acidity (33), which is
paramount to invasive metastasis, chemo resistance and immune
escape (34, 35). Secondly, while the mitochondrial electron
transport chain (ETC) and OXPHOS is required to sustain
tumor growth and ATP production when needed (33, 36, 37),
high levels of either oxygen and/or greater mitochondrial
biogenesis will drive tumorigenic processes as evidenced by
perioperative surgical, experimental models (6-8) and
investigation of stem cell metabolism (9, 10). While previous
therapeutic attempts have involved experimenting with
glycolytic inhibitors (38), a fairly new area of anti-cancer
therapy now includes deployment of mitochondria-targeted
therapeutics, however challenges in using classical inhibitors of
OXPHOS are not without counter-indication, as mitochondrial
inhibitors have a long history of contributing to degenerative
diseases such as Parkinson’s or Alzheimer’s disease (39, 40).

Figure 7. Whole transcriptomic DEG profile of the Control vector LS174T cells ± MPP+. The data is expressed in a volcano plot providing information on fold change (X-axis) and FDR p-Value (Y-axis). The graph contains select gene symbols for the most highly affected genes.
A rationale for the use of mitochondrial inhibitors to treat cancer was instituted by cause-effect linked observation where reduced tumor growth occurred in individuals using various drugs with mitochondrial inhibiting properties such as anesthetics. Several types of anesthetics (complex I, II, III and IV inhibitors), such as propofol, ropivacaine and bupivacaine reduce mitochondrial biogenesis, PGC-1alpha (41-43) and accelerate glycolysis exerting reduced tumor growth and augmented chemotherapeutic efficacy (44-47) similarly, silencing critical OXPHOS related genes such as

Figure 8. Whole transcriptomic DEG profile of the LDH-A/B DKO LS174T cells ± MPP+. The data is expressed in a volcano plot providing information on fold change (X-axis) and FDR p-Value (Y-axis). The graph contains select gene symbols for the most highly affected genes.
Table I. DAVID functional annotation pathway analysis of down-regulated DEGs caused by MPP+ when comparing Controls vs. Controls + MPP+ and LDH-A/B DKO vs. LDH-A/B DKO + MPP+. The data represents enrichment score, biological classifications, DEG count, p-Value, and Benjamin False Discovery p-Value.

| Cluster                                  | Enrichment Score | Count | p-Value      | Significance |
|------------------------------------------|------------------|-------|--------------|--------------|
| Annotation Cluster 1                     | 38.7             | 78    | 4.10E-56     | 9.30E-54     |
| Cell cycle & Mitosis                     |                  |       |              |              |
| Annotation Cluster 2                     | 22.82            | 55    | 9.30E-42     | 1.10E-39     |
| Chromosome, Chromatin, Centromeres, Kinetochores |            |       |              |              |
| Annotation Cluster 3                     | 22.77            | 29    | 4.80E-32     | 1.80E-30     |
| DNA Replication, G1/S Transition Mitosis |                  |       |              |              |
| Annotation Cluster 4                     | 18.97            | 36    | 1.50E-22     | 3.80E-21     |
| DNA Damage                               |                  |       |              |              |
| Annotation Cluster 5                     | 6.97             | 42    | 3.50E-08     | 3.80E-07     |
| ATP Binding                              |                  |       |              |              |
| Annotation Cluster 6                     | 6.69             | 13    | 8.00E-08     | 9.00E-06     |
| ATPase Domain Binding                    |                  |       |              |              |
| Annotation Cluster 7                     | 6.34             | 10    | 3.30E-08     | 7.50E-07     |
| Fanconi anemia pathway (DNA repair)      |                  |       |              |              |
| Annotation Cluster 8                     | 5.46             | 18    | 3.10E-15     | 1.90E-13     |
| Epigenetics: Chromatin, Methylation,     |                  |       |              |              |
| Nucleosome Remodeling, Histone Core &    |                  |       |              |              |
| folds, citrullination, H2A, H3.1, H3.2, H3, | | | | |
| Annotation Cluster 9                     | 5.23             | 8     | 2.50E-08     | 8.70E-07     |
| DNA Replication/MCM Helicase, MCM Complex|                  |       |              |              |
| Annotation Cluster 10                    | 5.13             | 14    | 5.00E-16     | 2.20E-14     |
| DNA strand elongation, replication,      |                  |       |              |              |
| damage repair, translation, DNA          |                  |       |              |              |
| Polymerase, nucleotide excision repair   |                  |       |              |              |
| Annotation Cluster 11                    | 4.62             | 13    | 1.80E-14     | 1.60E-12     |
| DNA strand replacement, recombination,   |                  |       |              |              |
| reciprocal meiotic recombination, lateral |                  |       |              |              |
| element, recombinase, double-strand      |                  |       |              |              |
| break reprocessing                       |                  |       |              |              |
| Annotation Cluster 12                    | 4.26             | 6     | 1.50E-06     | 5.30E-05     |
| Cyclins, P53 Signaling Pathway           |                  |       |              |              |
| Annotation Cluster 13                    | 3.67             | 19    | 5.60E-09     | 6.70E-08     |
| Microtubule movement, Kinesins-motor     |                  |       |              |              |
| regions, retrograde Golgi to ER          |                  |       |              |              |
| Annotation Cluster 14                    | 2.83             | 10    | 2.10E-08     | 5.00E-07     |
| Epigenetics: H4, histone Binding,        |                  |       |              |              |
| silencing at rDNA, TAF, beta catenin-TCF complex assembly | | | | |
| Annotation Cluster 15                    | 2.25             | 3     | 8.40E-04     | 4.10E-02     |
| HHH2, Helix-hairpin-helix motif, class 2, XPGN |            |       |              |              |
| Annotation Cluster 16                    | 2.09             | 4     | 4.00E-05     | 3.50E-04     |
| Purine, Pyrimidine, DNA Polymerses       |                  |       |              |              |
| Annotation Cluster 17                    | 2.08             | 7     | 1.30E-05     | 4.20E-04     |
| Protein Phosphatase (tyrosine)           |                  |       |              |              |

S100 calcium-binding protein A4 (S100A4) will reduce OXPHOS, complex I and ATP, accelerate glycolysis and attenuate invasive metastatic tumorigenesis in a three-dimensional growth models or in vivo (48). This pattern also extends to cardiac drugs (e.g. atovaquone; complex III inhibitor) (2) and anti-diabetic drugs such as the biguanide metformin or phenformin (complex I inhibitor), which attenuates mitochondrial respiration, amplifies glycolysis and results in reduced tumor growth in diverse models such as lymphoma (49-51). Phenformin has a history of causing lactic acidosis, which was in some cases fatal, justifying removal from the US drug market (52, 53). In the case of metformin, lactic acidosis is not as severe and is considered fairly safe (54) with potential drug repurposing application to treat diverse human cancers (55, 56). Oddly, in vitro experimental models using cancer cells to investigate the efficacy of metformin + glycolytic inhibitors such as 2-DG, require relatively high concentrations to confer symbiotic effects.
(high uM – low mM) (49, 57, 58). For this reason, there has been a recent surge in rationale and design of sophisticated conjugated cationic lipophilic mitochondrial carries that carry drugs such as metformin directly to the mitochondria in tumor cells (e.g. Mito-Met10), which can drastically alter the efficacy of a drug such as metformin by approximately 400 fold (59). The use of mitochondrial cationic lipophilic carriers, are central to today’s mitochondrial targeting therapies which work by taking advantage of tumor specific depolarized plasma membrane potentials/ acidic pH+ coupled to mitochondria negatively charged or hydrophilic polymer-stabilized nano-carries to access the mitochondria. These carrier backed systems include attaching diverse drugs to a cationic lipophilic carrier such as triphenylphosphonium (TPP+) or mitoquine (Mito-Q) coenzyme Q + TPP+, housing conjugated mitochondrial inhibitors (Complex I-III inhibitors, ATP synthase inhibitors, or uncoupling agents), chemotherapy drugs, antioxidants, some of these have been combined with glycolytic inhibitors such as 2-deoxyglucose (2-DG) (60, 61) (62-64). Other cations that can be used as cancer specific theranostic imaging or conjugated to a variety of other moieties including dequalinium (DQA), guanidine, Mito-carboxy proxyl (Mito-CP), pyridinium, 3-phenylsulfonylfuroxan, or (E)-4-(1H-indol-3-ylvinyl)-N-methylpyridinium iodide (F16) which can carry with them a variety of drugs (e.g. chlorambucil, vitamin derivatives, or metformin) (62, 65-68) to inhibit tumor growth (62, 69).

In the current study, we directly employ the use of MPP+ to inhibit the mitochondria because it a well-known complex I and IV inhibitor, with the capacity to both reduce transcripts that encode mitochondrial enzymes as well as eliciting kinetic inhibition of cytochrome oxidase (70-73). Here we treat colon cancer cells with MPP+ either without the LDH-A/B gene or a control set, the former
Figure 10. Affymetrix transcriptomic analysis of upregulated DEGs caused by MPP+ when comparing Controls vs. Controls + MPP+ and LDH-A/B DKO vs. LDH-A/B DKO + MPP+. The data represents FC (p<0.05), where DEGs were far greater in controls than LDH-A/B DKO (black - right) and greater in LDH-A/B DKO than controls on the left (Red).

Table II. DAVID functional annotation pathway analysis of up-regulated DEGs caused by MPP+ when comparing Controls vs. Controls + MPP+ and LDH-A/B DKO vs. LDH-A/B DKO + MPP+. The data represents enrichment score, biological classifications, DEG count, p-Value, and Benjamin False Discovery p-Value.

| Cluster                                      | Enrichment score | #Genes | p-Value | Significance |
|---------------------------------------------|------------------|--------|---------|--------------|
| Annotation Cluster 1                        | Enrichment Score: 3.08 Apoptosis & ER Stress | Count | 1.10E-05 | Benjamini    |
| Annotation Cluster 2                        | Enrichment Score: 1.72 BRLZ, Negative Regulation of Transcription | Count | 2.10E-06 | Benjamini    |
| Annotation Cluster 3                        | Enrichment Score: 1.09 Protein - Tyrosine Phosphatase | Count | 2.50E-02 | 1.00E+00     |
| Annotation Cluster 4                        | Enrichment Score: 0.86 GTPase Activator | Count | 9.30E-02 | 1.00E+00     |
Table III. Unique DEGs in the LDH-A/B DKO vs. LDH-A/B DKO + MPP. The data are expressed as Gene symbol, FC, and p-Value.

| Gene Symbol | Description | Fold change | p-Value | FDR p-Value |
|-------------|-------------|-------------|---------|-------------|
| AGPAT5 | 1-acylglycerol-3-phosphate O-acyltransferase 5 | 2.18 | 1.07E-05 | 8.00E-04 |
| DHCR24 | 24-dehydrocholesterol reductase | -2.14 | 1.17E-05 | 9.00E-04 |
| DHCR7 | 7-dehydrocholesterol reductase | -2.14 | 5.01E-05 | 2.30E-03 |
| ACAT2 | Acetyl-CoA acyltransferase 2 | -2.56 | 2.44E-06 | 3.00E-04 |
| ACAA2 | Acetyl-CoA acyltransferase 2 | -3.05 | 4.06E-07 | 1.00E-04 |
| ASC12 | Achaete-scute family bHLH transcription factor 2 | -3.24 | 1.14E-05 | 9.00E-04 |
| ACAD9 | Acyl-CoA dehydrogenase family, member 9 | -2.06 | 1.77E-05 | 1.20E-03 |
| ACADSB | Acyl-CoA dehydrogenase, short/branched chain | -2.23 | 1.43E-05 | 1.00E-03 |
| AHNAK2 | AHNAK nucleoprotein 2 | 5.27 | 2.34E-07 | 9.47E-05 |
| AARS | Alanyl-tRNA synthetase | 3.05 | 5.00E-04 | 1.04E-02 |
| ALDH1B1 | Aldehyde dehydrogenase 1 family, member B1 | -4.63 | 6.25E-08 | 4.07E-05 |
| ALDH18A1 | Aldehyde dehydrogenase 18 family, member A1 | -2.18 | 1.24E-05 | 9.00E-04 |
| ALDH3A1 | Aldehyde dehydrogenase 3 family, member A1 | -2.84 | 5.10E-03 | 5.75E-02 |
| AKR1C3 | Aldo-keto reductase family 1, member C3 | -2.2 | 5.00E-04 | 1.14E-02 |
| ASB4 | Ankyrin repeat and SOCS box containing 4 | -2.97 | 5.06E-08 | 3.59E-05 |
| ARMC3 | Armadillo repeat containing 3 | -3.61 | 1.00E-04 | 4.30E-03 |
| ARRD3 | Arrestin domain containing 3 | 4.55 | 2.85E-07 | 2.00E-04 |
| ATP5D | ATP synthase, H+ transporting, mitochondrial complex, delta subunit | -2.05 | 5.42E-06 | 5.00E-04 |
| BAAT | Bile acid-CoA:amino acid N-acyltransferase | -3.44 | 5.29E-07 | 1.00E-04 |
| CRAT | Carnitine O-acyltransferase | -2.07 | 2.01E-05 | 1.20E-03 |
| CPT1C | Carnitine palmitoyltransferase 1C | -2.11 | 5.05E-06 | 5.00E-04 |
| CPT2 | Carnitine palmitoyltransferase 2 | -2.16 | 1.12E-06 | 2.00E-04 |
| CASP5 | Caspase 5 | 6.98 | 6.10E-10 | 3.27E-06 |
| CEMP | Cell migration inducing protein, hyaluronan binding | -3.02 | 6.25E-09 | 1.08E-05 |
| CRBN | Cereblon | 2.57 | 4.28E-08 | 3.33E-05 |
| CFAPI26 | Cilia and flagella associated protein 126 | -7.14 | 4.06E-07 | 1.00E-04 |
| CKB | Creatine kinase, brain | -2.95 | 1.59E-05 | 1.10E-03 |
| CDK1 | Cyclin-dependent kinase 1 | -3.15 | 2.18E-06 | 3.00E-04 |
| CPEB3 | Cytoplasmic polyadenylation element binding protein 3 | 3.06 | 3.13E-06 | 4.00E-04 |
| DAGL1 | Diacylglycerol lipase, alpha | 2.16 | 1.50E-03 | 2.46E-02 |
| ERN1 | Endoplasmic reticulum to nucleus signaling 1 | 3.03 | 2.47E-05 | 1.40E-03 |
| FAM171B | Family with sequence similarity 171, member B | -3.56 | 1.92E-09 | 5.78E-06 |
| FAM216A | Family with sequence similarity 216, member A | -2.56 | 4.92E-08 | 3.55E-05 |
| FADS1 | Fatty acid desaturase 1 | -2.25 | 4.34E-06 | 5.00E-04 |
| FASN | Fatty acid synthase | -2.16 | 2.75E-05 | 1.50E-03 |
| GMNN | Geminin, DNA replication inhibitor | -3.06 | 1.29E-06 | 2.00E-04 |
| GPT3 | Glycerol-3-phosphate acyltransferase 3 | 2.08 | 2.43E-05 | 1.40E-03 |
| GMPR | Guanosine monophosphate reductase | -4.18 | 5.00E-04 | 1.14E-02 |
| HKDC1 | Hexokinase domain containing 1 | 3.72 | 2.40E-03 | 3.41E-02 |
| HIST2H2AA4; HIST2H2AA3 | Histone cluster 2, H2a4; histone cluster 2, H2a3 | -3.67 | 1.69E-08 | 2.02E-05 |
| HIST2H2BB; HIST2H3PS2 | Histone cluster 2, H2bb (pseudogene); histone cluster 2, H3, pseudogene 2 | -4.6 | 6.06E-08 | 4.07E-05 |
| ID3 | Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein | -2.74 | 1.73E-07 | 7.92E-05 |
| IFITM3 | Interferon induced transmembrane protein 3 | -3.63 | 5.73E-09 | 1.08E-05 |
| IDH2 | Isocitrate dehydrogenase 2 (NADP+), mitochondrial | -2.61 | 9.89E-07 | 2.00E-04 |
| KRT75 | Keratin 75, type II | 8.05 | 4.52E-10 | 3.22E-06 |
| LEMD1; BLACAT1 | LEM domain containing 1; bladder cancer associated transcript 1 (non-protein coding) | 3.11 | 1.77E-07 | 7.94E-05 |
| LINC01207 | Long intergenic non-protein coding RNA 1207 | 3.8 | 3.53E-07 | 1.00E-04 |
| MME | Membrane metallo-endopeptidase | -3.25 | 6.04E-06 | 6.00E-04 |
| MT1H | Metallothionein 1H | -3.18 | 6.01E-05 | 2.60E-03 |
| MIR1244-1; MIR1244-2; MIR1244-3 | microRNA 1244-1; microRNA 1244-2; microRNA 1244-3; microRNA 1244-4 | -3.74 | 6.75E-07 | 2.00E-04 |
| MSN | Moesin | 2.53 | 3.89E-08 | 3.29E-05 |
| NEDD4L | Neural precursor cell expressed, developmentally down-regulated 4-like, E3 ubiquitin protein ligase | 3.31 | 5.95E-09 | 1.08E-05 |
| NTS | Neurotensin | -5.78 | 8.02E-06 | 7.00E-04 |
| NID1 | Nidogen 1 | 4.77 | 2.58E-07 | 9.92E-05 |

Table III. Continued.
having no capacity to produce lactic acid or drive the alternative ATP SLP pathway. We expected to see complete energy collapse in the LDH-A/B DKO, as reported by others combining mitochondrial inhibitors with glycolytic inhibitors such as DG this was not the case (70, 74-76). Keep in mind, this is a unique model, where glycolysis is still functioning to the point of pyruvic acid, so in the true sense, the LDH-A/B DKO is not a full glycolytic inhibitor but does effectively shut down the required pyruvate to lactate conversion sustain the anaerobic production of ATP through SLP. The data from this study show an unexpected resistance to the toxin in the LDH-A/B DKO, which requires further explanation.

The data in this work establish efficacy of MPP+ to inhibit mitochondrial function, both biologically through suppressive respiration and an observed transcriptional loss of OXPHOS of complex I; NADH dehydrogenase, subunits 3, and 6 and complex III (cytochrome b). Moreover, the data in this work support our previous study in that the LDH-A/B DKO cell line triggered a metabolic response with heightened OXPHOS capacity (15). The baseline respiratory differential may have contributed to a resistance to MPP+ either by necessitating a higher required concentration of MPP+ to elicit damage, or possibly by extruding the drug equipped with high capacity ATPase multi-drug resistance pumps, as there is a clear relationship between higher OXPHOS stem cell metabolism, chemo-resistance, enhanced expression of ATP binding cassette multi-drug resistance pumps and poor clinical outcomes (77-79).

Some of the changes that accompany this resistance are reflected in the transcriptome when analyzing the differential between CV vs. CV + MPP+ and LDH-A/B DKO vs. LDH-A/B DKO + MPP+, such as heightened ATP binding cassette subfamily C member 3 (ABCC3) multi-drug resistance pump and a differential in the flow of intermediates from pyruvate (glycolysis) to Acetyl-CoA (TCA) or vice versa. MPP+ in

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Table III. Continued

| Gene Symbol | Description | Fold change | p-Value | FDR p-Value |
|-------------|-------------|-------------|---------|-------------|
| NDRG1       | N-myc downstream regulated 1 | 3.64 | 2.60E-03 | 3.63E-02 |
| PLCB4       | Phospholipase C, beta 4 | −3.09 | 8.76E-07 | 2.00E-04 |
| PEA15       | Phosphoprotein enriched in astrocytes 15 | 2.34 | 1.10E-07 | 6.18E-05 |
| PRTFDC1     | Phosphoribosyl transferase domain containing 1 | −3.17 | 1.97E-07 | 8.43E-05 |
| PIWIL1      | Piwi-like RNA-mediated gene silencing 1 | −4.49 | 1.52E-08 | 1.94E-05 |
| PLLP        | Plasminogen | −3.48 | 4.16E-08 | 3.29E-05 |
| PDK3        | Pyruvate dehydrogenase kinase, isozyme 3 | −2.09 | 2.00E-04 | 5.90E-03 |
| PDP1        | Pyruvate dehydrogenase phosphatase catalytic subunit 1 | 2.54 | 1.78E-07 | 7.94E-05 |
| REEP6       | Receptor accessory protein 6 | −4.56 | 3.11E-10 | 3.00E-06 |
| RNASEL      | Ribonuclease L (2,5-oligoisoadenylate synthetase-dependent) | 3.42 | 4.45E-06 | 5.00E-04 |
| RRM2        | Ribonucleotide reductase M2 | −6.31 | 5.15E-10 | 3.22E-06 |
| RNF144A     | Ring finger protein 144A | 3.68 | 8.72E-07 | 2.00E-04 |
| RNU5D-1     | RNA, U5 small nuclear 1 | −10.92 | 1.60E-07 | 7.50E-05 |
| RNU5F-1     | RNA, U5F small nuclear 1 | −6.38 | 9.97E-06 | 8.00E-04 |
| SAMHD1      | SAM domain and HD domain 1 | −3.97 | 7.19E-09 | 1.20E-05 |
| SLC25A37    | Solute carrier family 25 (mitochondrial iron transporter), member 37 | 2.54 | 1.24E-07 | 6.62E-05 |
| SLM1        | Solute carrier family 12 (sodium/potassium/chloride transporter), member 2 | −4.32 | 9.46E-10 | 4.52E-06 |
| SLC22A15    | Solute carrier family 22, member 15 | 4.07 | 4.00E-04 | 9.40E-03 |
| SLC25A37    | Solute carrier family 25 (mitochondrial iron transporter), member 37 | 2.54 | 1.24E-07 | 6.62E-05 |
| SORL1       | Spermidine synthase | −3.02 | 6.31E-06 | 6.00E-04 |
| SOAT1       | Sterol O-acyltransferase 1 | −2.87 | 6.49E-06 | 6.00E-04 |
| SOD2        | Superoxide dismutase 2, mitochondrial | −2.25 | 1.30E-03 | 2.25E-02 |
| TESCC       | Tescalcin | −3 | 3.12E-07 | 1.00E-04 |
| TK1         | Thymidine kinase 1, soluble | −3.12 | 2.96E-06 | 4.00E-04 |
| TIMELESS    | Timeless circadian clock | −4.11 | 1.38E-08 | 1.86E-05 |
| TMEM98-AS1  | TMEM98 antisense RNA 1 | 3.6 | 2.71E-06 | 3.00E-04 |
| TMEM71; LRRC6| Transmembrane protein 71; leucine rich repeat containing 6 | 3.11 | 1.07E-07 | 6.07E-05 |
| UBE2T       | Ubiquitin conjugating enzyme E2T | −3.74 | 5.18E-09 | 1.08E-05 |
| USP18       | Ubiquitin specific peptidase 18 | −3 | 9.39E-07 | 2.00E-04 |
| XDH         | Xanthine dehydrogenase | 3.25 | 1.36E-07 | 6.92E-05 |
| ZC3H6       | Zinc finger CCCH-type containing 6 | 3.33 | 6.59E-06 | 6.00E-04 |
| ZNF25       | Zinc finger protein 25 | 3.22 | 5.63E-06 | 5.00E-04 |
| ZNF555      | Zinc finger protein 555 | 6.76 | 6.40E-08 | 4.07E-05 |
the CV resulted in a unique (CV set only) elevation of phosphoenolpyruvate carboxykinase 1 (PKC1), indicative of a response to nutrient deprivation (80) with capacity to redirect carbons from oxaloacetate back into glycolysis to carry out gluconeogenesis. In contrast, the LDH-A/B DKO exposed to MPP+ responded with up-regulation of positive regulator of pyruvate dehydrogenase (PDH), PDH phosphatase catalytic subunit 1 (PDP1) with a down-regulation of negative PDH feedback; PDH kinase, isozyme 3 (PDK3), effect that would push pyruvate to enter the mitochondria, and its conversion to acetyl-CoA. While the LDH-A/B DKO MPP+ treatment group show greater pyruvate to Acetyl-CoA production while there is no conversion of pyruvate to lactic acid, the transcriptome suggests a pooling or accumulation of Acetyl-CoA, since these changes coincide with a reduction in transcripts that code for proteins which enable Acetyl-CoA to be used to drive biosynthetic processes (fatty acid, cholesterol and ketone synthesis).

Examples of these include specific losses in the LDH-A/B DKO MPP+ group (not in the CV + MPP+ group), including down-regulation of 1) acyl-CoA dehydrogenase (ACAD9, ACADSB) (initial steps in the fatty acid β-oxidation pathway) 2) acetyl-CoA acyltransferase 2 (ACAA2) (final steps required for the B-oxidation spiral (ACAA2). Lower electron transport chain activity, coincided with lower expression of genes (TCA and fatty acid oxidation) which would generate equivalents (NADH and FADH2) , no longer being accepted to generate an electrochemical gradient for ATP production. In addition, acetyl-CoA which is the central carbon currency to fuel energy related processes is also met with a down-regulation of its route through biosynthetic processes including: down-regulated transcripts required for the initiation step of cholesterol/ ketone synthesis (ACAT2, the final step of cholesterol synthesis (DHCR 7, DHCR 24) and fatty acid synthesis (FAS).
FA beta-oxidation pathways would also be reduced since the mitochondria no longer accept reducing equivalents (NADH and FADH2) from these pathways to generate an electrochemical gradient for ATP production (9).

The pooling of acetyl-CoA seems to be a unique central aspect in the LDH-A/B DKO + MPP+ (not present in the CV vs. CV + MPP+), as there is also a loss of transcripts that encode proteins required to carry out anabolic substrate use of acetyl-CoA to produce sterols, fatty acids, and ketones. These include losses to 1) acetyl-CoA acetyltransferase 2) (ACAT2) (the first enzyme in the cholesterol and ketone synthesis pathway which is required to convert two molecules of acetyl-CoA to acetoacetyl-CoA), 2) the final step for cholesterol biosynthesis 24-dehydrocholesterol reductase, 7 dehydrocholesterol reductase (DHCR 7 & 24) and 3) reduction of fatty acid synthase (FAS) needed to carry out condensation reactions from acetyl-CoA and malonyl-CoA and a corresponding loss to fatty acyl desaturase 1 (which catalyzes the removal of two hydrogen atoms from a fatty acid, creating a carbon/carbon double bond). These changes coincided with an increase in glycerol-3-phosphate (GPAT3) O-acyltransferase which catalyzes the chemical reaction where by acetyl-CoA + sn-glycerol 3-phosphate is converted to CoA + 1-acyl-sn-glycerol 3-phosphate required for the organization of phospholipids to sustain biological membrane structures. Acetyl-CoA is the central carbon currency of energy metabolism. It is synthesized from pyruvate in mitochondria, enters into the TCA cycle for conversion to citric acid cycle (citrate synthase), and excess citrate is exported to the cytoplasm by a mitochondrial citrate carrier, where it is reconverted back to acetyl-CoA by ATP-citrate lyase (ACLY) then available for fatty acid, ketone, cholesterol synthesis or acetylation reactions that involve both protein and epigenetic cellular function including histone and chromatin remodeling, which controls transcription of mRNA (81, 82). The central functional role of ACLY in driving lipid catabolic tumorigenic processes is the rationale behind the development of inhibitors (e.g., hydroxycitrate, bempedoic acid) in oncology research (83, 84). Clearly, acetyl-CoA has a central role in cancer metabolism and also essential to carrying out epigenetic histone modifications/lysine acetylation (85). Future studies will be required to examine how acetyl-CoA is being utilized in the absence of a functional glycolytic pathway, why it is accruing and how it is being used.

The transcriptome data also reflects the importance of epigenetic changes elicited by MPP+ in both CV and LDH-A/B DKO, evidenced by the sheer number of down-regulated histone cluster transcripts as well as the massive bidirectional DEGs in non-protein coding microRNA and long-intergenic transcripts. MPP+ also evoked changes in a number of METTL transcripts, which are believed to contribute to nucleoside modifications in diverse types of RNA and DNA (86, 87), however very little is known on these particular epigenetic modifications.

**MPP+ and cell cycle.** Other than a loss of OXPHOS by MPP+ as anticipated (88, 89) the most significant effect to both groups was a loss of transcripts otherwise required for cell cycle. In both groups we the MPP+ mediated down-regulation of cell cycle genes (CDC20, CDC25A, CDC25C, CDC45, CDC6, CDC 7), cell cycle associated genes (CDC2A, CDC3A, CDC7A and CDCA8), cyclins (cyclin A2, B1, B2, D, D2, E1, E2, F, L1), cyclin-dependent kinases (CDK1, CDK13, CDK14) and marker of proliferation Ki-67 (MIK67). The effects of MPP+ on both cell lines also resulted in down-regulation of DNA replicative processes including the following: mini-chromosome maintenance complex components (MCMs 2, 3, 4, 5, 6, 7, 8, 9, 10) origin recognition complex subunits (ORCs 1, 2, 5, 6), kinesin family member genes (KIFs 11, 14, 15, 18A, 20A, 20B, 23, 4A) centromere proteins (CENPE E, F, H, I, J, K, M, N, O, P, Q, U, W) and centrosomal proteins of kDa (128 kDa, 135 kDa, 152 kDa, 162 kDa, 192 kDa, 19 kDa, 295 kDa, 55 kDa, 76 kDa, 78 kDa, 83 kDa and 97 kDa). Down-regulation also extended to loss of transcripts required replicative DNA processes including; DNA polymerases (POLs A1, A2, D2, E2, Q, D4), DNA primases (PRIMs 1 and 2), DNA repair transcripts (DNA crosslink repair genes: DCLRIs 1A,1B and 1C), DNA replication helicase/nuclease gene 2i; GINS complex subunits (Psf1 homolog) (GINS 1, 2, 3 and 4); Fanconi anemia complementation group (FANC A, B, C, D2, L1, M and J); spindle and kinetochore associated complex subunits (SKA1,3) and topoisomerases (TOPs 2A, 2B, 2C). These findings are in alignment with others showing the effects of MPP+ in vitro in exerting cytostatic effects, secondary to cell death initiated by glucose depletion as a result of accelerated glycolysis (88, 90). Whatever the consequence, there is no doubt that MPP+ caused a significant amount of stress on both cell lines, reflected by up-regulation of DNA-damage-inducible, alpha (CADDA45A) beta (GADD45B) gamma (GADD45H), commonly the aftermath of strain associated with the loss of cell cycle progression (91) particularly inability to pass the G2-M phase (92-94). Refer to the data located in NIH Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE149289.

**Conclusion**

The findings from this study suggest a complex metabolic survival response to a specific mitochondrial toxin (MPP+) in colon cancer cells that lack LDH-A/B transcripts and the related functional SLP glycolytic anaerobic ATP energy yielding pathway. While this study provides a framework for further analysis, cancer cells without LDH may shift toward aerobic OXPHOS creating counter indication when using mitochondrial targeting drugs, which may necessitate higher concentration.
Availability of Data and Material

The dataset has been deposited to NIH Gene Expression Omnibus located at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149289.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors’ Contributions

NM conducted studies on basic cell metabolic profiles. EM assisted with these studies and carried out microarray work. RB was involved with mechanistic troubleshooting and study design and KS oversaw and guided this project.

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