Complex I inhibition shifts mitochondrial dynamics and regulates cancer cell survival, providing a synthetic lethal interaction

Authors: Firoz Khan Bhati[1], Uday Saha[2,3], Sandeep Singh[2,3] and Manoj Kumar Bhat[1]*

Authors' affiliations:

1. Biotechnology Research and Innovation Council - National Centre for Cell Science (BRIC- NCCS), Savitribai Phule Pune University Campus, Ganeshkhind, Pune, 411 007, Maharashtra, India.
2. Biotechnology Research and Innovation Council - National Institute of Biomedical Genomics (BRIC-NIBMG), P.O.: N.S.S., Kalyani 741251, West Bengal, India.
3. Regional Centre for Biotechnology, NCR Biotech Science Cluster, 3rd Milestone, Faridabad-Gurugram Expressway, Faridabad - 121 001, Haryana (NCR Delhi), India.

"*" Denotes Corresponding author

Email address of authors

Firoz Khan Bhati: firozbhati@live.com

Uday Saha: us1@nibmg.ac.in

Sandeep Singh: ss5@nibmg.ac.in

Manoj Kumar Bhat: manojkbhat@nccs.res.in

***Corresponding author:** Dr. Manoj Kumar Bhat, DBT-Distinguished Scientist, BRIC-NCCS, Savitribai Phule Pune University Campus, Ganeshkhind, Pune, India.

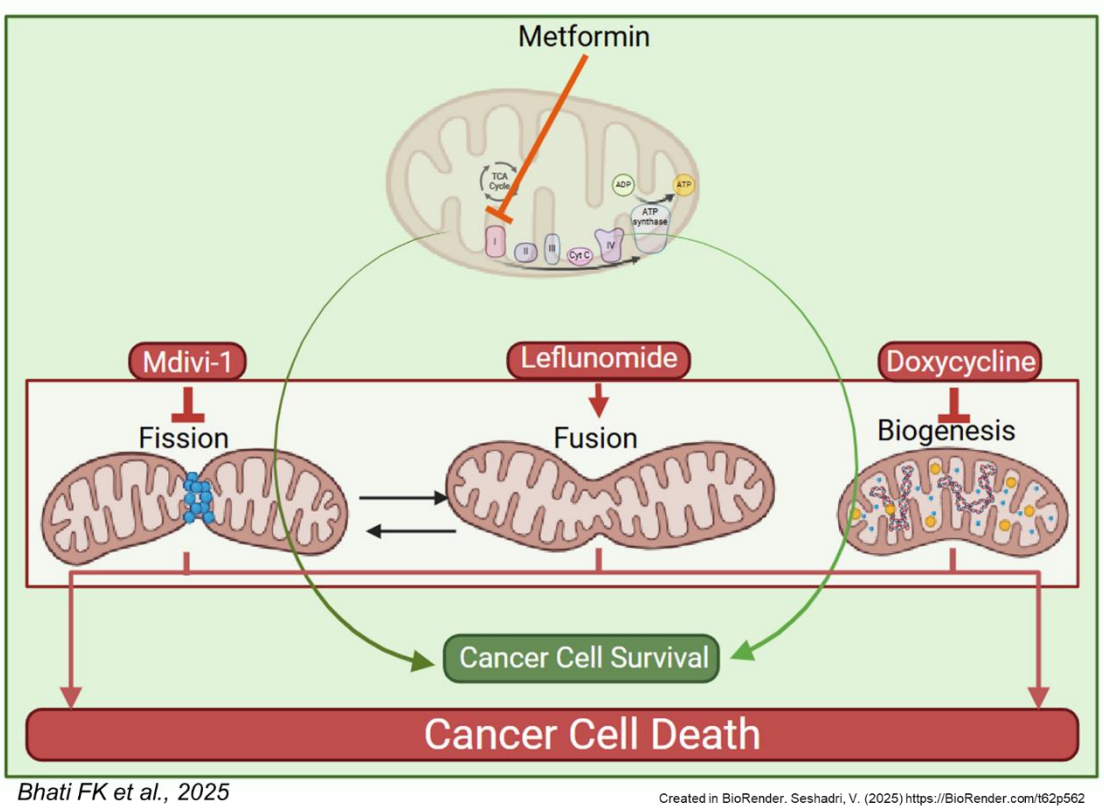
Tel.: +91-20-25708066; Fax: +91-20-25692259.

Abstract

Use of strong complex I inhibitors in clinical trials for cancer treatment failed to give desired outcomes due to drug-induced toxicity, which rekindled the focus towards metformin: a known mild mitochondrial complex I inhibitor. Metformin has been extensively explored for its anticancer activity, and the results obtained have indicated mixed outcomes. Our results indicate that alterations in mitochondrial dynamics following metformin treatment are associated with cell survival and tumor progression. Interestingly, targeting these alterations induces death in metformin-treated cancer cells.

Key Words: Complex I inhibitors, Metabolic flexibility, mitochondrial dynamics, cancer cell survival, synthetic lethality

Graphical Abstract



Metformin treatment induces changes in mitochondrial dynamics, thereby enhancing the survival of cancer cells. Targeting these metformin-induced alterations can result in cell death.

Introduction

Cancer cells exhibit metabolic plasticity to meet their energy demands and biosynthetic needs throughout disease progression. Therefore, a deeper mechanistic understanding of metabolic plasticity can lead to the development of novel actionable therapeutic targets [1,2]. Mitochondrial metabolic plasticity contributes to drug resistance and evasion of cell death [3,4]. Various pharmacological inhibitors and clinically relevant drugs have been explored to disrupt mitochondrial metabolism [5,6]. Mitochondrial Complex I, a key component of the electron transport chain, plays a crucial role in cancer cell proliferation, resistance to cell death, and metastasis [7]. Metformin, an antidiabetic drug is generally well-tolerated, and exhibits minimal drug related toxicity in cancer patients. It known to inhibit mitochondrial complex I in cancer cells and extensively studied for its anticancer properties [7–13]. Also, metformin's utility is being evaluated in combination with chemotherapy, radiotherapy, and immunotherapy in various studies [14]. However, clinical trials on metformin, as an anticancer agent have yielded inconsistent results, underscoring the need for more investigations.

Metformin treatment induces energy stress which drives cancer cells to rewire their metabolism, a phenomenon known as drug induced metabolic flexibility. It has been reported that metabolically non-flexible cancer cells exhibit sensitivity to metformin and those with high metabolic adaptability may continue to survive and proliferate [15]. Cancer cells respond to metformin by upregulating glycolysis or enhancing oxidative phosphorylation. Specifically, in breast cancer tumor enhanced mitochondrial response to metformin has been reported, which correlates with a higher tumor proliferation score, indicating it’s crucial role in determining metformin sensitivity [16].

Previous studies from our laboratory correlated the induction of energy stress to mitochondrial biogenesis as an adaptive survival mechanism [17]. Moreover, in melanoma cells, metformin drive a glycolytic phenotype, revealing a potential vulnerabilities that can be exploited to enhance its anticancer efficacy [18]. Put together, these findings suggest that metformin alone is unlikely to be an ideal cytotoxic agent because of the metabolic flexibility in cancer cells. However, exposure of cancer cells to metformin creates vulnerabilities which are exploitable through synthetic lethal interactions.

We investigated the role of metformin treatment-associated alterations in mitochondrial dynamics as a marker of metabolic flexibility in HCC cells. Our data suggest that targeting metformin associated mitochondrial changes offers an opportunity to overcome the metabolic adaptability and enhance anticancer efficacy in HCC cells.

Materials and Methods

Cell culture and cell lines

Human hepatocellular carcinoma cell line Huh-7 was a gift by Dr. Ralf Bartenschlager (University of Heidelberg, Germany), MC-38 (Murine Origin, Colon Adenocarcinoma) was gifted by Dr. Ruben Harnandez (CIMA, Spain), 4T1 tdTomato positive (Murine Origin Triple Negative Breast Cancer) was obtained from Imperial Life Science(P) Ltd, HCT116 (Human Origin, Colon Adenocarcinoma). B16F10 (Murine Origin Melanoma), Hep-3B, Hep-G2 (Human Origin, Hepatocellular Carcinoma), HT-29, and HCT-15 (Human Origin, Colorectal Carcinoma) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in our in-house BRIC-NCCS, India cell repository. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA), 100 µg/mL streptomycin and 100 U/ml penicillin (Invitrogen Life Technologies, CA, USA) and maintained in temperature-controlled (37 °C), humidified CO_2 (5%) incubator (Thermo Fisher Scientific, OH, USA).

Antibodies and chemicals

Antibody against Actin (1:1000) (Cat # Sc-1615),; Vinculin (7F9) (1:1000) (Cat # sc-73614); LDH-A (F-3) (Cat # sc-137244),; TFAM (Cat # sc-376672),; TOM-20 Alexa 488 (F-10) (1:200) (Cat # sc-17764 AF488),; and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, CA, USA. CD-45 APC-eFluor™ 780 (Cat # 30-F11) (1:200) was purchased from Invitrogen Life Technologies, CA, USA. Metformin (Cat # 151691) was purchased from MP Biomedicals LLC, OH, USA. Doxycycline (Cat # D9891),; Propidium Iodide (Cat # p-4170),; MTT (Cat # USB-19265) and Crystal Violet (Cat # C-3886) were purchased from Sigma, MO, USA. Leflunomide (Cat # HY-B0083),;

Mdivi-1 (Cat # HY-15886) is purchased from MedChemExpress, NJ, USA. *Mito Tracker Deep Red FM* (Cat # M22426) were purchased from Invitrogen OR, USA.

Fetal bovine serum was purchased from Hyclone, UT, USA, or Gibco, NY, USA. Penicillin and streptomycin were purchased from Invitrogen Life Technologies, CA, USA.

MTT assay

Cells (3 x 10^3 cell/well) were seeded in 96 well plate and kept for 24 h in the incubator. Treatment with drugs was given for various time points as per the experimental requirements. At the end of the experiment, culture media was removed and 50 µL of 1 mg/ml MTT in media without FBS was added in each well. Cells were kept for 3 h in the incubator. Thereafter, MTT was removed and 100 µl of isopropanol was added to solubilize formazan crystals and incubated for 10 min at room temperature. The absorbance was taken at 570nm.

Crystal violet cell biomass assay

Appropriate number of cells were seeded in 12 or 24 well plates and allowed to grow for 24 h. Cells were treated with drugs for 48 h and washed thrice with ice chilled Phosphate Buffered Saline (PBS). Thereafter, 3% PFA was added and kept for 20 min at RT in dark for fixing. Subsequently, 3 washes of 1xPBS were given and crystal violet (0.05% prepared in PBS) was added. The cells were incubated for 1 h with crystal violet stain at RT [19]. Crystal violet stain was washed and pictures were taken using OLYMPUS E-330 digital SLR camera. Images were processed for quantitative analysis by ImageJ Software. Data was plotted by using Graph Pad Prism 7.

Propidium Iodide (PI) exclusion cell death assay

Appropriate number of cells were seeded in 12 well plate and after 24 h they were treated with drugs and/ or inhibitors, as per the experimental design, for various time points. After completion of treatment period, cells were tripsinized and centrifuged at 500 g for 5 min. Supernatant was discarded and cell pellets were washed with PBS. The cell pellets were resuspended in 250 µL of PBS and transferred to the FACS tubes. Immediately before subjecting cells to FACS analysis, cells were incubated with PI by adding 10 µg/mL to each tube for 15 min at RT. Subsequently, cells were analyzed on BD FACSCanto II by using PE channel, and 10000 events / tube were acquired. Data was analyzed using FlowJo Software. The data was plotted as % cell death using GraphPad Prism.

Cell death assay using annexin V and PI

Cells were seeded and after 24 h these were treated with drugs along with respective controls. At the end of treatment duration, cells were tripsinized and washed with PBS. They were resuspended in 1xbinding buffer (provided in by the manufacturer in the kit 1 x 10^6 cells/mL). Cells were further incubated with 0.2 µl/50 µL Annexin V antibody in binding buffer at room temperature for 15 min. Subsequently, 10 µg/mL PI was added to cells in binding buffer for next 15 min. After the completion of incubation, 200 µL of binding buffer was added and cells were acquired using FACS. Data was analyzed by FlowJo Software and percentage change in apoptotic cells was calculated and plotted using GraphPad Prism. Data was represented as total apoptotic cells which is a sum of annexin V positive, PI positive and dual positive cells.

RNA extraction

Cells were treated with drugs and inhibitors after 24 h of seeding. At the end of incubation duration, media was removed and cells were washed with 1X PBS. In each well, 1 mL of

Trizol was added and incubated for 5 min. Cell lysates were collected in 1 ml tubes and homogenized by repeated pipetting. Samples were immediately proceeded for next step and phase separation was done by adding chloroform (0.2 mL per 1 mL Trizol) followed by 3 min of incubation. Subsequently, the samples were centrifuged at 12000 g at 4°C for 15 min. Upper colorless aqueous phase was collected and transferred to new tubes in which 0.5 mL of isopropanol was added. Thereafter, the samples were incubated for 10 min at room temperature and centrifuged at 12000 g for 10 min at 4°C. Supernatant was discarded, and the pellets were resuspended in 1 mL of 75% ethanol, vortexed briefly and centrifuged at 7500 g for 5 min at 4°C. Supernatant was decanted and the pellets were dried in a laminar hood for 10 min. The dried pellets were resuspended in 25 µL of RNAse free water containing 0.1 mM EDTA. The tubes containing samples were heated at 55 ºC for 10 min to solubilize the RNA on a heat block. The RNA concentration was estimated by measuring the absorbance at 260 nm using Nanodrop (Thermo fisher Scientific, USA). The optical density 260/280 was also measured to ensure protein-free RNA.

cDNA preparation

The RNA isolated was used for the preparation of cDNA. The reaction mixer containing 500 µg/mL oligo (dt) primer, dNTPs, RNAse OUT, first strand buffer and 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) was added to tubes in which 2.5-5 µg of isolated RNA was added as per the recommended protocol by the manufacturer. After the completion of reaction, cDNA was stored at -20°C or used for PCR.

Gene	Primer Sequence	Product size (bp)
Human *DNM1L*	(F)CTAGTTACTGCCTACCTGAGTA (R)CAGTTTATAGCCAGTCTCCAC	93
Human *MFN1*	(F)CCGATGAAGTAAACGCCTTA (R)GCAAGGGATCAGTGTATGTAG	110
Human *MFN2*	(F)AAACCCAGTGTCCATCTTTC (R)CCCACTTCAACCAGTAACAA	107
Human *FIS1*	(F)GCTGTGTCCAAGTCCAAATC (R)GGAGAACAGGGAAAGGACA	96
Human *MFF*	(F)CGCCACTTCTAATCCTCATC (R)GCATCTACAACAGTCAGGTC	99
18S rRNA	(F) CGCCGCTAGAGGTGAAATTC (R) CATTCTTGGCAAATGCTTTCG	67

Mitochondrial density measurement

By using Mito Tracker Deep Red FM: Cells were seeded in appropriate plates and incubated for 24 h. Treatments were given as per the experimental design. On completion of incubation, cells were washed by using warm DMEM (without FBS) and 100 nM Mito Tracker Deep Red FM solution was added to each well and further incubated for 30 min at 37°C. Thereafter, cells in wells were washed twice with DMEM (without FBS) and once with PBS. These were tripsinized and resuspended in 250 µL of PBS. Cells were immediately analyzed by FACS. Data was analyzed for the median change in the intensity of dye accumulation inside the cells and plotted using GraphPad Prism.

By using antibody against TOM20: Cells were seeded in the appropriate culture plate and allowed to adhere for 24 h. Treatments were given as per the experimental requirement. After completion of treatment duration, cells were tripsinized and washed once with PBS. These were fixed by using 4% PFA at room temperature for 10 min, washed once with PBS, and resuspended in ice cold methanol for the permeabilization on ice for 10 min. Cells were centrifuged at 500 g for 10 min and supernatant was discarded. Pellets were washed with ice cold PBS. The cell pellets were resuspended in PBS containing translocase of outer mitochondrial membrane 20 (TOM-20) antibody and incubated for 1 h on ice with intermittent tapping at every 15 min. At the end of the incubation, cells were washed with PBS, centrifuged at 500 g for 10 min and resuspended in 200 µL of 0.1% PFA in PBS. Cells were acquired using FACS. Data was analyzed for the median change in the intensity per cell and was plotted using GraphPad Prism.

Animal experiment

Female Balb/c and male NOD/SCID mice were procured from Experimental Animal Facility (EAF) of the BRIC-NCCS, Pune, India. All mice were housed and maintained in animal quarters under controlled environmental condition of 22 ± 2 °C with a light and dark cycle of 12 h, along with free access to clean drinking water and standard rodent pellet food *ad libitum*, unless otherwise mentioned.

Isograft tumor model: Isograft were developed by injecting $3x10^5$ 4T1 cells in mammary fat pad of 6-8 weeks old female BALB/c mice. Measurable tumor developed after 7 days following injection of cells in animals. Mice were randomized in two groups. Mice in group one was treated with 250 mg/kg/day metformin in drinking water whereas, mice in other

group were used as control. Metformin in drinking water was replaced every 3rd day as previously described [10]. Mice were treated with metformin for 18 days.

Xenograft tumor model: Xenograft were developed by injecting $5x10^6$ Hep-3B cells subcutaneously in male NOD/SCID mice. Experiment was performed twice, once only treatment of metformin was given and, second time metformin, doxycycline (DXY) and leflunomide (LEF) were given as single drug or in combination with metformin. A measurable tumor was developed after 22 days of cell injection. Before administration of drugs, mice bearing tumor ≈100 mm^3 were randomized into required groups. Doxycycline (50 mg/kg/day) and leflunomide (25 mg/kg/day) was administered intraperitoneally. Doxycycline was dissolved in saline whereas leflunomide initially dissolved in DMSO and further resuspended in corn oil which resulted in a mixture of 5%DMSO and 95% corn oil. Vehicle of these drugs were administered in control group.

Tumor was measured by using a digital vernier caliper at 3 days intervals. Tumor volumes were calculated by using the formula: 0.523 x (Length) x $(Width)^2$. When the tumor size reached around 50-100 mm^3, tumor bearing mice were randomly distributed into control and 5 experimental groups each containing 4 to 6 animals (Experimental group-1; Metformin, group-2; Leflunomide, group-3; Metformin and Leflunomide, group-4; Doxycycline, group-5; Metformin and Doxycycline). After completion of treatment duration mice were sacrificed and tumors were excised. Tumor weights were measured and pictures taken. Tumors tissues were further processed for the preparation of single cell suspension for the FACS analysis. Part of single cell suspension was utilized for western blot analysis and RT-PCR.

All the animal experiments were performed as per the requirement and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and after obtaining permission from the Institutional Animal Ethics Committee (IAEC; IAEC/2018/B-335, IAEC/2024/B-473).

RNAseq data analysis

Raw Bulk RNA seq count data was downloaded from GEO datasets of metformin treated cancer cells or primary cells from normal individuals treated with metformin (GSE190076, GSE208245, GSE181521, GSE137317, GSE153792). Non-confirmed or unmapped reads were removed prior to analysis. Genes having low counts i.e. less than 5 in at least 60% of the samples were not considered. R (R version 4.4.0) package edgeR (Version 4.2.1) was used to normalize the counts by the TMM method to obtain CPM values. To compare the treatment groups, CPM values were log-transformed and data was plotted using GraphPad Prism

Hematoxylin and eosin Staining

Organs (heart, kidney, lungs, liver) and tumor tissue were excised from the mice and stored in 10% PFA. The preserved samples were outsourced for processing and histopathological analysis (Chaitanya Laboratories, Pune, India). After processing, samples were embedded in paraffin blocks and sectioning were done. During staining, tissue sections were deparaffinized and hydrated with xylene and graded alcohol. Then, they were stained with hematoxylin to highlight nuclei followed by rinsing with alcohol. Following this, cytoplasmic structures were stained with eosin. After further rinsing and dehydration with alcohol, the sections were mounted on coverslip and allowed to dry. Finally, the prepared slides were observed under a microscope and images were taken

for detailed histological and pathological analysis. The images were analyzed by pathologist and toxicity grading score were calculated as No Abnormality Detected (NAD), Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4).

Mito stress assay for mitochondrial function

Cells (1.5×10^4 to 3×10^4 cells/well) were plated into 24-well XF Cell culture plates (Cat # 100,777–004; Agilent Technologies, CA, USA) and allowed to grow overnight in a 5% CO2 incubator at 37 °C. Cells were then treated with vehicle or metformin and Mdivi-1 as a single drug or in combination for 24 h in 10% FBS-containing media and assays were performed. After treatment, cells were washed with their respective assay medium and incubated in a humidified non-CO2 incubator at 37 °C for 45 min with 500 µl of assay medium (XF base or DMEM medium). Experiments were performed in Agilent XF base media (102,353–100) as per protocol provided by the Agilent Seahorse XF Cell Mito Stress Test Kit user guide. Oligomycin, carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone & antimycin were injected through port A, B, and C respectively in each well. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was measured by using Seahorse XFe24 analyzer Agilent (CA, USA). Data was obtained and plotted is included.

Statistical analysis

Statistical analysis (student's 2-tailed unpaired t-test and one-way or two-way ANOVA) was performed by using Graph Pad Prism Software for comparison of different groups. In majorities of the *in vitro* experiments, bars represent experimental variations within the wells, unless otherwise mentioned. The significance in tumor volume were calculated by

applying Kruskal-Wallis's test followed by Dunn's multiple comparisons test. The values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P< 0.001$).

Results

Metformin has minimal effect on tumor progression in preclinical models

Previously, it has been reported that administering 250 mg/kg/day of metformin to mice resulted in a steady-state serum concentration of approximately 10μM [10]. This serum concentration of metformin is comparable to levels attainable in humans by a dose of 500 mg/day. The maximum approved dose of metformin for humans is 2500 mg/day [20]. To assess the impact of metformin on tumor growth, we utilized the 4T1 (a triple negative murine breast cancer cells) and Hep-3B (a hepatitis B associated hepatocellular carcinoma cells) tumor models. The 4T1 cells were subcutaneously injected in the mammary fat pad of BALB/c female mice and Hep-3B cells were subcutaneously injected in right upper flank of male NOD/SCID mice. BALB/c mice have intact immune system whereas NOD/SCID mice are immunocompromised as immune cells are dysfunctional. Tumor bearing mice were randomly assigned to metformin group and control group. The treatment group of 4T1 tumor bearing mice received 250 mg/kg/day metformin and Hep-3B tumor bearing mice received 250 / 500 mg/kg/day metformin through drinking water. The schematic representation of animal experiment layout is depicted in Figure-1A. Tumor volume was monitored every 3rd day by a digital vernier calipers. In both cases mice were treated for 18 days after the appearance of palpable tumor. Mice weight and tumor growth were closely monitored throughout the experiment. Weight of mice remained unchanged in all treatment groups compared to control, indicative of minimal drug associated adverse effect in animals (Figure-1C and F). In both of these tumor models it was observed that metformin treatment has insignificant effect on the growth of tumors, which is clearly evident by determination of tumor volume and tumor images

(Figure-1). These findings indicate that metformin treatment alone does not impede tumor growth.

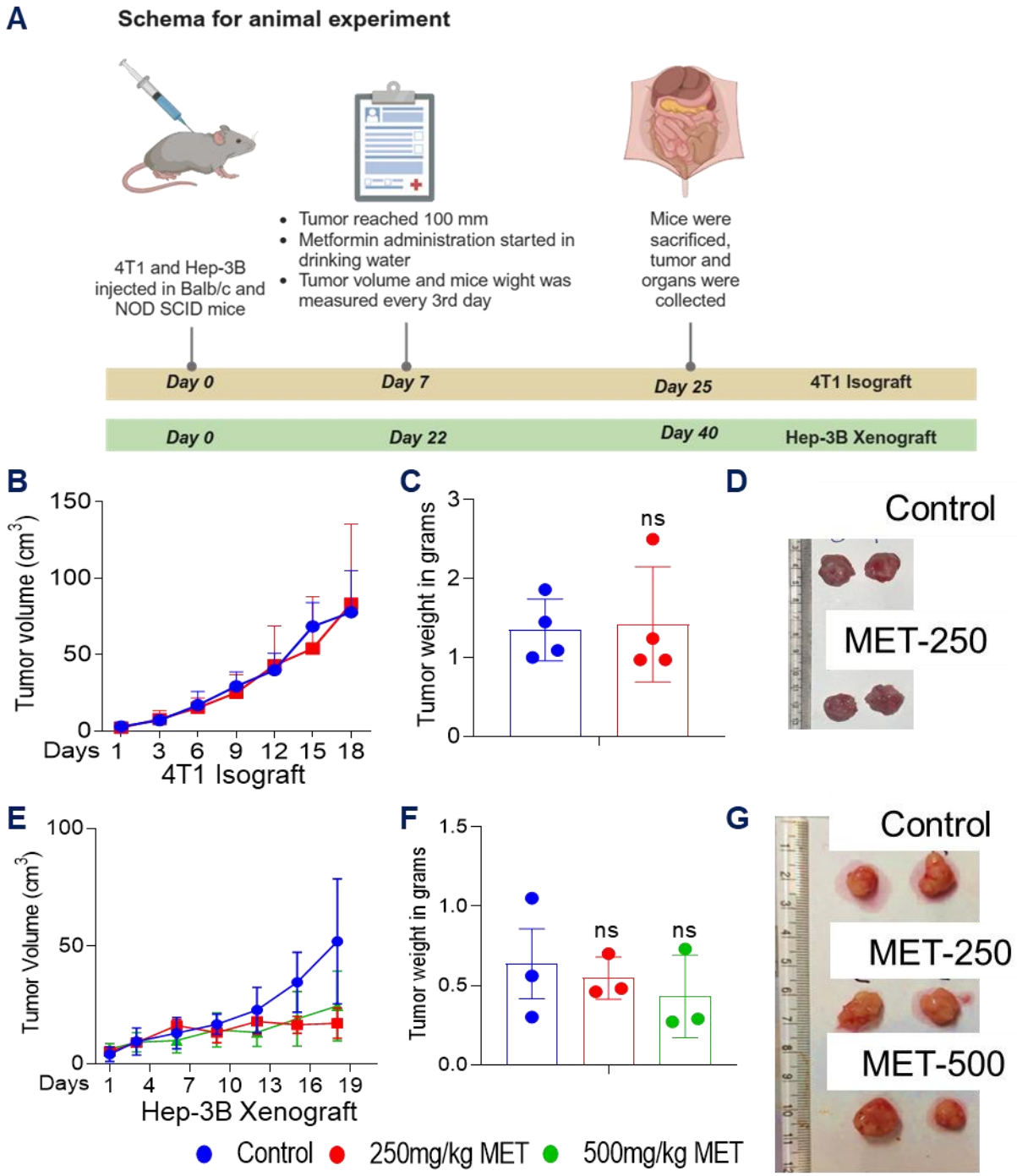


Figure 1: Effect of metformin on the growth of 4T1 isograft and Hep-3B xenograft tumors. *4T1 cells were injected in mammary fat pad of Balb/c mice and Hep-3B cells were injected in upper right flank of NOD/SCID mice. Balb/c mice were given 250 mg/kg/day metformin and NOD/SCID were given 250 or 500 mg/kg/day metformin in drinking water. At the end of the experiment tumor were excised and tumor weight were measured; (A) Schema of animal experiment (Created in BioRender. Seshadri, V. (2025) https://BioRender.com/t62p562), (B) 4T1 tumor volume measured by digital vernier caliper, (C) Excised 4T1 tumor weight, (D) 4T1 tumor photograph, (E) Hep-3B tumor volume, (F) Hep-3B tumor weight, (G) Hep-3B tumor photograph. Data was plotted using GraphPad Prism. The significance in tumor volume change were calculated by applying Kruskal-Wallis test followed by Dunn's multiple comparisons test. Respective p-values are denoted by "*", where if a p-value <0.05(*), <0.01(**), <0.001(***) and <0.0001(****).*

Metformin treatment causes growth arrest in cancer cells

To evaluate the effect of metformin on the cancer cells, we selected a range of concentrations from 0.1 to 10 mM. The lower concentration of 0.1 mM is close to physiologically relevant concentration, while a concentration of around ≥ 5 mM is required to effectively inhibit mitochondrial complex I in vitro [20]. Therefore, in this study, the cells were treated with 0.1, 1, and 10 mM of metformin.

To evaluate the consequence of acute and chronic treatment of metformin, cancer cells were treated for 24,48,72 and 248 h with 0.1, 1- and 10-mM metformin. The effect was evaluated by MTT and crystal violet assay as per schema (Figure-2A). A concentration

and time dependent inhibition in growth was detected in HT-29, HCT-116, Hep-3B and Hep-G2 cells by MTT assay (Figure-2B).

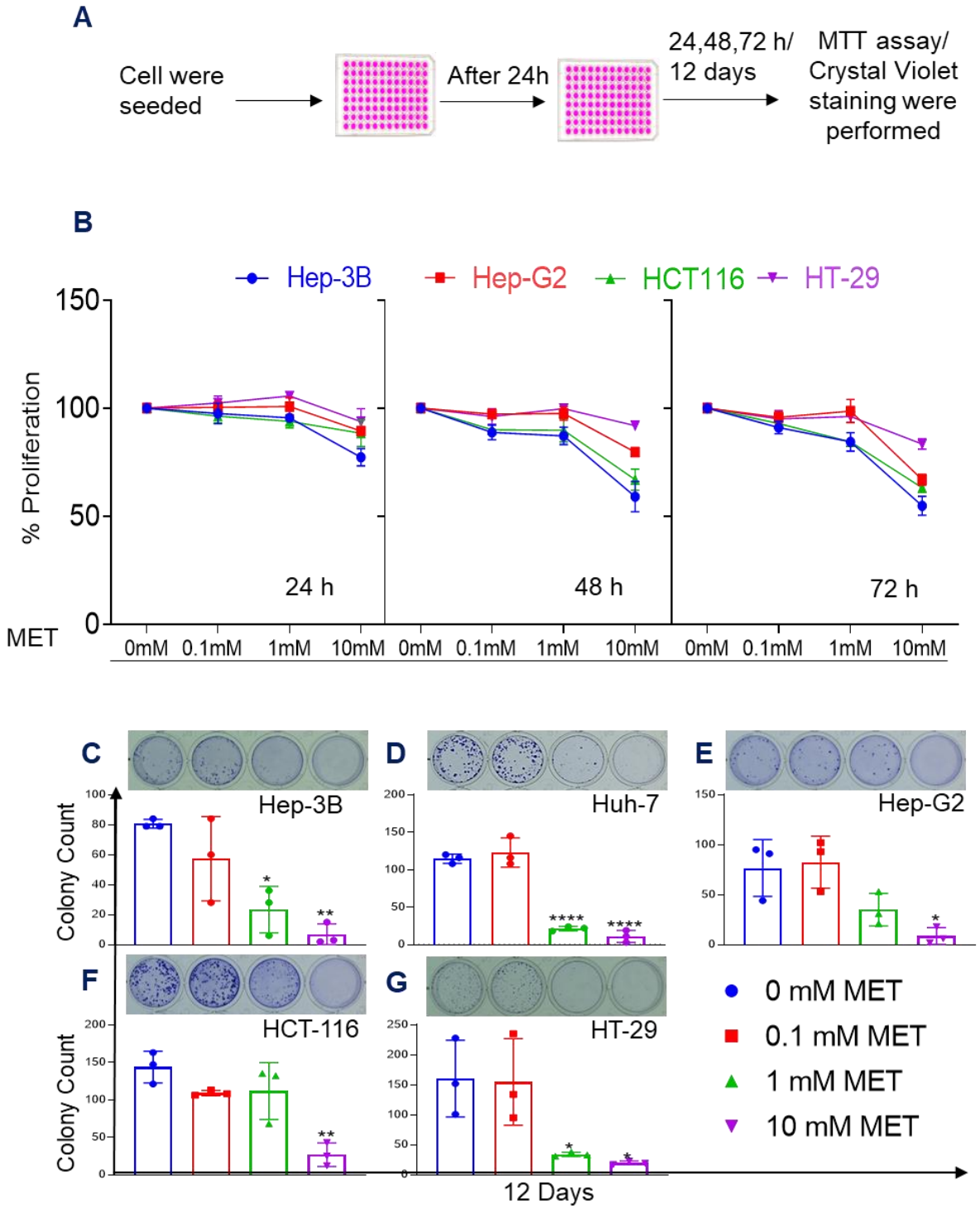


Figure 2: Effect of metformin on cancer cell growth and survival. (*A) Schema representing in-vitro experimental layout, (B) HCC and CRC cells were treated with 0.1, 1, 10 mM metformin for 24, 48 and 72 h and MTT assay was performed as per the protocol mentioned in the methodology section, (C) Hep-3B, (D) Huh-7, (E) Hep-G2, (F) HCT-116 and (G) HT-29 cells were treated with 0.1, 1, 10 mM of metformin for 12 days and stained with crystal violet. For statistical analysis, a paired Student's t-test was performed, with p-values denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Also, to evaluate the consequences of chronic exposure of cells to metformin on colony inhibition, colorectal carcinoma (CRC) and hepatocellular carcinoma (HCC) cells were treated for 12 days by replacing old media with new media containing metformin every 3rd day. After completion of treatment period, cells were processed, stained with crystal violet and colonies were counted. A dramatic reduction in colony-forming ability was observed in all tested cells upon exposure to 10 mM metformin. However, lower concentrations, which are closer to physiologically relevant levels, failed to significantly impact colony count, even with chronic exposure. These findings suggest that at low-doses metformin does not impede the colony formatting ability of cancer cells (Figure 2C-G).

Metformin treatment modulates mitochondrial dynamics

The data mentioned above indicate that higher concentrations of metformin treatment restrict cancer cell growth in vitro and it does not inhibit tumor growth significantly. This prompted us to investigate cellular events those may be contributing to the

ineffectiveness of metformin.

Earlier, our group has reported that energy stress in cancer cells promotes mitochondrial biogenesis, which is linked to the survival [17,21]. This finding aligns with several reports on the relevance of alterations in mitochondrial dynamics (biogenesis, fission, fusion and mitophagy) in supporting cancer cell survival under adverse conditions such as nutrient deprivation, hypoxia, and drug exposure [22]. Mitochondrial fusion and fission play a vital role in regulating mitochondrial homeostasis. These processes are crucial for maintaining mitochondrial health, ensuring efficient energy production, and enabling cellular adaptation to stress. Fusion, mediated by proteins such as Mito Fusin 1 (MFN-1) and Mito Fusin 2 (MFN-2), allows mitochondria to merge and share contents, thereby preserving mitochondrial integrity. In contrast, fission is regulated by Dynamin-related protein 1 (DRP-1) and facilitated by proteins like FIS-1 and MFF to promotes the division of mitochondria to isolate the damaged sections for degradation. [23]. These mitochondrial dynamics, enable mitochondria to respond to cellular stress, which may contribute to survival of cancer cell under metabolic constraints.

Thus, alteration in mitochondrial dynamics were probed into. To assess if metformin treatment is promoting mitochondrial biogenesis, HCC and CRC cells were evaluated for the status of mitochondrial biomass by staining with *Mito Tracker Deep Red FM dye*. Thereafter, single-cell dye accumulation was measured using FACS, and data was analyzed to calculate Median Fluorescence Intensity (MFI). Notably, metformin treatment causes a significant increase in mitochondrial dye accumulation in Hep-3B, Huh-7, HCT116, HT-29, and HCT15 cells, whereas, in Hep-G2 cells, no noticeable change was detected (Figure-3A).

Increase in the accumulation of dye, as detected by FACS and by confocal microscopic analysis, was noted. Also, an increase in TOM-20 protein levels was detected in Hep-3B cell treated with metformin (Figure-3B and C). Confocal images were acquired and analyzed for the changes in mitochondrial morphologies after metformin treatment by image J plugin mitochondrial analyzer. Image analysis has shown a significant increase in the circularity and decrease in the perimeter and area of single mitochondrial structure in metformin treated Hep-3B cells. These parameters suggest an increase in mitochondrial fission in the cells treated with metformin compared to control (Supplementary Figure-S6). Moreover, the effect of metformin treatment on mRNA levels of genes involved in mitochondrial dynamics were evaluated by probing into the status of MFF-1, FIS1, DRP-1, MFN-1 and MFN-2 genes. The result obtained indicate that metformin treatment increases mitochondrial fission associated genes in Hep-3B cells (Figure-3D and E). This was also validated by western blotting and an increased expression of DRP-1 was detected (Figure-3F).

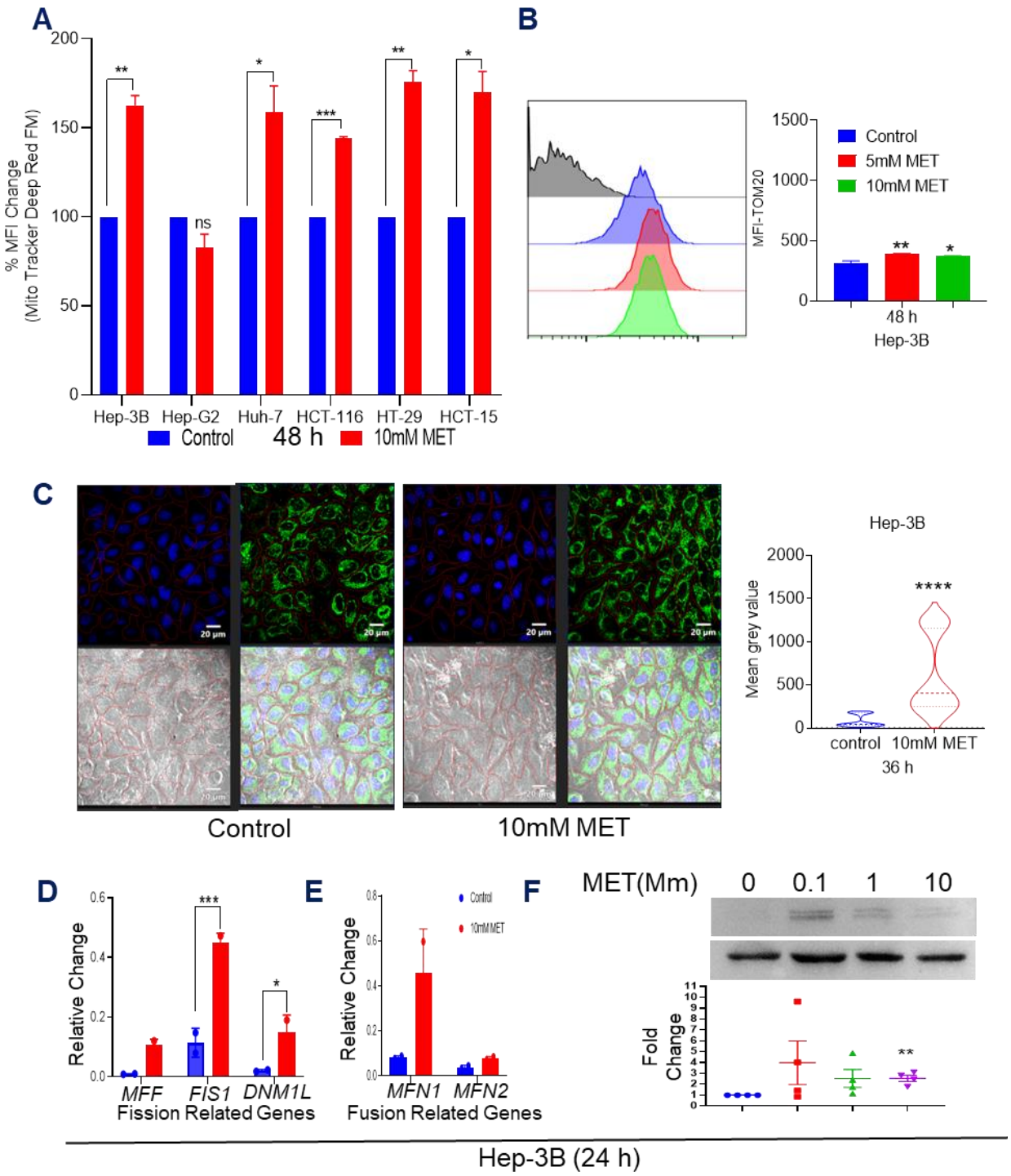


Figure 3: Effect of metformin on mitochondrial dynamics in human cancer cells in in-vitro. *(A) HCC and CRC cells were treated with 10 mM metformin for 48 h and stained with Mito Tracker Deep Red FM dye; data are represented as MFI, (B) Hep-3B cells were*

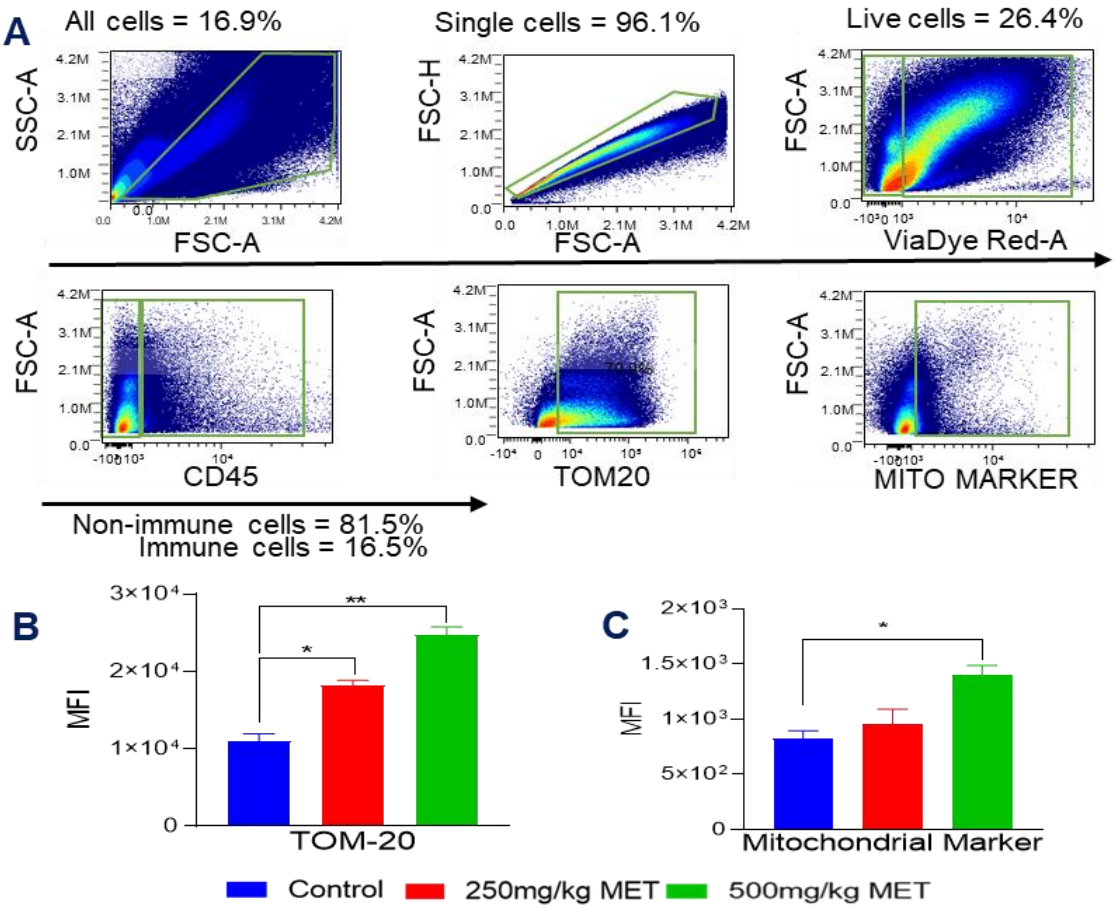
*treated with metformin for 48 h and stained with the mitochondrial protein TOM-20. Data was acquired using FACS, and MFI was plotted after analysis with FlowJo and GraphPad Prism, (C) Hep-3B cells were treated with metformin for 36 h and stained with TOM-20, confocal images were analyzed for changes in single-cell MFI using Cell Sense software , (D) Hep-3B cells were treated with metformin for 24 h and assessed for expression of mitochondrial dynamics-related genes by RT-PCR, (D) Fission-related gene expression in Hep-3B, (E) Fusion-related gene expression in Hep-3B. (F) DRP-1 expression in Hep-3B cell treated with metformin for 24 h. For statistical analysis, a paired Student's t-test was performed, with p-values denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Metformin treatment influences mitochondrial dynamics in liver cancer cells

Systemic effect of metformin on the mitochondrial markers was evaluated in a single cell suspension of excised tumors (from Figure 1). This single cell suspension was probed with antibodies for mitochondrial markers as well as for CD45 (a pan immune cell marker). The processed cells were gated for CD45 negative population and analyzed for the status of mitochondrial markers (Figure-4A). Interestingly, an increase in TOM-20 levels and also pan mitochondrial marker staining was observed in metformin treated tumor cells compared to control (Figure-4B and C). However, the level remained unchanged in 4T1 tumor cells (Supplementary Figure-S1). This finding indicate that metformin alters mitochondrial dynamics *in-vivo* in Hep-3B xenografted tumor cells. Additionally, a part of single-cell suspension was used for the preparation of whole cell lysate for western blotting analysis to probe into the status of mitochondrial transcription factor A (TFAM)

expression. An increase in TFAM expression was observed in metformin-treated conditions compared to control (Figure-4D). TFAM is a transcription factor which regulates protein associated with mitochondria and mitochondrial biogenesis [24]. No significant change was observed in the expression of LDH-A, a rate limiting enzyme in glycolysis (Figure-4D).

Furthermore, publicly available RNAseq datasets were analyzed to evaluate metformin induced changes in mitochondrial dynamics related genes. It was observed that metformin alters mitochondrial dynamics associated genes (Supplementary Figure-S3). Collectively, our findings indicate that metformin treatment per se does not influence tumor size. However, it alters mitochondrial dynamics, which points towards its involvement in the survival of tumor cells.


A
All cells = 16.9%
Single cells = 96.1%
Live cells = 26.4%
SSC-A
FSC-A
FSC-H
ViaDye Red-A
CD45
TOM20
MITO MARKER
Non-immune cells = 81.5%
Immune cells = 16.5%
B
MFI
TOM-20
C
Mitochondrial Marker
Control
250mg/kg MET
500mg/kg MET

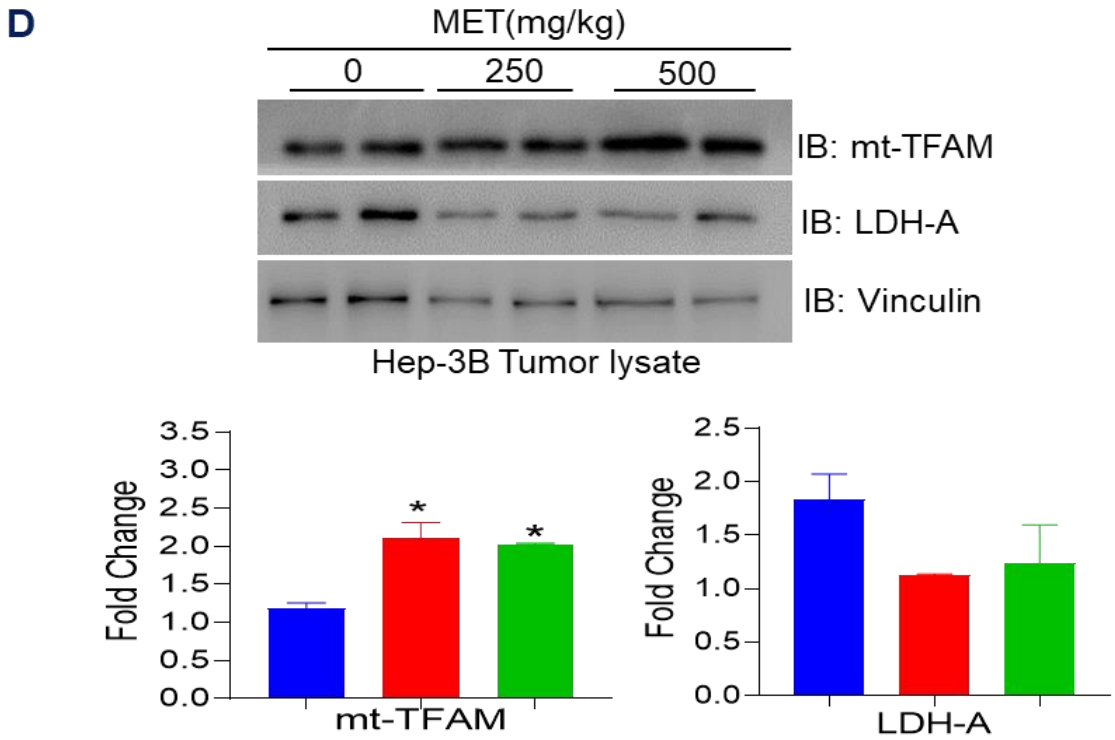

D
MET(mg/kg)
0
250
500
IB: mt-TFAM
IB: LDH-A
IB: Vinculin
Hep-3B Tumor lysate
Fold Change
mt-TFAM
LDH-A

Figure 4: Effect of metformin on mitochondrial biomass in Hep-3B xenografted tumor cells. *Hep-3B cells were injected into NOD/SCID mice, and treatment with 250 mg/kg/day or 500 mg/kg/day metformin was administered through drinking water. At the end of the experiment, tumors were excised, and single-cell suspensions were prepared. Cells were stained with respective markers an analyzed by using FACS. (A) Shows gating strategies for the evaluation of mitochondrial markers in single-cell suspension cells, (B) The MFI of TOM-20, (C) Mitochondrial marker MFI were calculated by analyzing data using SpectroFlo and OMIQ software. Lysates were prepared from cell suspension and western blot were performed as per the protocol mentioned in the methodology section, images were acquired using UVTECH ChemiDoc and processed using ImageJ Software. The mean gray values were calculated and plotted. (D) Representative blot and mean intensity is showing expression of mt-TFAM and LDH-A levels, Graphs and statistical analysis was done using GraphPad Prism. For statistical analysis, a paired Student's t-test was performed, and p-values are denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Targeting metformin induced changes in mitochondrial dynamics promotes cytotoxicity in cancer cells

Above mentioned findings indicate that metformin treatment modulates mitochondrial biogenesis and fission. To investigate relevance of these processes in cancer cell survival, the use of pharmacological agents which specifically target mitochondrial dynamics were explored. Doxycycline, an inhibitor of mitochondrial biogenesis [25], diminishes formation of new mitochondria, while Mdivi-1, a selective DRP-1 inhibitor, was

used to directly inhibit mitochondrial fission, thereby limiting the division and separation of mitochondria. Additionally, leflunomide was utilized to promote mitochondrial fusion. Notably, leflunomide-induced fusion also exerts an indirect inhibitory effect on fission, as these processes are inversely correlated.

(A) Targeting metformin induced biogenesis promotes cytotoxicity in cancer cells

The role of mitochondrial biogenesis towards the ability of cancer cells to overcome the metabolic stress actuated by metformin was explored. Therefore, Hep-3B and Huh-7 cells were treated with either metformin, doxycycline, or a combination of metformin and doxycycline. After completion of the treatment period, cells were processed and analyzed for changes in mitochondrial biomass and cell death by FACS analysis. In Hep-3B cells the combined treatment effectively abrogated the increase in mitochondrial biomass (Supplementary Figure-S2). Whereas, the combination augmented cells death in Hep-3B and Huh-7 cells (Figure-5A ad B).

(B) Targeting metformin promoted fission enhances cytotoxicity in cancer cells

The importance of metformin-induced mitochondrial fission on cancer cell survival was probed into by treating the cells with Mdivi-1 and leflunomide to promote fusion. HCC cells were treated with Mdivi-1, a mitochondrial division inhibitor, in combination with metformin, and cell death was assessed. Our results indicate that metformin treatment when coupled with the DRP-1 inhibitor, significantly augmented cell death compared to individual drug treatments in Hep-3B and Huh-7 cells (Figure – 5C and D). Furthermore, Hep-3B cells were treated with metformin alone or in combination with Mdivi-1 for 24 h

and subjected to the bioenergetic phenotype analysis which measured oxygen consumption rate (Supplementary Figure-S7A) and extra cellular acidification rate (Supplementary Figure-S7D). The data indicated a decreased basal respiration rate (Supplementary Figure-S7B), Proton leak (Supplementary Figure-S7C), and stressed extracellular acidification rate. Whereas, a significant increase in stressed oxygen consumption rate was detected in combination compared to single treatment (Supplementary Figure-S7E). Overall single drug treatment stretches metabolic flexibility which is neutralized when combine treatment of metformin and Mdivi-1 was given (Supplementary Figure-S7F).

In second strategy fission were targeted through the use leflunomide (DHODH inhibitor). DHODH is an enzyme located in the inner mitochondrial membrane that catalyzes the rate-limiting step in de novo pyrimidine nucleotide synthesis which generates uridine monophosphate [26]. Our results indicate that combined treatment of metformin with leflunomide enhances death in HCC cells (Figure-5E and F).

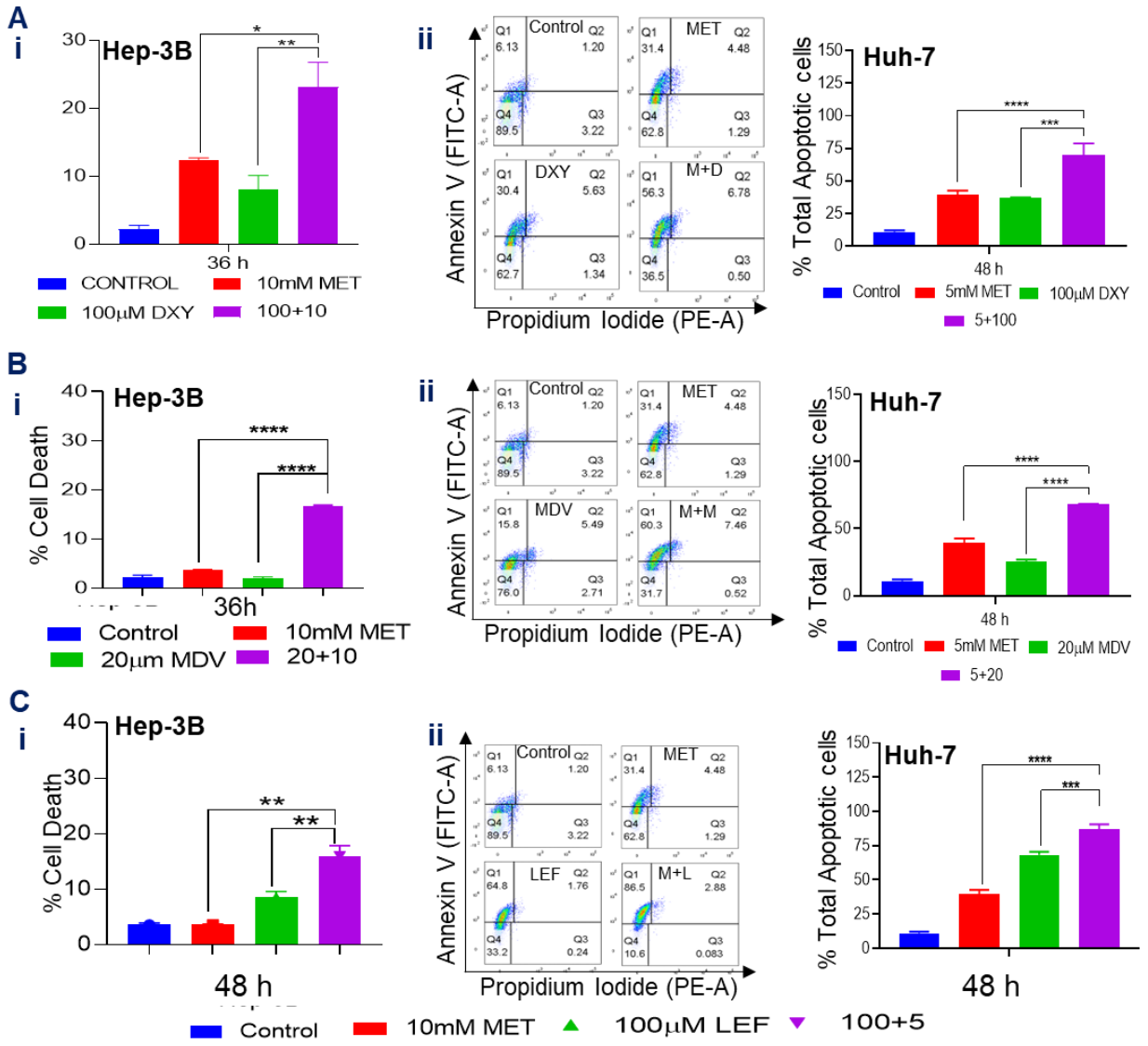


Figure 5: Effect of targeting metformin-induced mitochondrial dynamics on cancer cell survival in vitro. *An equal number of Hep-3B and Huh-7 cells were seeded, and treatment with metformin as single agent or in combination with mitochondrial dynamics modulator was given. After the end of incubation time,, Hep-3B cells were subjected to, propidium iodide (PI) exclusion cell death assay and Huh-7 cells were evaluated by Annexin V /PI cell death assay, (A) (i) Hep-3B cells, (ii) Huh-7 cells were treated with*

*metformin and DXY (Doxycycline) individually or in combination with the respective controls for 36 h, and 48 h respectively and subjected to cell death assay (B)) (i) Hep-3B cells, (ii) Huh-7 cells treated with metformin and MDV(Mdivi-1) as a single agent or in combination, along with the respective controls for 36 h and 48 h respectively and subjected to cell death assay . (C) (i) Hep-3B cells, (ii) Huh-7 cells were treated with metformin and LEF as a single agent or in combination, along with the respective controls for 48 h AND subjected to cell death assay. Data were analyzed using FlowJo Software, and the percentage of positive cells was denoted as dead cells. Graphs and statistical analysis were performed using GraphPad Prism. A one-way ANOVA was performed. Respective p-values are denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Targeting metformin promoted changes in mitochondrial dynamics arrest growth of tumor

Increase in the cell death in HCC cells treated with metformin in combination with doxycycline as well as leflunomide led us to evaluate implication of these combination in-tumor xenografted mice. For this, Hep-3B cells were xenografted in NOD/SCID mice and experiment were performed as shown in Figure-6A. Tumor volumes of mice were measured every 3rd day. Mice were randomized in six different groups. No noticeable change in body weight of mice across all groups was detected indicative of no gross symptoms of toxicity or possible adverse side effects in animals treated with drugs (Figure-7C). Importantly, a significant inhibition in tumor growth was detected in the combination of metformin with leflunomide and doxycycline (Figure-7B and D). Moreover,

sections of tumor tissues and organs (heart, kidney, lungs, liver) from mice treated with single drug or combination were H&E stained for the histopathological analysis. The treatment of metformin, doxycycline, leflunomide and their combination with metformin exhibited minimal toxicity on heart, kidney, lungs and liver of the animals. Doxycycline treatment resulted in mild toxicity in liver as is evident by histopathological observations which was reduced in the presence of metformin (Supplementary Table-1).

Interestingly, in the animal treated with combination of Metformin + leflunomide/ Metformin + Doxycycline, a significant degenerative and narcotic changes in tumor sections compared to single drug treatment group was detected (Supplementary Figure-S5 and Supplementary Table-1).

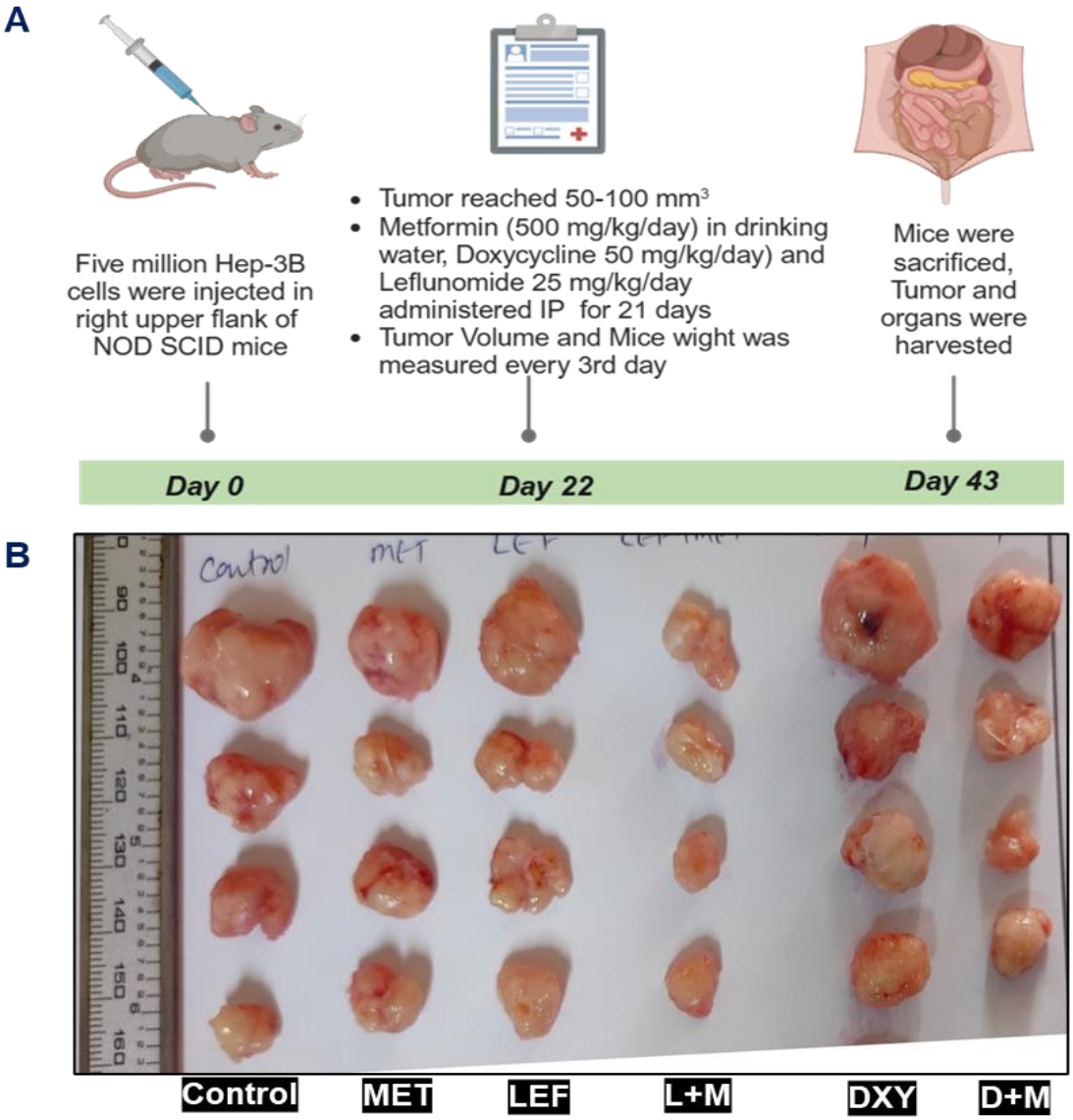

A
Five million Hep-3B cells were injected in right upper flank of NOD SCID mice
Tumor reached 50-100 mm3
Metformin (500 mg/kg/day) in drinking water, Doxycycline 50 mg/kg/day) and Leflunomide 25 mg/kg/day administered IP for 21 days
Tumor Volume and Mice wight was measured every 3rd day
Mice were sacrificed, Tumor and organs were harvested
Day 0
Day 22
Day 43
B
Control
MET
LEF
L+M
DXY
D+M
C
Mice Weight (g)
Days
D
Control
MET
LEF
M+L
DXY
M+D
Tumor Volume (mm3)
Days
**
*

Figure 6: Effect of targeting metformin-induced mitochondrial dynamics on the growth of Hep-3B xenograft tumors*. Hep-3B cells were injected into NOD/SCID mice. Subsequently, treatment with metformin (500 mg/kg/day), doxycycline (50 mg/kg/day), and leflunomide (25 mg/kg/day) were administered as single agents or in combination with metformin in the appropriate vehicle, as described in the methodology section. At the end of the experiment, tumors were excised, and tumor weights were measured. (A) Schematic representation of Hep-3B xenograft tumors in NOD/SCID mice. (B) Photograph of Hep-3B tumors at experiment termination. (C) Weight of Hep-3B tumor bearing mice, (D) Hep-3B tumor volume measured using a digital vernier caliper. Data was plotted using GraphPad Prism. Respective p-values are denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Discussion

Use of Metformin as an anticancer agent has given mixed outcome in preclinical and clinical settings when given as single drug or in combination with other therapies [14,17,27,28]. Preclinical testing approaches have been refined to account for varying doses and administration routes of metformin. Given the high clearance rate and tolerability of metformin in mice, some models more accurately mimic human pharmacokinetics than others [29,30]. In humans, the attainable serum concentration of metformin is approximately 10-40 μM [20]. Additionally, our result, together with previously published work from our laboratory and existing literature, indicate that serum attainable metformin concentrations are insufficient to cause cell death in vitro (Figure-2). Notably, Even higher concentrations of metformin transiently inhibit growth, without inducing cell death in colon cancer cells [31,32]. Metformin, in-vitro and in-vivo inhibits cell proliferation and tumor growth by abrogating function of mitochondrial complex I. However, metformin treatment does not cause cell death in-vitro due to availability of excess nutrients which support metabolic flexibility of cancer cells [10]. In this study metformin was administered at a dose of 250mg/kg/day in 4T1 isograft and 250 /500 mg/kg/day in Hep-3B xenograft tumors. In Hep-3B tumor xenograft administration of 500 mg/kg/day of metformin did not reduce tumor growth compared 250 mg/kg/day in HCT-116 xenograft model. This suggest that complex I inhibition by metformin it not translating into tumor growth inhibition [18,33]. These findings indicate that cancer cells can adapt to metformin treatment thereby limiting its effectiveness.

Metformin actuated alterations in cancer cell metabolism has been proposed to hold therapeutical potential. Recently It has been demonstrated that energy stress in cancer

cells is associated with increased mitochondrial biogenesis, as an adaptive response that can influence survival outcomes [18,34]. Moreover, increase in mitochondrial density has been proposed to potentially modulate metformin's pro-apoptotic effect in cancer cells [35]. However, the studies evaluating mechanism linking the role of metformin induced alteration in mitochondrial dynamics towards cancer cells survival and subsequently targeting these for growth inhibition are lacking. Therefore, the impact of metformin on mitochondrial dynamics was investigated. Interestingly, our findings from in-vitro and in-vivo investigations indicate that metformin treatment increases mitochondrial biogenesis and fission in cancer cells (Figures 3 and 4). Moreover, upon analyzing publicly available RNAseq data, alterations in the expression of genes related to mitochondrial dynamics and mitochondrial encoded genes was detected in HCC cells. Parallelly, no significant change in these genes were detected in PBMCs of normal individuals administered with metformin (Supplementary Figure S4). Additionally, RNAseq data analysis from the Cancer Genome Atlas database correlated enhanced expression of mitochondrial fission related genes with the cell cycle pathway, tumor stemness maintenances, TP53 mutation, immune infiltration and disease progress in HCC [36]. Based on above mentioned findings, we hypothesized that changes in mitochondrial dynamics by metformin is likely to support survival of cancer cells. On these lines, our results indicate that combining metformin with modulators of mitochondrial dynamics potentiate cancer cells death (Figure-5). Moreover, in Hep-3B xenograft model co-administration of metformin with either with doxycycline or with leflunomide causes inhibition of tumor growth. These data indicate that metformin promoted alteration in mitochondrial dynamics is a contributory factor towards the survival of cancer cells (Figure-6). Moreover, these combinations

cause inhibition of tumor growth by inducing death in the tumor cells (Figure-6 and Supplementary Table-1), with minimal adverse effects on vital organs and survival of mice. On these lines the use of anticancer agents such as cytarabine, etoposide, doxorubicin, bleomycin, zalcitabine (ddC), and arsenic trioxide because of their known inhibitory effect on mitochondrial biogenesis in combination with metformin or other complex I inhibitors are likely to positively influence therapeutical outcomes [37]. Also, a synergistic effect of metformin with doxorubicin and etoposide has been reported, which further strengthen this proposition [38,39].

Metformin has been reported to promote mitochondrial fission in hepatic stellate cells [40]. It increases mitochondrial fission and enhances mitochondrial respiration specifically in hepatocytes of obese mice [41]. Based on these reports, we examined whether in HCC cells metformin promotes mitochondrial fission by evaluating the expression levels of genes such as MFF-1, FIS-1, and DRP-1. Also, the morphology of mitochondria was evaluated in metformin treated Hep-3B cells. The findings indicate that metformin treatment significantly upregulates expression of these genes (Figure-3) and skew the mitochondrial dynamics toward fission (Supplementary Figure-S6). Interestingly, combine inhibition of mitochondrial complex I and DRP-1 or induction of mitochondrial fusion by FDA-approved anti-arthritic drug leflunomide promotes HCC cells death compared to single drug treatment (Figure-5). Moreover, combination treatment resulted in the abrogation of metabolic plasticity as reflected by cellular metabolic phenotype analysis, is likely to play a role in diminishing the survival of cancer cells. (Supplementary Figure-S7). Interesting, targeting fission in metformin-treated tumors significantly reduced tumor growth (Figure-6).

Upregulation of oncogenic signaling pathways such as RAS/RAF/MEK/ERK axis, and complex I inhibitors (rotenone and phenformin) are known to promote mitochondria fission in cancer cells [42]. Inhibitors of RAS/RAF/MEK/ERK axis causes an increase in mitochondrial fusion. Therefore, it plausible that a combination of complex I inhibitors together with inhibitors of this axis are likely to amplifying the therapeutic impact against cancer cells in which metabolic plasticity is driven by mitochondrial fission.

In conclusion, our study underscores metformin's impact on mitochondrial dynamics, revealing potential therapeutic targets. Combining metformin with inhibitors of mitochondrial biogenesis or fission in preclinical and clinical settings can potentially enhance metformin's anticancer efficacy.

Acknowledgements: F.K.B. and U.S. acknowledges University Grants Commission (UGC), New Delhi, India for research fellowship. F.K.B also acknowledges Science and Engineering Research Board, Department of science and technology for senior research fellowship. The authors thank Biotechnology Research and Innovation Council - National Centre for Cell Science (BRIC- NCCS), Pune, India, and Savitribai Phule Pune University, Pune, India for their support.

Author contributions: F.K.B: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. M.K.B.: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. U.S.: Writing – review & editing, Methodology, Formal Analysis. S.S: Resources, Methodology, Formal Analysis, Writing – review & editing.

Funding

This work was supported by an intramural grant from the Biotechnology Research and Innovation Council- National Center for Cell Sciences (BRIC-NCCS), funded by the Department of Biotechnology (DBT), Government of India and a research grant to MKB by the Science and Engineering Research Board (SERB sanction order number CRG/2020/005264 dated- 29.05.2023), Government of India. The funding agencies were not involved in the study design, data collection, interpretation, analysis, decision to publish, or writing of the manuscript

References:

[1] S. McGuirk, Y. Audet-Delage, J. St-Pierre, Metabolic Fitness and Plasticity in Cancer Progression, Trends Cancer 6 (2020) 49–61. https://doi.org/10.1016/j.trecan.2019.11.009.

[2] M. Tufail, C.-H. Jiang, N. Li, Altered metabolism in cancer: insights into energy pathways and therapeutic targets, Mol. Cancer 23 (2024) 203. https://doi.org/10.1186/s12943-024-02119-3.

[3] J. Chiche, M. Rouleau, P. Gounon, M.C. Brahimi-Horn, J. Pouysségur, N.M. Mazure, Hypoxic enlarged mitochondria protect cancer cells from apoptotic stimuli, J. Cell. Physiol. 222 (2010) 648–657. https://doi.org/10.1002/jcp.21984.

[4] A.D. Scheid, T.C. Beadnell, D.R. Welch, Roles of mitochondria in the hallmarks of metastasis, Br. J. Cancer 124 (2021) 124–135. https://doi.org/10.1038/s41416-020-01125-8.

[5] S. Fulda, L. Galluzzi, G. Kroemer, Targeting mitochondria for cancer therapy, Nat. Rev. Drug Discov. 9 (2010) 447–464. https://doi.org/10.1038/nrd3137.

[6] S. Mukherjee, G.K. Bhatti, R. Chhabra, P.H. Reddy, J.S. Bhatti, Targeting mitochondria as a potential therapeutic strategy against chemoresistance in cancer, Biomed. Pharmacother. 160 (2023) 114398. https://doi.org/10.1016/j.biopha.2023.114398.

[7] F.A. Urra, F. Muñoz, A. Lovy, C. Cárdenas, The Mitochondrial Complex(I)ty of Cancer, Front. Oncol. 7 (2017). https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2017.00118.

[8] R. Masoud, G. Reyes-Castellanos, S. Lac, J. Garcia, S. Dou, L. Shintu, N. Abdel Hadi, T. Gicquel, A. El Kaoutari, B. Diémé, F. Tranchida, L. Cormareche, L. Borge, O. Gayet, E. Pasquier, N. Dusetti, J. Iovanna, A. Carrier, Targeting Mitochondrial Complex I Overcomes Chemoresistance in High OXPHOS Pancreatic Cancer, Cell Rep. Med. 1 (2020). https://doi.org/10.1016/j.xcrm.2020.100143.

[9] Y. Zhou, J. Zou, J. Xu, Y. Zhou, X. Cen, Y. Zhao, Recent advances of mitochondrial complex I inhibitors for cancer therapy: Current status and future perspectives., Eur. J. Med. Chem. 251 (2023) 115219. https://doi.org/10.1016/j.ejmech.2023.115219.

[10] W.W. Wheaton, S.E. Weinberg, R.B. Hamanaka, S. Soberanes, L.B. Sullivan, E. Anso, A. Glasauer, E. Dufour, G.M. Mutlu, G.S. Budigner, N.S. Chandel, Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis, eLife 3 (2014) e02242. https://doi.org/10.7554/eLife.02242.

[11] K. Vasan, M. Werner, N.S. Chandel, Mitochondrial Metabolism as a Target for Cancer Therapy, Cell Metab. 32 (2020) 341–352. https://doi.org/10.1016/j.cmet.2020.06.019.

[12] R. Alghandour, M.A. Ebrahim, A.M. Elshal, F. Ghobrial, M. Elzaafarany, M.A. ELbaiomy, Repurposing metformin as anticancer drug: Randomized controlled trial in advanced prostate cancer (MANSMED), Urol. Oncol. Semin. Orig. Investig. 39 (2021) 831.e1-831.e10. https://doi.org/10.1016/j.urolonc.2021.05.020.

[13] H.E. Barakat, Hussein ,Raghda R. S., Elberry ,Ahmed Abdullah, Zaki ,Mamdouh Ahmed, M. and Elsherbiny Ramadan, Factors influencing the anticancer effects of metformin on breast cancer outcomes: a systematic review and meta-analysis,

Expert Rev. Anticancer Ther. 22 (2022) 415–436. https://doi.org/10.1080/14737140.2022.2051482.
[14] S.R. Lord, A.L. Harris, Is it still worth pursuing the repurposing of metformin as a cancer therapeutic?, Br. J. Cancer 128 (2023) 958–966. https://doi.org/10.1038/s41416-023-02204-2.
[15] S. Andrzejewski, P.M. Siegel, J. St-Pierre, Metabolic Profiles Associated With Metformin Efficacy in Cancer, Front. Endocrinol. 9 (2018). https://www.frontiersin.org/journals/endocrinology/articles/10.3389/fendo.2018.00372.
[16] S.R. Lord, W.-C. Cheng, D. Liu, E. Gaude, S. Haider, T. Metcalf, N. Patel, E.J. Teoh, F. Gleeson, K. Bradley, S. Wigfield, C. Zois, D.R. McGowan, M.-L. Ah-See, A.M. Thompson, A. Sharma, L. Bidaut, M. Pollak, P.G. Roy, F. Karpe, T. James, R. English, R.F. Adams, L. Campo, L. Ayers, C. Snell, I. Roxanis, C. Frezza, J.D. Fenwick, F.M. Buffa, A.L. Harris, Integrated Pharmacodynamic Analysis Identifies Two Metabolic Adaption Pathways to Metformin in Breast Cancer, Cell Metab. 28 (2018) 679-688.e4. https://doi.org/10.1016/j.cmet.2018.08.021.
[17] B. Chaube, P. Malvi, S.V. Singh, N. Mohammad, B. Viollet, M.K. Bhat, AMPK maintains energy homeostasis and survival in cancer cells via regulating p38/PGC-1α-mediated mitochondrial biogenesis, Cell Death Discov. 1 (2015) 15063. https://doi.org/10.1038/cddiscovery.2015.63.
[18] B. Chaube, P. Malvi, S.V. Singh, N. Mohammad, A.S. Meena, M.K. Bhat, Targeting metabolic flexibility by simultaneously inhibiting respiratory complex I and lactate generation retards melanoma progression, Oncotarget 6 (2015) 37281–37299.
[19] S.S. Mayengbam, A. Singh, H. Yaduvanshi, F.K. Bhati, B. Deshmukh, D. Athavale, P.L. Ramteke, M.K. Bhat, Cholesterol reprograms glucose and lipid metabolism to promote proliferation in colon cancer cells, Cancer Metab. 11 (2023) 1–22. https://doi.org/10.1186/s40170-023-00315-1.
[20] L. He, F.E. Wondisford, Metformin Action: Concentrations Matter, Cell Metab. 21 (2015) 159–162. https://doi.org/10.1016/j.cmet.2015.01.003.
[21] B. Chaube, M.K. Bhat, AMPK, a key regulator of metabolic/energy homeostasis and mitochondrial biogenesis in cancer cells, Cell Death Dis. 7 (2016) e2044. https://doi.org/10.1038/cddis.2015.404.
[22] Y. Ma, L. Wang, R. Jia, The role of mitochondrial dynamics in human cancers, Am. J. Cancer Res. 10 (2020) 1278–1293.
[23] L.-C. Tábara, M. Segawa, J. Prudent, Molecular mechanisms of mitochondrial dynamics, Nat. Rev. Mol. Cell Biol. (2024). https://doi.org/10.1038/s41580-024-00785-1.
[24] J.-H. Koh, Y.-W. Kim, D.-Y. Seo, T.-S. Sohn, Mitochondrial TFAM as a Signaling Regulator between Cellular Organelles: A Perspective on Metabolic Diseases, Diabetes Metab. J. 45 (2021) 853–865. https://doi.org/10.4093/dmj.2021.0138.
[25] C. Scatena, M. Roncella, A. Di Paolo, P. Aretini, M. Menicagli, G. Fanelli, C. Marini, C.M. Mazzanti, M. Ghilli, F. Sotgia, M.P. Lisanti, A.G. Naccarato, Doxycycline, an Inhibitor of Mitochondrial Biogenesis, Effectively Reduces Cancer Stem Cells (CSCs) in Early Breast Cancer Patients: A Clinical Pilot Study, Front. Oncol. 8 (2018) 452. https://doi.org/10.3389/fonc.2018.00452.

[26] R.D. Alamri, M.A. Elmeligy, G.A. Albalawi, S.M. Alquayr, S.S. Alsubhi, S.H. El-Ghaiesh, Leflunomide an immunomodulator with antineoplastic and antiviral potentials but drug-induced liver injury: A comprehensive review, Int. Immunopharmacol. 93 (2021) 107398. https://doi.org/10.1016/j.intimp.2021.107398.
[27] A. Vancura, I. Vancurova, Metformin induces protein acetylation in cancer cells, Oncotarget 8 (2017) 39939–39940. https://doi.org/10.18632/oncotarget.17829.
[28] J. Wen, Z. Yi, Y. Chen, J. Huang, X. Mao, L. Zhang, Y. Zeng, Q. Cheng, W. Ye, Z. Liu, F. Liu, J. Liu, Efficacy of metformin therapy in patients with cancer: a meta-analysis of 22 randomised controlled trials, BMC Med. 20 (2022) 402. https://doi.org/10.1186/s12916-022-02599-4.
[29] F. Zhang, S. Han, W. Song, Anticancer effects of metformin in experimental animal models of different types of cancer: a systematic review and meta-analysis, Lab. Anim. Res. 38 (2022) 22. https://doi.org/10.1186/s42826-022-00131-6.
[30] N.S. Chandel, D. Avizonis, C.R. Reczek, S.E. Weinberg, S. Menz, R. Neuhaus, S. Christian, A. Haegebarth, C. Algire, M. Pollak, Are Metformin Doses Used in Murine Cancer Models Clinically Relevant?, Cell Metab. 23 (2016) 569–570. https://doi.org/10.1016/j.cmet.2016.03.010.
[31] A. Mogavero, M.V. Maiorana, S. Zanutto, L. Varinelli, F. Bozzi, A. Belfiore, C.C. Volpi, A. Gloghini, M.A. Pierotti, M. Gariboldi, Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production, Sci. Rep. 7 (2017) 15992. https://doi.org/10.1038/s41598-017-16149-z.
[32] X. Sui, Y. Xu, J. Yang, Y. Fang, H. Lou, W. Han, M. Zhang, W. Chen, K. Wang, D. Li, W. Jin, F. Lou, Y. Zheng, H. Hu, L. Gong, X. Zhou, Q. Pan, H. Pan, X. Wang, C. He, Use of Metformin Alone Is Not Associated with Survival Outcomes of Colorectal Cancer Cell but AMPK Activator AICAR Sensitizes Anticancer Effect of 5-Fluorouracil through AMPK Activation, PLOS ONE 9 (2014) e97781. https://doi.org/10.1371/journal.pone.0097781.
[33] D.Y. Gui, L.B. Sullivan, A. Luengo, A.M. Hosios, L.N. Bush, N. Gitego, S.M. Davidson, E. Freinkman, C.J. Thomas, M.G. Vander Heiden, Environment Dictates Dependence on Mitochondrial Complex I for NAD+ and Aspartate Production and Determines Cancer Cell Sensitivity to Metformin, Cell Metab. 24 (2016) 716–727. https://doi.org/10.1016/j.cmet.2016.09.006.
[34] D. Benjamin, D. Robay, S.K. Hindupur, J. Pohlmann, M. Colombi, M.Y. El-Shemerly, S.-M. Maira, C. Moroni, H.A. Lane, M.N. Hall, Dual Inhibition of the Lactate Transporters MCT1 and MCT4 Is Synthetic Lethal with Metformin due to NAD+ Depletion in Cancer Cells, Cell Rep. 25 (2018) 3047-3058.e4. https://doi.org/10.1016/j.celrep.2018.11.043.
[35] C. Loubiere, S. Clavel, J. Gilleron, R. Harisseh, J. Fauconnier, I. Ben-Sahra, L. Kaminski, K. Laurent, S. Herkenne, S. Lacas-Gervais, D. Ambrosetti, D. Alcor, S. Rocchi, M. Cormont, J.-F. Michiels, B. Mari, N.M. Mazure, L. Scorrano, A. Lacampagne, A. Gharib, J.-F. Tanti, F. Bost, The energy disruptor metformin targets mitochondrial integrity via modification of calcium flux in cancer cells, Sci. Rep. 7 (2017) 5040. https://doi.org/10.1038/s41598-017-05052-2.

[36] L. Zhang, X. Zhang, H. Liu, C. Yang, J. Yu, W. Zhao, J. Guo, B. Zhou, N. Jiang, MTFR2-dependent mitochondrial fission promotes HCC progression, J. Transl. Med. 22 (2024) 73. https://doi.org/10.1186/s12967-023-04845-6.
[37] S.B. Panina, J. Pei, N.V. Kirienko, Mitochondrial metabolism as a target for acute myeloid leukemia treatment, Cancer Metab. 9 (2021) 17. https://doi.org/10.1186/s40170-021-00253-w.
[38] F. Jalali, F. Fakhari, A. Sepehr, J. Zafari, B.O. Sarajar, P. Sarihi, E. Jafarzadeh, Synergistic anticancer effects of doxorubicin and metformin combination therapy: A systematic review, Transl. Oncol. 45 (2024) 101946. https://doi.org/10.1016/j.tranon.2024.101946.
[39] S.F. Teixeira, I. dos S. Guimarães, K.P. Madeira, R.D. Daltoé, I.V. Silva, L.B.A. Rangel, Metformin synergistically enhances antiproliferative effects of cisplatin and etoposide in NCI-H460 human lung cancer cells, J. Bras. Pneumol. Publicaçãо Of. Soc. Bras. Pneumol. E Tisilogia 39 (2013) 644–649. https://doi.org/10.1590/S1806-37132013000600002.
[40] Y. Su, C. Hou, M. Wang, K. Ren, D. Zhou, X. Liu, S. Zhao, X. Liu, Metformin induces mitochondrial fission and reduces energy metabolism by targeting respiratory chain complex I in hepatic stellate cells to reverse liver fibrosis, Int. J. Biochem. Cell Biol. 157 (2023) 106375. https://doi.org/10.1016/j.biocel.2023.106375.
[41] Y. Wang, H. An, T. Liu, C. Qin, H. Sesaki, S. Guo, S. Radovick, M. Hussain, A. Maheshwari, F.E. Wondisford, B. O'Rourke, L. He, Metformin Improves Mitochondrial Respiratory Activity through Activation of AMPK, Cell Rep. 29 (2019) 1511-1523.e5. https://doi.org/10.1016/j.celrep.2019.09.070.
[42] S.R. Veiga, X. Ge, C.A. Mercer, M.I. Hernández-Álvarez, H.E. Thomas, J. Hernandez-Losa, S. Ramón y Cajal, A. Zorzano, G. Thomas, S.C. Kozma, Phenformin-Induced Mitochondrial Dysfunction Sensitizes Hepatocellular Carcinoma for Dual Inhibition of mTOR, Clin. Cancer Res. 24 (2018) 3767–3780. https://doi.org/10.1158/1078-0432.CCR-18-0177.