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SU92-MSZGW-F23-034

FOS

Mitochondrial Division Inhibitor 1 Augments The Anti-Metastatic Activity Of Chemotherapeutic Drug In Colorectal Cancer

SUPERIOR UNIVERSITY

A Thesis Submitted In Parttial Fulfillment of the

Requirement for the Degree of

MASTER OF PHILOSOPHY
IN
ZOOLOGY

Submitted By

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Supervised By

Dr Tahir Mehmood

DEPARTMENT OF BIOLOGICAL SCIENCES

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I, Raheela Shehzadi a student of **" MS/M.phil Zoology "** at **"Faculty of Sciences"** the Superior University, Lahore, hereby declare that this thesis titled, "**Mitochondrial Division Inhibitor 1-Induced Mitochondrial Dynamics Augments the Anti-Metastatic Activity of Chemotherapeutic Drug in Colorectal Cancer**" is my own research work and has not been submitted, published, or printed elsewhere in Pakistan or abroad. Additionally, I will not use this thesis to obtain any degree other than the one stated above.

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This is to certify that the research work presented in this thesis, titled "**Mitochondrial Division Inhibitor 1-Induced Mitochondrial Dynamics Augments the Anti-Metastatic Activity of Chemotherapeutic Drug in Colorectal Cancer"** was conducted by **"Raheela Shehzadi"** under the supervision of **"Dr Tahir Mehmood."**

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Sciences, The Superior University, Lahore in partial fulfillment of the requirements for the degree of Master of Science/Master of Philosophy in the field of "**MS Zoology"** in Faculty of Sciences at The Superior University, Lahore.

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DEDICATION

To the *ALLAH Almighty* **WHO** Is the creator of this universe, the cherisher and sustainer of this world...!!

Lord of all things, Master of the "Day of Judgment" and have the power of all things.

TO

Holy Prophet HAZRAT MUHAMMAD (PBUH)

WHO Is the greatest social reformer, the city of knowledge, who has guided his "Umma" to seek knowledge from cradle of grave...!!!

TO

My **beloved parents**, whose prayers, sacrifices, and unwavering belief in me have been my foundation, your love reflects the mercy of Allah in my life. To my beloved son, **Muhammad Bazaid Sultan,** whose radiant smile and boundless curiosity remind me daily of the blessings bestowed upon me, you are my heart's joy and my reason to strive harder.

To my siblings, (**Aneela, Mazhar, Tanzeela)**, whose laughter, encouragement, and shared moments of faith have kept me grounded and hopeful, you are my companions in this world.

And to my husband, **Irfan Hussain**, my partner in faith and life, whose steadfast support, patience, and love have been a source of strength through every challenge, you are my blessing and my anchor.

This work, a humble step in seeking knowledge as ordained by Allah, is dedicated to you all with deepest gratitude and love, for you have walked this path with me in spirit and heart.

RAHEELA SHEHZADI

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May Allah reward you all abundantly for your contributions to this humble effort in seeking knowledge, as He elevates those who strive in His path.

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LIST OF ABBREVIATIONS

AMPK	AMP-Activated Protein Kinase
CRC	Colorectal Cancer
Drp-1	Dynamin-Related Protein 1
FIS 1	Mitochondrial Fission 1
HCT116	Human Colorectal Carcinoma Cell Line 116
Mdivi-1	Mitochondrial Division Inhibitor 1
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MMP	Mitochondrial Membrane Potential
MMQ	Mitochondrial Mass Quantification
OPA1	Optic Atrophy
pDrp-1	Phosphorylated Dynamin-Related Protein 1
pAMPK	Phosphorylated AMP-Activated Protein Kinase
ROS	Reactive Oxygen Species

ABSTRACT

Colorectal cancer is one of the most prevalent causes of cancer-related death, and the 5-year survival rate is reduced to less than 20 percent in case of metastasis. The overdivision of mitochondria under the stimulation of the protein dynamin-related protein 1 (Drp1) enhances the mobility, diffusion and resistance to drugs of colorectal cancerous cells. Gemcitabine, a commonly used chemotherapy drug, struggles to effectively treat metastatic colorectal cancer due to resistance. Mitochondrial division inhibitor 1 (Mdivi-1), which inhibits Drp1, could enhance gemcitabine's ability to curb metastasis by limiting mitochondrial division and related markers. Hence, the present study was designed to study the anti-metastatic activity of mitochondrial division inhibitor 1 along with gemcitabine against HCT116 cells. HCT116 colorectal cancer cells were treated with Mitochondrial division inhibitor 1 (5–50 μM) alone or in combination with gemcitabine. Mitochondrial mass quantification, reactive oxygen species (ROS) levels and mitochondrial membrane potential were quantified via flow cytometry. Expression of dynamin-related protein 1 (Drp1) and its phosphorylated form, along with AMP-activated protein kinase (AMPK) activity, were assessed through western blot analysis. Results showed Mitochondrial division inhibitor 1 (Mdivi-1), reduced cell viability and movement in a dose-dependent way, increased mitochondrial mass and reactive oxygen, and altered mitochondrial membrane potential. It also boosted mitofusin-2 (Mfn2) and activated AMPK. Combined with gemcitabine, Mdivi-1 enhanced anti-metastatic properties, with cells invasion considerably reduced as opposed with gemcitabine alone, without losing its toxicity towards cancer cells. Such findings can suggest that Mdivi-1 increases the sensitivity of colorectal cancer cells to gemcitabine through mitochondrial dysfunction. This combination could provide a promising solution to enhance the results with metastatic colorectal cancer, which is an essential gap in the treatment. Nevertheless, additional clinical studies are necessary to prove its usefulness and safety, taking into account such possible side effects as the inhibition of mitochondrial complex I.

KEYWORDS: Mdivi-1, gemcitabine, colorectal cancer, HCT116 cells, mitochondrial dynamics, DRP1, pDRP1, Mfn2, AMPK, pAMPK, mitochondrial fission and fusion, metastatsis -

CHAPTER NO 1

INTRODUCTION

Colorectal cancer (CRC) is a severe disease that begins at the colon or rectum, typically as precancerous lesions known as adenoma in the inner lining of the big intestine. I got to know that it is one of the most common cancers in the world and also one of the biggest killers. Among men, it ranks as the third after lung and prostate cancer and among women, it ranks just after breast cancer. International statistics indicate that it ranks second in terms of cancer deaths [1].

In the US alone, it was estimated in 2020 that approximately 147,950 cases of the CRC (104,610 of the cases were in the colon and 43,340 were in the rectum). Majority of diagnoses occur more in individuals older than 50 years, with approximately 17,930 cases (12 %) projected in individuals below the age of 50. The same year, an estimated 53,200 deaths were related to CRC, with 3,640 (7%) of them being among those who were below 50 years old [2]. Incidence reaches a peak in 65-74 years of age, and it is a little higher in women. Metastasis, or the location of cancer in other body organs, is the primary cause of mortality. Early detection of the disease will indeed enhance better survival [3].

In the last ten years, CRC has struck younger people more frequently as the rates increase by 1-2 per cent annually in those who are under 50. This change is commonly attributed to lifestyle changes that are modifiable such as excess body weight, sedentary lifestyle, high consumption of processed foods and red meat, smoking and general population changes such as the aging population. Common symptoms of CRC are persistent stomach pain, bowel alterations, bloody diarrhea, unexplainable loss of weight, nausea, vomiting, persistent fatigue, loss of appetite, and abdominal swelling [4].

CRC rates are highest in high-income neighborhoods including Australia, New Zealand, Europe, and North America but are smaller in such localities as Africa and South-Central Asia. These variations are believed to be caused by differences in eating, everyday lifestyle, environmental conditions and genetic predisposition to cancer [5].

The tumor spread, which is also referred to as the invasion-metastasis sequence, is a process that entails cancer cells detaching themselves out of the initial tumor and

developing secondary tumors in other locations. It begins with invasion of local tissues in which malignant cells invade the surrounding extracellular matrix and then invades the blood or lymph vessel (invasion). After this, the cells circulate systemically and leave the vessels (extravasation) to the tissue of remote organs. This process culminates in the colonization stage where such cells develop visible tumors and this stage completes the metastatic process [6,7].

In addition to the direct genetic alterations, non-coding RNAs, microRNAs (miRNAs) are of great importance in spreading CRC. These molecules regulate the activity of genes and influence components of the tumors environment, such as immune system, blood vessel development and connective tissue cells. An example is that some miRNAs can also interact with cells in the vascular lining, tumor-supporting fibroblasts and precursor stem cells, all of which cause disease progression [8].

As a preventative measure against CRC, regular screening tests such as colonoscopy are advisable after every ten years in people at average risk, as well as healthy lifestyles such as consumption of foods with a lot of fiber, sufficient consumption of processed meat, tobacco avoidance, and healthy body weights. In more risky populations, such preventive medications as aspirin can be used in consultation with the specialist. The management is based on the extent of the spread of the disease and the specifics of the tumor: at the early stages, it is usually possible to operate on it with the help of such interventions as the removal of the polyp or partial bowel resection. In more developed or extensive cases, follow-up chemotherapy is common with more rectal tumors getting radiation as well as drugs [9,10].

Treatment of CRC is based on chemotherapy which is effective, yet obstacles like treatment tolerance and resistance that arise are impeding. The most important drugs are 5-fluorouracil (5-FU) and oxaliplatin, but both are resistant to the development of drug resistance, which happens in the form of gene mutations, silencing by chemical changes in the epigenetic environment, and alterations in the supportive environment of the tumor [11,12]. Treatment evasion revolves around chemical modifications made to DNA such as methylation. This has the capability to silence tumor protective genes, promoting the development of disease and drug resistance. Such compounds as 5-aza-2 -deoxycytidine are under investigation to activate these genes and reestablish treatment responsiveness in CRC.

Moreover, some of the tumor cells which possess stem-like properties contribute to resistance; they are able to self-replicate indefinitely and are resistant to conventional therapy. To overcome this, it needs accuracy treatments, immunobased therapy, and superior delivery techniques of small particles to direct the drugs and minimize side effects. These inventions will enhance the accuracy of the drugs and reduce the damage to the healthy tissue, but tumors tend to develop new mechanisms of resistance, reducing the long-term effects. New evidence indicates that chemotherapy may be enhanced with the use of inhibitors such as Mdivi-1 to make tumor cells more vulnerable [13-15].

1.1 MITOCHONDRIAL DYNAMICS

Mitochondria, often referred to as the powerhouses of the cell, are important in the generation of ATP and also in serving as the communication hubs that keep the cells stable and also in regulating programmed cell death. They produce reactive molecules such as oxygen radicals and have different shapes such as compact spheres or long filaments. The organelle health, shape and positioning of mitochondria rely on constant fusion (joining), fission (splitting), relocation and selective removal (mitophagy) processes. Mitochondria are fused and DNA, proteins and nutrients are shared to repair defects and maintain energy gradients. The outer-membrane mitofusins 1 and 2 (Mfn1 and Mfn2) and inner-membrane optic atrophy 1 (OPA1) are considered core players. On the other hand, fission splits them to facilitate quality control, to separate problematic components, and to evenly redistribute them during cell splits, primarily through the coordination of dynamin related protein 1 (Drp1), which is directed to the outer membrane by anchors such as mitochondrial fission factor (Mff) and fission protein 1 (Fig1).

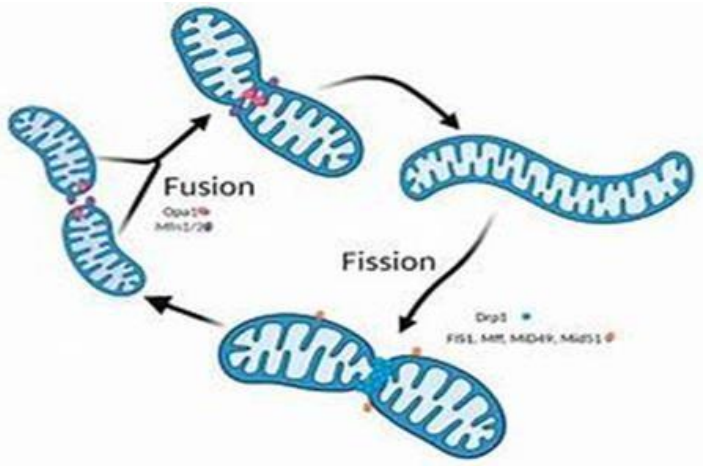


Figure 1.1: Diagram illustrating the mitochondrial dynamics process, showing the cycle of fission (mediated by Drp1, Fis1, Mff, MiD49, MiD51) and fusion (mediated by Opa1, Mfn1/2) in cells (1)

Mitophagy is the process of the autophagic disposal of impaired or dysfunctional mitochondria by the autophagic machinery. Such a system is needed to maintain the integrity of mitochondria and shut down defective mitochondria that may result in the imposition of cellular stress leading to a range of pathologies. Another cause of mitochondrial defects, which may result in the development of a variety of disorders, are disruptions in mitochondrial dynamics, such as asymmetrical fusion and fission, dysfunctional intracytoplasmic transport, and impaired mitophagy [16,17].

Mitochondrial fragmentation in a variety of cancers has been associated with the elevated expression of Drp1. Nefactorial Tumorigenesis is a multidimensional event that is triggered by genetic mutations that convert the normal cells into malignant ones, which have hallmarks of cancer, including uncontrolled proliferation and apoptotic resistance. These changes include oncogenic mutations, genomic rearrangements, and post-translational modifications disabling signaling cascades, metabolic pathways, and the work of ion channels, therefore, enhancing tumor development. Malignant cells are reprogrammed to metabolism which is characterized by high glucose uptake and glycolysis even in the presence of oxygen, a process initially reported by Otto Warburg and presumably caused by damaged mitochondrial respiratory capacity.

DRP1 inhibition can suppress tumour growth, which highlights the importance of the mitochondrial dynamics as an effective therapeutic approach. The new data puts a significant importance on mitochondria in stabilising tumour microenvironment

reconfiguring metabolic processes to address increased energy needs. It has been identified to cause cell death in various malignancies such as lymphoblastic leukaemia, glioblastoma, nutrition-resistant renal cell carcinoma, pancreatic cancer, breast cancer, and colon cancer, which is the result of suppressed mitochondrial respiration and oxidative phosphorylation (OXPHOS). The high levels of DRP1 in cancerous cells, therefore, are a therapeutic target [10,18-20].

1.2 MITOCHONDRIAL DIVISION INHIBITOR 1(MDIVI-1)

Mdivi-1 is a quinazolinone derivative that is a small molecule and inhibits dynamin-related protein 1 (DRP1), thus hindering mitochondrial fission in hepatic cells. This activity makes Mdivi-1 one of the possible anticancer agents in humans. Mdivi-1 prevents mitochondrial fragmentation, especially in staurosporine-induced apoptosis, to promote the generation of long mitochondrial networks and preventing programmed cell death, which indicates its potential therapeutic value. It is important to note that although Mdivi-1 is protective against excitotoxic injury of the nervous system, it enhances cisplatin induced cytotoxicity in cholangiocarcinoma cells and suppresses the proliferation of a variety of tumour types by regulating oxidative metabolism.

Inhibition of DRP1 by pharmacological methods, or genetic silencing of it, has been shown to induce apoptosis and tumour suppression of brain tumour-initiating cells. In the same way, silencing of DRP1 or increasing mitofusin 1 (MFN1) that facilitates mitochondrial fusion has been linked to a decrease in the metastasis of breast cancer. MFN1, MFN2 and optic atrophy-1 (OPA1) are proteins of fusion that promote angiogenesis, oxygen use, and ATP production in hepatocellular carcinoma, which promotes tumour progression. On the other hand, OPA1 silencing in ovarian cancer cells leads to the impairment of mitochondrial integrity, depletion of membrane potential, cytochrome c release, and apoptosis. Notably, it does not seem that Mdivi-1 disrupts proliferation or survival of non-cancerous human cells (e.g., fibroblasts and epithelial cells), demonstrating some selective antitumour activity.

The compound, Mdivi-1, which specifically interacts with DRP1, is known to have the ability to reorganize the mitochondrial architecture and energy metabolism. Research has shown that it can inhibit the proliferation of both thyroid and lung carcinoma cells, induce spontaneous cell death in pancreatic cancer stem cells and increase the amount of reactive oxygen species (ROS) in breast carcinoma cells. Outside the field of

oncology, Mdivi-1 has been used to investigate mitochondrial pathologies in diabetic cardiomyopathy, myocardial infarction, cardiac hypertrophy, Alzheimer disease and Huntington disease. Mdivi-1 when used with cisplatin amplifies DNA damage, increases the levels of Noxa proteins, and disrupts mitochondrial activity, leading to a Bax - and Bak -independent loss of mitochondrial outer membrane (MOMP) and a strong induction of the intrinsic apoptotic pathway. These results make Mdivi-1 and its thioquinazolinone analogs the candidates as a complementary addition to the platinum-based cancer therapy [18,21-23].

In spite of these promising findings, the exact effect of Mdivi-1 on colorectal cancer has not been well characterised. The current paper will shed light on the role of Mdivi-1 in modulating the mitochondrial activity of colorectal cancer cells in proliferation, motility and invasiveness using the HCT116 cell line as a model. Protective effects have been proven in preclinical research such as models of heart failure and cardiac arrest with some limitations. In mouse experiments, the excess mitophagy, tissue scarring, and cell death were alleviated, and the cardiac work was promoted (measured through echocardiography) through DRP1 inactivation in response to Mdivi-1 (e.g., 50mg/kg). It also facilitated the growth of angiogenesis by up-regulating pro-angiogenic (VEGF) and down-regulating anti-angiogenic factors [24]. Mdivi-1 at 1 μM concentration prevented chemotherapy-induced cardiotoxicity by enhancing cardiac output, heart rate, and blood flow, infarct size, and mitochondrial integrity without impairing antineoplastic effect of doxorubicin [25]. Mdivi1 (1 mg/kg 14 injections) decreased the fibrosis of a post-myocardial infarction by inhibiting DRP1 in fibroblasts, boosting antioxidant reaction (i.e., Hmox1), and improving cardiac performance in murine models, thus slowing down the progression of heart failure [26]. Mdivi-1 (0.24mg kg during resuscitation) in a cardiac arrest model induced by eight minutes of KCl infusion then resuscitation was found to increase 72-hour survival (65 versus 33 percent in controls), decrease time to spontaneous recovery and decrease post -resuscitation recovery [27].

The cardioprotective effect of Mdivi-1 in models of ischaemia-reperfusion (I/R) (decreased apoptosis and maintained mitochondrial morphology) is encouraging in terms of cardiac arrest, in which I/R injury is severe; but there are limited direct studies on it [28]. Preclinical studies have demonstrated that Mdivi-1 has the ability to protect against heart failure by reducing fibrosis, improving function, and reducing

cardiotoxicity, and cardiac arrest by improving survival and myocardial repair. However, its clinical utility has not been authenticated, and some of its complications such as inhibition of complex I and the generation of ROS highlights the importance of conducting more studies especially, human experiments to determine its effectiveness and safety.

1.3 GEMCITABINE

The nucleoside form of the drug is called Gemcitabine (2',2'-difluorodeoxycytidine; dFdC), a man-made analogue of deoxycytidine, which is characterized by the addition of two atoms of fluorine into its deoxyribose ring. The use of any chemotherapeutic agent has a long history of over fifteen years of use as reported in the literature [29]. Unlike other nucleoside analogs like cytosine arabinoside (AraC), gemcitabine has specific physicochemical characteristics that make it have a characteristic activity profile [30]. It acts as a prodrug and requires intracellular activation to be able to execute its therapeutic action; it is selectively and actively transported into cells and then phosphorylated by the cellular kinases to produce its active metabolites. The triphosphate analog of gemcitabine is competitive with the polymerases of the DNA in that it resembles dCTP, thus interfering with the replication of the DNA molecules. Gemcitabine is also endowed with a unique cellular pharmacology, metabolic processing and a mechanism of action. It is regularly used together with other chemotherapeutic agents to increase the effectiveness of the treatment in general [31].

The antineoplastic action of gemcitabine is associated with the predominant effect on DNA synthesis in S phase of cell cycle, which interrupts the G1-S transition [32]. Being a pyrimidine nucleoside analog it acts as an antimetabolite and has developed to be a foundation of modern chemotherapy. In the clinic, it has been used as a treatment of non-small cell lung cancer, pancreatic cancer, bladder cancer, breast cancer and more recently in ovarian cancer. Gemcitabine is the first-line monotherapy of choice in the advanced pancreatic carcinoma with a better overall survival, quality of life, and pain reduction in comparison with 5-fluorouracil (5-FU) [33].

This has resulted in gemcitabine being the treatment regimen of choice in patients with locally advanced or metastatic pancreatic cancer. The use of gemcitabine is not recommended in the treatment of heart failure and cardiac arrest; however, it has been linked to infrequent cardiotoxic effects, which can trigger the diseases. Gemcitabine

can cause congestive heart failure and cardiomyopathy with a low left ventricular ejection fraction (LVEF) (case reports (i.e., LVEFs of 15% to 40%), pharmacovigilance data (484 cases of heart failure reported in VigiBase)) in heart failure. There is the rare probability of direct causation of cardiac arrest; but gemcitabine can indirectly result in cardiac arrest by causing acute coronary syndrome or arrhythmia, as atrial fibrillation, especially in individuals with underlying cardiovascular risk factors. There are low cardiovascular adverse drug reactions (CV- ADRs) (~1-percent), but 82-percent of CV-ADRs are severe, with up to 17-percent resulting in cardiovascular mortality. The mechanisms will probably be endothelial damage, oxidative stress, or inflammation, but these pathways are not fully understood. Management involves the immediate withdrawal of gemcitabine and standard heart-failure management; recovery good recovery is often seen though there is an increased risk of recurrence of 57% in the case of rechallage. The existing state of knowledge is restricted to a case study and drug pharmacovigilance trials and so there is an urgent requirement to conduct additional research to explore the mechanistic nature and best management of gemcitabine-related cardiotoxicity. Close attention is also essential especially in patients who have cardiovascular risk factors or those who take combination regimens [36,37].

Colorectal carcinoma (CRC) has become a significant health issue in the world because of its high propensity to metastasis and resistance to orthodox chemotherapeutic agents, which therefore restricts therapeutic effectiveness. A key cause of these predicaments is the wayward conduct of mitochondria - organelle of a cell that produce energy. Mitochondrial excessive fission that affects mitochondrial dynamics and leads to tumor cell survival, metastasis and drug resistance that leads to increased proliferation, migration and apoptotic escape in CRC.

Mdivi-1, which is a selective mitochondrial fission inhibitor, provides an encouraging approach to therapeutic intervention through alleviating excessive fission and, as a result, restoring homeostasis in mitochondria and potentially sensitizing cancer cells to chemotherapy. In this regard, Mdivi-1 combined with gemcitabine which is a commonly used chemotherapeutic agent in CRC can contribute to the prevention of metastasis and resistance to drugs.

AIMS AND OBJECTIVES:

The aim of this study was to explore how Mdivi.1-induced mitochondrial dynamics influenced the anti-metastatic activity of chemotherapeutic drugs in colorectal cancer, focusing on mitochondrial fission, apoptosis and metastatic potential.

This study has following objectives;

1. To investigate Mdivi.1-induced cytotoxicity in HCT116 cells,
2. To investigate gemcitabine-induced cytotoxicity in HCT116 cells.
3. To investigate gemcitabine-induced anti-proliferative activity in the presence of Mdivi.1 in HCT116 cells.
4. To investigate gemcitabine-induced MMP in the presence of Mdivi.1 in HCT116 cells.
5. To investigate gemcitabine-induced ROS activity in the presence of Mdivi.1 in HCT116 cells.

This study investigated how Mdivi.1-induced modulation dynamics of mitochondria could augment the anti-metastatic effects of gemcitabine in colorectal cancer, aiming to improve treatment outcomes for this aggressive disease.

CHAPTER NO 2

LITERATURE REVIEW

Mdivi- 1, a specific antagonist of Dynamin -Related Protein 1 (DRP1), regulates mitochondrial fission and has a potential of improving the efficacy of chemotherapy in colorectal cancer (CRC). This review outlines the role of aberrant DRP1-mediated fission and evidence of its involvement in the proliferation and chemoresistance in CRC, and assesses the potential of Mdivi-1 to act synergistically with gemcitabine to enhance the antimetastatic treatment effects.

Ahn and coworkers (2024) used the RNA sequencing (RNA -seq) and methyl-capture sequencing (MC-seq) methods to investigate the far-reaching effects of Mdivi-1, an inhibitor of mitochondrial division, on SH-SY5Y human neuroblastoma cells. Mdivi-1 is mainly known to induce mitochondrial fusion, but in this study its different effects on physiology of cells were established. The p53 gene network was upregulated, and genes related to the establishment of the DNA replication cycle were downregulated, which was accompanied by a G1 -phase arrest demonstrated by RNA -seq data. MC-seq revealed weak changes in the DNA methylation, which were mostly located in CpG islands. In-depth analysis indicated the activation of the p53 pathway and the inhibition of a pathway related to Parkinson disease. In addition, Mdivi-1 attenuated copy number and transcription of mitochondrial DNA, raised reactive oxygen species (ROS) levels and induced mitochondrial-nucleus signaling, but caused no direct DNA damage, furthering the perception of the molecular action of Mdivi-1 and the interaction of nuclear and mitochondrial compartments [15].

Dai, Wang, et al. (2020) hypothesized that Mdivi-1 may suppress the cell growth of cancer by blocking the oxidative metabolism, regardless of its impact on the mitochondrial fusion, which is a novel understanding of its effect on the metabolism in addition to its well-known as a DRP1 blocking activity [19].

Silva-Pavez et al. (2024) reported that mitochondrial fission, which is controlled by DRP1, contributes to maintaining the oncogenic cellular homeostasis and that Mdivi-1 inhibition increases the sensitivity of cells to the chemotherapeutics and reduces cell proliferation. Mdivi-1 causes cell detachment in breast cancer cells and leads to the survival of floating cells with diminished proliferative and clonogenic abilities, but

retains migratory and invasive potentials on reattachment. Notably, this disengagement is not mediated by DRP1 but rather is instigated by the repression of the mitochondrial complex I which is contingent on glucose metabolism and pentose phosphate pathway. These results suggest a subtle, DRP1-independent effect of Mdivi-1 that could pose a challenge to clinical practice in that cells that are viable and may be invasive emerge into circulation [20].

Zhang, L, and Sun, L et al. (2023) examined that Mdivi-1 imposes a strong therapeutic potential in thyroid cancerous cells by inhibiting cell proliferation, inducing apoptosis, decreasing cell invasiveness, and reversing epithelial-mesenchymal transition by using the NF- 0 pathway, which is independent of DRP1. This finding highlights its potential as a specific therapy agent of thyroid cancer [21].

Qian, W, Wang, J, et al. (2014) emphasized the challenge of overcoming resistance to platinum-based anticancer agents like cisplatin which often result into failure of the treatment. They found that thioquinazolinone derivatives especially Mdivi-1 have potential to reverse intrinsic and acquired cisplatin resistance in tumor cell lines and in primary ovarian tumors of cisplatin-nonresponsive patients. The synergistic effect of cisplatin and Mdivi-1 causes rapid apoptosis. It is interesting to note that this combination bypasses cisplatin resistance in a DRP1-independent mechanism because DRP1 is the mitochondrial fission protein that is usually targeted by Mdivi-1. Mdivi-1 causes replication defects, and in combination with cisplatin it significantly increases replication stress, increasing the efficacy of therapy of resistant cancer cells [22].

Mehmood, T, Nasir, Q, and others (2025) have shown that Mdivi-1 suppresses the expression of DRP1, enhances the level of mitofusin-2 (Mfn2) and mitochondrial volume without intensifying oxidative phosphorylation. These changes lead to less invasion and metastatic spread in HCT116 colorectal cancer cells. According to their results, the regulation of the mitochondrial dynamics might be one of the potential approaches to inhibiting migration and metastasis of the colorectal cancer cells [23].

The conclusion of Tatlipinar et al. (2024) is that the potent chemotherapeutic agent gemcitabine that is approved for the treatment of small-cell lung, pancreatic, bladder, and breast cancer has a wider antitumor activity than cytarabine despite their similar structure and mechanism. In one example involving the HCT116 cell line of the CRC, gemcitabine used alone or combined with the autophagy-inhibitor, chloroquine, caused

cell-cycle arrest at the G1 and G2 levels and strongly induced apoptosis, which increased cell death compared to gemcitabine monotherapy. On the other hand, the gemcitabine, when combined with rapamycin, an autophagy activator, did not cause any significant cell-cycle changes. These results suggest that gemcitabine is likely to complement chloroquine in enhancing the treatment effects of colorectal cancer [35].

Wu, Z, Xiao, C, et al. (2023) highlighted that mitochondrial fusion-fission processes play a critical role in the occurrence and development of colorectal cancer. Increased fission or fusion activity may support metabolic changes in tumor cells and, as such, induce oncogenic signaling pathways to promote cell growth, invasion, migration, and therapeutic resistance. Extreme fission can cause apoptosis but balanced fusion can protect against oxidative damage. Mitochondrial dynamic disturbance may, therefore, be promoting and inhibiting factors in colorectal cancer progression [38].

Aishwarya, R, Alam, S, and co-authors (2020) studied the different effects of Mdivi-1, a small-molecule inhibitor of DRP1 -mediated mitochondrial division, on mitochondrial trafficking, energy production, electron transport, and large-scale autophagy in cardiomyocytes. Their results indicated that Mdivi-1 lowers the level of DRP1, causes the enzymatic cleavage of long form OPA1(L-OPA1) and changes the abundance of oxidative phosphorylation (OXPHOS) complex proteins, which are the factors inducing superoxide accumulation in mitochondria. These modifications were linked to the decline in mitochondria energy activity. Moreover, Mdivi-1 inhibited massive autophagy, which was indicated by a sequestered autophagic flux, which could be reinstated by increasing the expression of Atg7 expression; high p62 abundance was essential in this reinstatement. The paper suggests the role of Mdivi-1 in the impact on mitochondrial and cellular homeostasis of cardiomyocytes by the way of its influence on the processing of DRP1 and L-OPA1 [39].

Han, XJ, Yang, ZJ, and others (2014) found that hypoxic conditions increase DRP1 expression, mitochondrial division and motility in metastatic MDA-MB-231 breast cancer cells but not in non-metastatic MCF -7 cells. DRP1 inhibition using Mdivi-1 or suppression of its expression alleviates these responses, such as cisplatin-induced cell death, mitochondrial damage, and ROS generation in MDA -MB 231 cells. Inhibition of DRP1-mediated mitochondrial fission could thus help in preventing breast cancer metastasis and enhancing the success of chemotherapy [40].

Song et al. (2024) came to a conclusion that the active component of Cnidium monnieri is osthole, which was provided the opportunity to prevent the development of colorectal cancer by regulating mitochondrial metabolism and autophagic flux. Osthole demonstrated effectiveness in reducing the growth of colorectal tumours, improving intestinal damage, and functional recovery of goblet cells and down-regulating Claudin1 and Axin1 expression using an azoxymethane/dextran sodium sulfate (AOM/DSS) mouse model in combination with in vitro cell culture. It was found that the compound selectively killed colorectal carcinoma cells, blocked the ASC/caspase -1/IL-1b inflammasome pathway, impaired mitochondrial function and inhibited ATP production by blocking oxidative phosphorylation and glycolysis. Osthole also inhibited autophagic flux, thus facilitating apoptosis in HCT116 and HT29 cells, and the TiRNA HisGTG regulation was found to be one of the factors that limited its antineoplastic effect. Such results highlight the potential of osthole as a management approach of colorectal cancer via autophagy- and mitochondria-mediated processes [41].

According to Caldeira et al. (2021), mitochondria play a major role in maintaining the functionality of pulmonary epithelial cells, which is important in executing such processes as the production of surfactants, mucociliary clearance, secretion of mucus, cellular senescence, innate immunity, and tissue repair. The pathogenesis of chronic lung diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and asthma, is closely related to perturbations in mitochondrial activity, both in morphology and dynamics, and in mitochondrial redox signalling, mitophagy and endoplasmic reticulum interactions. The review notes that mitochondrial dysfunctions can be targeted using mitochondria-based therapeutics, and such a direction can provide new strategies in the treatment of pulmonary disorders [42].

The study by Natu and Nagaraju (2023) was carried out in the context of pancreatic ductal adenocarcinoma (PDAC), which is a malignancy with low survival odds and high resistance to chemotherapy. The first-line therapeutic agent in the treatment of PDAC is gemcitabine, which has low treatment efficacy because of resistance through tumour microenvironment (TME) interactions with cellular intracellular mechanisms. Preclinical trials suggest that the TME can be adjusted to TME schedules of gemcitabine to address the resistance. Low-dose and frequent metronomic therapy is promising and has the potential to overcome some of the challenges of conventional

PDAC treatment schedules. The present review focuses on the mechanism of action of gemcitabine, combination therapy processes, TME interactions, as well as the use of gemcitabine in metronomic therapy of PDAC [43].

According to Akita et al. (2014), Tumour necrosis factor related apoptosis inducing ligand (TRAIL) although it has potential in therapy, has resistance in some tumour cells that reduce its clinical effects. Since the dynamics of mitochondria, which are dictated by the proportions of fission and fusion, determine cell survival, the article investigated their effect on the TRAIL-induced apoptotic process. The findings proved that mitochondrial division inhibitor-1 (Mdivi-1), which is a Drp1 inhibitor, modulates mitochondrial morphology, increases TRAIL-induced apoptosis of malign cells (melanoma, lung carcinoma, and osteosarcoma) but spares normal cells. Mdivi-1 caused dose- and time-dependent cytotoxicity and enhanced TRAIL sensitivity by caspase-dependent, mitochondrial and endoplasmic reticulum stress pathways at 12.5 0 -mM concentrations. In addition, Mdivi-1 augmented mitochondrial oxidative stress and favoured hyperfusion, and genetic silencing of Drp1 elicited similar sensitisation consequences. Taken together, these results imply that the blockage of mitochondrial morphology processes can be used to selectively eliminate tumour cells and enhance the use of TRAIL therapy [44].

Parker et al. (2016) targeted the role of mitochondrial dynamics in the tumour radioresponsiveness. The purpose of the study was to describe the state of mitochondria in cancer cells, regulate the dynamics of the mitochondrion, and evaluate the changes in cell viability under the influence of irradiation. Through the staining of MitoTracker Red and microscopic studies, researchers assessed mitochondrial dynamics, respiratory capacity, and membrane potential in different cancer cell lines. Mitochondrial dynamics regulation of mitochondrial fusion by fission inhibitor Mdivi-1 engendered cytotoxicity and further augmented radiosensitivity substantially. These changes were linked to a cell-cycle arrest, increased apoptosis and amplified cytogenetic damage, but DNA repair mechanisms were not affected. The authors came to the conclusion that mitochondrial dynamics can be used as a targeted approach to cancer treatment and can increase the efficacy of radiotherapeutic approach. Though a new system of scoring mitochondrial fragmentation was invented, no firm relationship between mitochondrial status and radiosensitivity was observed [45].

According to Lorusso et al. (2006), gemcitabine which is a new agent that has shown promising activity and favourable toxicity profile has proven to be effective as a single agent in the treatment of platinum-resistant ovarian cancer. Gemcitabine and carboplatin are synergistic, thus making the combination interesting to consider in the treatment of recurrent ovarian cancer that is platinum-sensitive. Also, its use in combination with agents like paclitaxel is actively under investigation to help further clarify the use of gemcitabine in treating previously treated and untreated ovarian cancer patients [46].

Masuo et al. (2023) examined gemcitabine-resistant pancreatic cancer cell lines (MIA PaCa -2 and Panc-1) and found that they had increased the number of mitochondria, which facilitated oxidative phosphorylation (OXPHOS) and up-regulation of anti-apoptotic proteins BCL2 and BCL-xL. This was accompanied with reduced mitochondrial fission 1 protein, resulting in decreased mitophagy and reduced AMP/ATP ratio which inhibited AMPK activity and triggered HSF1-heat shock protein pathway, therefore, increasing stress tolerance. The mitochondrial dynamics and protein expression changes to gemcitabine resistance in pancreatic ductal adenocarcinoma, a disease with early resistance to chemotherapeutic drugs and adverse prognosis. There was synergy between combination therapy using gemcitabine and BCL2/HSF1 inhibitors, implying the attainment of a plausible therapeutic approach to overcome acquired resistance [47].

Woo et al. (2020) emphasized that mitochondrial dynamics is involved in apoptosis induced by cisplatin, and specifically, they talked about the fission protein Drp1. Mdivi-1 (mitochondrial fission inhibitor) at low concentrations (<10 mM) also did not affect apoptosis by itself but in combination with cis platin, was able to stimulate apoptosis in cancer cells, specifically in the renal carcinoma cell line Caki -1 but not in normal cells. The combination triggered caspase 3 mediated apoptosis and down-regulated c-FLIP expression, the overexpression of which counteracted apoptotic effect. These results support the fact that Mdivi± can be used as a cisplatin sensitiser in oncologic treatment [48].

Han et al. (2019) also found out that in cancer cells in the hypoxic (less than 1% of O 2) environment, hypoxia induces mitochondrial fission and cisplatin (CDDP) resistance by generating reactive oxygen species (ROS) that decrease inhibitory phosphorylation

of Drp1 (Ser637) and Mfn1 levels, promoting fission. Antioxidants of N-acetylcysteine and Trolox also weaken this reaction, and hypoxia is replicated by the use of hydrogen peroxide. Mitochondrial fission inhibition by Mdivi-1 or si-Drp1 enhances the sensitivity of the CDDP in hypoxic ovarian cancer cells and tumour sphere in patients with advanced stage. These findings imply that outcome in the attack of hypoxia-induced mitochondrial homeostasis, especially Drp1 functions, might enhance the effectiveness of anticancer treatments in ovarian cancer [49].

Kinoshita et al. (2024) examined the role of the mitochondrial dynamics in acute myeloid leukemia (AML) and especially the fission-related genes DNM1L and MFF. Their result showed that high level of MFF expression is associated with poor clinical outcomes. The authors measured the effects of shRNA-mediated DNM1L and MFF knockdown in AML cell lines with the following results: the cellular proliferation was reduced, mitochondrial networks were enlarged, and mitochondrial respiration was impaired, but the glycolytic activity did not significantly change in most cases except when the cells were deprived of DNM1L. In mouse models of immunodeficiency (NOG) transplantation model of AML cells with reduced DNM1L or MFF showed statistically significant prolongation of survival when compared to controls. Moreover, DNM1L-encoded protein DRP1 is pharmacologically inhibited using Mdivi-1 which reduced cell proliferation and oxidative phosphorylation in the AML cells. These results indicate the vital importance of mitochondrial fission in AML development and the importance of mitochondrial dynamics as a possible therapeutic intervention [50].

Emerging data suggest that Mdivi-1 has cardioprotective properties due to its inhibitory effect on Drp1-induced mitochondrial fission. As an example, Ding et al. (2022) demonstrated that Mdivi-1 suppresses cardiac fibrosis after infarction of the heart, particularly in the infarct border zone, by inhibiting the effects of oxidative stress [51].

On the same note, Maneechote et al. (2021) found that Mdivi-1 reduces myocardial ischemia/reperfusion injury through maintenance of mitochondrial architecture and inhibition of apoptosis [52].

To concur with these findings, Sharp et al. (2015) have found out that Mdivi-1 treats a murine heart arrest model and improves survival, which further supports its ability as a therapeutic intervention in cardiac injury [53].

Alam et al. (2018) recommended that gemcitabine may cause cardiotoxicity which may result in heart failure in cancer patients. Three patients who were on gemcitabine monotherapy to treat pancreatic cancer developed congestive cardiac failure in a case series [54].

Nabeel & Ali., 2023 concluded that Although gemcitabine is generally considered relatively safe, with only a few reported cases of cardiac adverse effects, several cases indicate a link between gemcitabine and the development of heart failure and cardiomyopathy. A case involved a 33-year-old female who developed dilated cardiomyopathy while receiving gemcitabine for ovarian serous adenocarcinoma [55].

Lisi et al., 2020 suggested that a 55-year-old female with sarcoma developed fatal heart failure after treatment with pazopanib, after previous treatment with gemcitabine [56].

Mohebali et al., 2016 investigated that The risk of cardiotoxicity may be increased when gemcitabine is used in combination with other cardiotoxic agents or in patients with pre-existing cardiac conditions [57].

Although previous studies have identified the involvement of mitochondrial dynamics in the development of colorectal cancer (CRC) and the ability of Mdivi-1 to increase the efficacy of chemotherapy through DRP1 inhibition of fission, much is still unclear. Scant research has focused on the synergistic interaction of Mdivi-1 and gemcitabine in CRC, especially with reference to metastatic capability and drug resistance using the HCT116 cell model. Additionally, the time-dependent dynamics of their interaction, such as the transition from synergistic to additive effects, and the mechanisms involved, like antioxidant responses or off-target effects of Mdivi-1 (e.g., mitochondrial complex I inhibition), are not well understood. Furthermore, there are insufficient systematic in vivo studies validating these conclusions in physiological CRC models, restricting insights into clinical translatability and systemic effects.

CHAPTER NO 3

METHODOLOGY

3.1 MATERIAL REQUIRED:

"Cell line and culture protocols"

The human CRC line HCT116 was maintained in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 , supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin.

3.1.1 DRUGS:

- Mdivi-1 (Santa Cruz Biotechnology, Waltham, MA, USA)
- Gemcitabine (Sigma-Aldrich, St. Louis, MO, USA, or equivalent commercial source)

3.1.2 MITOCHONDRIAL STAINS:

- MitoTracker Deep Red (Invitrogen, Carlsbad, CA, USA)
- TMRM (tetramethylrhodamine methyl ester, Abcam, USA)
- MTT assay (MTT; Bio Basic Inc., Markham, Canada)

3.1.3 REAGENTS:

Antibodies: The following primary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA): anti-Drp1, anti-phospho-Drp1 (Ser616), anti-Mfn2, anti-AMPK-α, anti-phospho-AMPK-α, anti-Cox-2, anti-iNOS, anti-MMP9, and anti-β-actin.

Other reagents: Additional reagents included 2',7'-dichlorofluorescin diacetate (DCFDA; Sigma-Aldrich) for reactive oxygen species (ROS) detection, Matrigel (Corning), phosphate-buffered saline (PBS), trypsin-EDTA, lysis buffer, protease/phosphatase inhibitor cocktail, and a Bradford protein assay kit.

Equipment: Biosafety cabinet, CO_2 incubator, fluorescence microscope, flow cytometer, microplate reader, Western blot apparatus, transwell chambers, centrifuge, pipettes, cell culture plates (6-well, 96-well), and sterile consumables

3.2 CELL CULTURE

HCT116 colorectal cancer cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air.

3.3 TREATMENTS

HCT116 cells were exposed to varying concentrations of Mdivi-1 (10, 25, 50, 75, and 100 μM) and gemcitabine (500 nM, 1, 2.5, 5, and 10 μM) for 24, 48, 72, and 96 hours. Treatments were applied both individually and in combination (Mdivi-1 plus gemcitabine).

3.4 MTT ASSAY

The assessment of cell survival relied on the MTT method (from Bio Basic Inc., Markham, Canada), which is a dye-based technique that measures enzyme activity in mitochondria. This involves the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, converted by active mitochondrial enzymes in living cells into a purple, non-soluble formazan product. Since such conversion is directly related to the metabolic well-being of a cell culture, the method is frequently used to measure the negative effects of compounds on cell cultures cultivated in laboratories or on patient samples [58].

3.5 CELL VIABILITY ASSAY

Cell viability is a ratio of actively functional and energy-producing cells in a population and is a vital parameter in the experimentation of cultured cells. These tests are typical of the cellular responses to different substances or treatment [59]. The MTT assay was also applied in this study to calculate viability. To summarize, the cells of HCT116 at a concentration of 10,000 cells/well were seeded in 96-well plates and exposed to control (0.1% DMSO), Mdivi-1 (25 μM/cell), gemcitabine (1 μM/cell), or both control and

Mdivi-1. After exposure, 10 µL of MTT reagent (prepared at 5mg/mL) was introduced to each well after which it was allowed to incubate after 4 hours. The successive crystals were dissolved by using DMSO and the optical density was measured at 570nm.

3.6 WOUND HEALING ASSAY

HCT116 cells were cultured in 12-well plates until reaching 80–90% confluency. A wound was created by scratching with a sterile 200 µL pipette tip, and floating cells were removed by washing with serum-free DMEM/F12 medium. The cells were then treated with Mdivi-1, gemcitabine, a combination of Mdivi-1 and gemcitabine, or vehicle, all in the presence of 10% FBS, for 24 hours at 37 °C. After treatment, the medium was replaced with 1% FBS, and cells were incubated for an additional 24 hours. Cell migration was observed and recorded under a microscope (Zeiss, AX10, USA), with images captured at 0, 24, and 48 hours for analysis. Data were expressed as the colony formation rate relative to the percentage of the control.

3.7 TRANSWELL MIGRATION ASSAY:

HCT116 cells were cultured in 12-well plates with DMEM/F12 medium supplemented with 10% FBS and treated with Mdivi-1, gemcitabine and combination of gemcitabine with Mdivi-1 or vehicle for 16 h. Post-treatment, cells were trypsinized, and 5,000 cells in 200 µL of DMEM/F12 medium with 1% FBS were seeded into the upper chamber of Transwell inserts (8-µm pores; Corning Costar, Corning, NY, USA). The lower chamber contained 600 µL of DMEM/F12 medium with 10% FBS as a chemoattractant. After 24 and 48 h of incubation, non-migrated cells in the upper chamber were removed with a cotton swab and washed with D-PBS. Migrated cells on the underside were fixed with 4% paraformaldehyde, stained with crystal violet, and counted in five random fields under a Zeiss AX10 microscope (USA). Migration was expressed as the percentage of migrated cells relative to the control.

3.8 MITOCHONDRIAL MASS QUANTIFICATION ASSAY

Mitochondrial mass gives information about the total mitochondrial amount of cells and is usually indicative of cellular state, or functionality or novel mitochondrial generation. The mitochondrial mass was measured in this study by the use of MitoTracker Deep

Red dye. To perform the procedure HCT116 colorectal cancer cells were planted into 6-well plate and given a period of 24 h incubation to ensure that the cells are equilibrated in a nutrient rich medium. Treatments were made up of control (0.1% DMSO), Mdivi-1 25 μM, gemcitabine 1 μM, or both, and administered either 48 or 72 h. Cells were treated, washed with PBS, detached with 0.25 percent trypsin-EDTA, and stained in the dark with 10 nM of MitoTracker Deep Red (Invitrogen) at 37 °C to kill the stains. The cells were then resuspended in 500 μL PBS and assessed using flow cytometry (BD Biosciences, Becton Dickinson) using a 644 nm laser and 665 nm detector. Results were plotted as numbers of the average fluorescence signal compared to the control. There were three different runs of all experiments and each of the experiments was carried out in triplicate.

3.9 MITOCHONDRIAL MEMBRANE POTENTIAL ASSAY

MMP which is basically the electrical gradient across the inner membrane of the mitochondrion is pivotal in energy processes, including oxygen uptake, generation of energy molecules, and the generation of reactive oxygen species. To measure it, a positively charged fluorescent probe tetramethylrhodamine methyl ester (TMRM; Abcam, USA) was used. The cells of HCT116 were counted with the help of haemocytometer and then plated in 6 well plates with 2 mL of the growth medium in each well. Treatments were done immediately after adherence, and they were either 0.1 percent DMSO, Mdivi-1 25 μM, gemcitabine 1 μM, or a combination of both, and were 48 or 72 h long. Then 2 mL that had 100 nM TMRM was added and cells incubated at 37 °C and 30 minutes. PBS was used to rinse cells, and they were detached by treating with 0.25% trypsin-EDTA and centrifuged at 300 x g in 5min. Pellets were resuspended in 500 μL of PBS and brought to a final concentration of about 1×10^6 cells/mL to be analysed uniformly. A flow cytometer (excitation at 543nm, emission at 565nm) was used to detect the signal, and 10 000 events were acquired on each sample. The data were analysed using FlowJo software and the values given in terms of mean fluorescence intensity as compared to the controls. Biological replications of the experiment were done thrice and mean and standard deviation were reported.

3.10 REACTIVE OXYGEN SPECIES ANALYSIS

Mitochondrial reactive oxygen species (mROS), which are mainly in the form of superoxide are indicators of stress, mitochondrial activities, and cellular mechanism to therapeutic interventions. In order to determine mROS, HCT116 cells were counted on a hemocytometer and seeded at 5,000 cells per well on 100 µL of growth medium in black 96 2 well plates that had transparent bases. The cultures were then incubated with the vehicle control (0.1% DMSO), Mdivi-1 25 µM, gemcitabine 1 µM or a combination of both after incubating the cells overnight. The cultures were then incubated with either 48 or 72 hrs of incubation. The medium was substituted with 100 µL of 10 µM 2,7-dichlorofluorescin diacetate (DCFDA; Sigma-Aldrich) at the end of the exposure time, and plates were incubated at 37 °C in 30minutes. After two rounds of rinsing with phosphate-buffered saline (PBS), the fluorescence was measured using a plate reader (excitation 485nm, emission 535nm). The resulting fluorescence intensities were normalized against the control, in terms of percentage changes and each of the experimental conditions was replicated three independent assays with three inner replicas.

3.11 WESTERN BLOTTING

To examine proteins related to mitochondria and cellular pathways, Western blotting was carried out. In short, HCT116 cells were quantified using a hemocytometer and allocated to 6-well plates with 2 mL growth medium each. After settling overnight, exposures were control (0.1% DMSO), Mdivi-1 at 25 µM, gemcitabine at 1 µM, or combined, for 48 or 72 hours. Cells were then chilled on ice and broken open in RIPA lysis solution enhanced with 1% PhosSTOP and enzyme blockers (Roche, Mannheim, Germany) to maintain protein stability. Protein amounts were quantified via Bradford and Lowry techniques. Uniform loads (20 µg each) were run on 10% SDS-PAGE gels and moved to nitrocellulose filters. Filters were sealed with 5% skim milk in TBST, then exposed overnight at 4 °C to first-line antibodies targeting Drp1 (dilution 1:1000), p-Drp1 (Ser616, 1:1000), Mfn2 (1:1000), AMPK-α (1:1000), p-AMPK-α (1:1000). After TBST cleaning, filters received HRP-linked anti-rabbit secondaries for 1 hour at ambient temperature. Signals were revealed with ECL Plus kit and imaged on a Bio-Rad ChemiDoc™ system. Emphasis was on proteins tied to mitochondrial balance and spread indicators under Mdivi-1 and gemcitabine influence.

3.12 STATISTICS

Findings are given as means with standard deviations (SD) from no fewer than three separate trials. Differences between control and treatment groups were tested with repeated-measures ANOVA, followed by Tukey's multiple comparison adjustment, using GraphPad Prism. Significance was set at $p < 0.05$.

CHAPTER NO 4

RESULTS

4.1 Effect of Mdivi-1, gemcitabine and co-treatment of Mdivi-1 with gemcitabine on cell viability in HCT116 cells

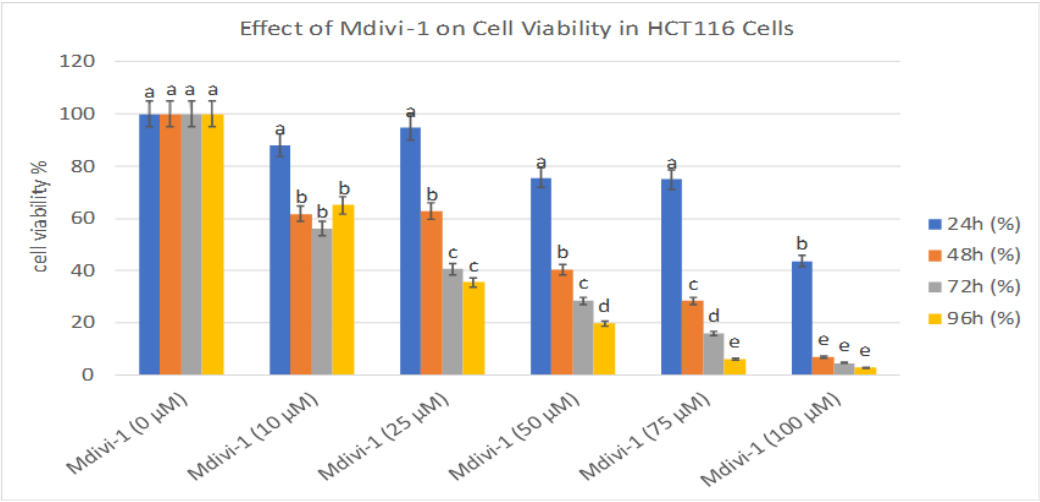
4.1.1 EFFECT OF MDIVI-1 ON CELL VIABILITY IN HCT116 CELLS

To assess Mdivi-1's impact on HCT116 cell growth, cells were exposed to escalating doses of Mdivi-1 (10–100 μM) over 24, 48, 72, and 96 hours, with cell viability measured via the MTT assay. As depicted in Figure 2A, Mdivi-1 decreased viability in a manner dependent on both dose and duration. At lower doses (10–25 μM), a modest decline in viability was noted, which intensified with extended exposure. Higher doses (50–100 μM) caused a significant viability drop, nearing total cell death at 100 μM after 72–96 hours. Dose-response analysis determined an IC_{50} of approximately 25 μM at 48 and 72 hours, guiding the choice of this concentration and time points for further mechanistic studies.

4.1.2 Effect of Gemcitabine on HCT116 Cell Viability

HCT116 cells were treated with varying gemcitabine concentrations for 24, 48, and 72 hours, and the MTT assay was used to evaluate its impact. Results from Figure 2B indicated that gemcitabine reduced cell viability in a dose- and time-dependent fashion. For subsequent mechanistic investigations, an IC_{50} of 1 μM gemcitabine was selected for 48 and 72-hour incubation periods.

A



B

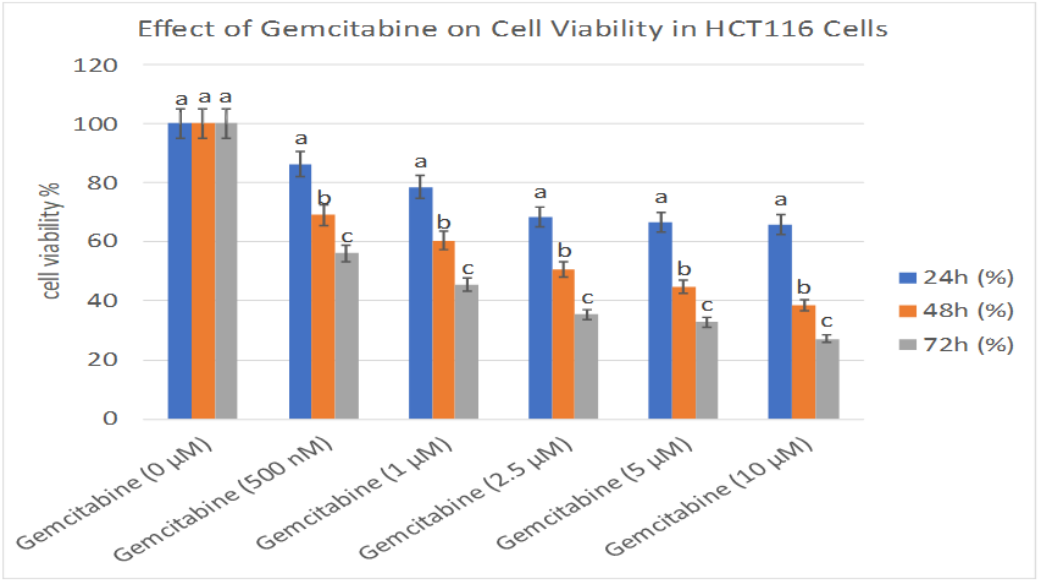
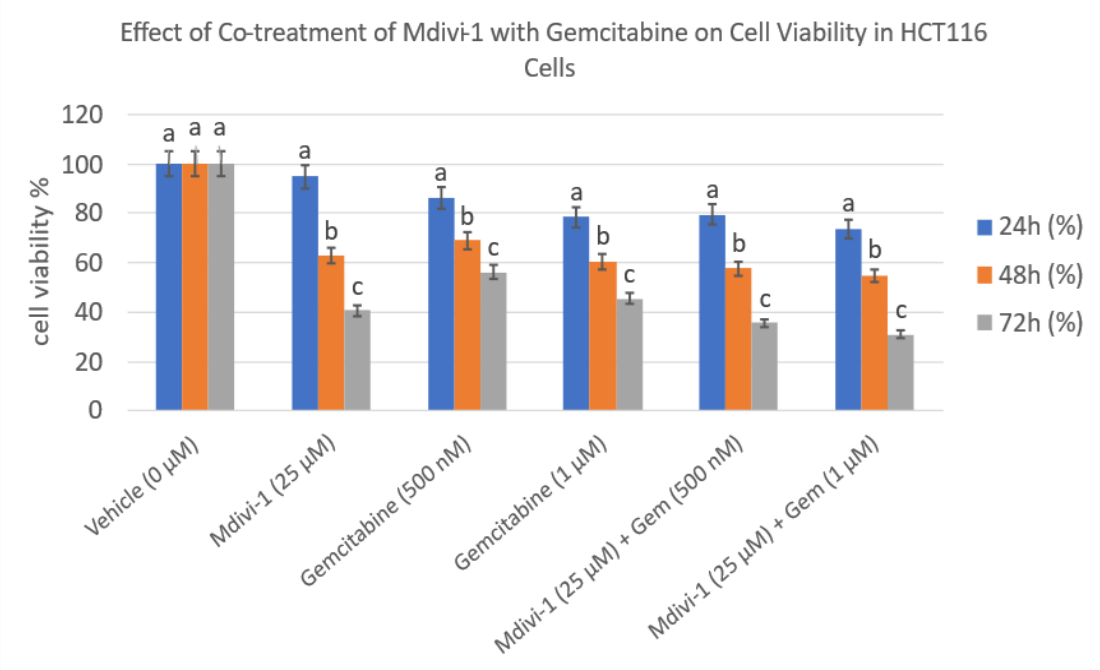


***Figure* 4.1.A Impact of Mdivi-1 on HCT116 Cell Viability**HCT116 cells were exposed to a range of Mdivi-1 concentrations (0, 10, 25, 50, 75, and 100 µM) over 24, 48, 72, and 96 hours. Mdivi-1 triggered cytotoxicity that increased with both dose and time, with an IC50 estimated at around 25 µM after 48 and 72 hours. Statistical evaluation using one-way ANOVA indicated highly significant differences ($P < 0.0001$).**4.1.B Impact of Gemcitabine on HCT116 Cell Viability**HCT116 cells were treated with various gemcitabine doses (0, 500 nM, 1 µM, 2.5 µM, 5 µM, and 10 µM) for 24, 48, and 72 hours. Gemcitabine demonstrated growth suppression that varied with dose and duration, with an IC50 of approximately 1 µM at 48 and 72 hours. One-way ANOVA confirmed statistically significant differences ($p < 0.0001$). Groups marked with distinct superscript letters (a, b, c, d, e) showed significant differences from one another, as determined by Tukey's post hoc test ($p < 0.05$).

4.1.3 EFFECT OF THE CO-TREATMENT OF MDIVI-1 WITH GEMCITABINE ON CELL VIABILITY IN HCT116 CELLS

Mdivi-1 was administered at an IC50 concentration of 25 µM and gemcitabine at an IC50 concentration of 1 µM to HCT116 cells, and efficacy assessed after 48 hours and 72 hours post-incubation. The data showed that Mdivi-1 had a significant effect in enhancing gemcitabine induced cytotoxicity at 24 h and at 48 h, which is a sign of synergistic action of the two agents at the time. This potentiation indicates that Mdivi-1 has the potential to sensitise tumour cells to gemcitabine impact on mitochondrial fission, and could potentially sensitise tumour cells to the antitumor effects of gemcitabine. However, at 72 hours, the joint treatment got an additive effect instead of a synergetic effect meaning that the agents still had a combined cytotoxic effect but the interaction was reduced compared to previous time points. These findings highlight the temporal interaction between Mdivi and gemcitabine, which is strongly synergistic after 24 and 48 h and with an additive effect after 72 h, which is probably due to changes in the responsiveness of the cells or the pharmacokinetics of the drug with time.



`***Figure 4.1.C:*** **Impact of Co-treatment with Mdivi-1 and Gemcitabine on HCT116 Cells:** HCT116 cell were exposed to a vehicle control or to Mdivi-1 (25 µM) plus gemcitabine (500 nM or 1 µM) and combinations thereof after 24, 48 and 72 h. The MTT assay was used to determine cell viability expressed as a percentage

of that of the vehicle control. Combinations of the two agents caused more cell death than either agent but had a strong additive effect at 72 h. One-way ANOVA showed significant differences ($p<0.0001$) and those that were differentiated by superscript (a,b,c,d,e) were significantly different (Tukey post-hoc, p).

4.2 THE COMBINE EFFECT OF MDIVI-1 AND GEMCITABINE ON MITOCHONDRIAL MASS QUANTIFICATION OF HCT116 CELLS

The mass quantification of mitochondria is the measurement of the overall amount or number of mitochondria in an individual cell which is regularly performed by such methods as the fluorescence microscopy of mitochondrial-specific dyes, mitochondrial proteins by western blot, or mitochondrial DNA content by quantitative polymerase chain reaction. Such measurements are proxies of the mitochondrial bio-genesis, turnover and the capacity of the cell to generate energy. Under experimental conditions, determining mitochondrial mass is necessary to determine cellular metabolic activity, mitochondrial health, or stressor reactions including oxidative damage and disease [65].

MitoTracker Deep Red, a cell permeable fluorescent probe that specifically targets the mitochondria of living cells is commonly used in this application. Cells are put in culture media containing MitoTracker Deep Red (10 to 100 nM) and allowed to incubate with the dye between 15 to 30 minutes under optimal conditions after which fluorescence intensity is measured using flow cytometry; high mean fluorescence intensity will indicate high mass of the mitochondria. Mdivi-1 (25 μM) was used in combination with gemcitabine (1 μM) in a test of its effect on mitochondrial mass of HCT116 cells at 48 and 72 hours after the incubation period. Mitochondrial mass was measured with the help of Mitotracker Deep Red that is the fluorescent dye specifically staining mitochondria, thus, allowing to estimate precisely the content of mitochondria. The findings have shown that mitochondrial mass was significantly increased after co-treatment with Mdivi-1 and gemcitabine after 48 and 72 hours as compared to their untreated controls. These observations indicate that, the combined therapeutic regimen changes mitochondrial dynamics in a time-dependent fashion that may be contributing to the changes in mitochondrial mass and the possible effects on the cytotoxic and apoptotic effects of the HCT116 cells at the time points examined.

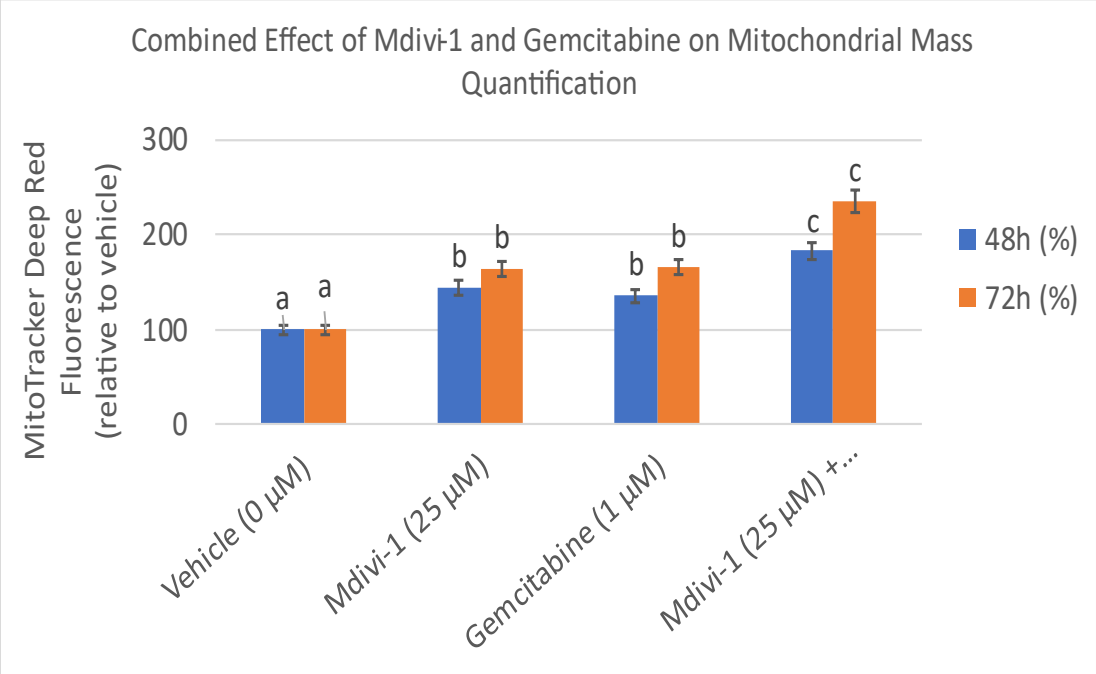


Figure 4.2: **Effect of Mdivi-1 and Gemcitabine Combination on Mitochondrial Mass Quantification** Mitochondrial mass was measured in HCT116 cells exposed to a control, Mdivi-1 (25 µM), gemcitabine (1 µM), or their combination for 48 and 72 hours, using MitoTracker Deep Red staining. Fluorescence intensity readings were scaled by a factor of 100 for graphical representation and shown as fold change compared to the control. The combined treatment notably elevated mitochondrial mass relative to individual treatments or the control. One-way ANOVA indicated highly significant differences ($P < 0.0001$). Groups marked with distinct superscript letters (a, b, c) exhibited significant variations from one another, as verified by Tukey's post hoc test ($p < 0.05$).

4.3 THE COMBINED EFFECT OF MDIVI-1 AND GEMCITABINE ON MITOCHONDRIAL MEMBRANE POTENTIAL OF HCT116 CELLS

Mitochondrial membrane potential (ΔΨm) is the electrochemical potential difference that is across the inner mitochondrial membrane, which is set up by the proton gradient that is generated during the electron transfer chain in cellular respiration. It is a vigorous stimulator of adenosine triphosphate (ATP) production and is an indicator of mitochondrial integrity. In a laboratory setting, ΔΨm is an essential biomarker of mitochondrial health, dissipation of which is involved in the induction of apoptosis, the pathophysiology of neurodegenerative diseases and an assortment of metabolic impairments. Measurement of ΔΨm gives a quantitative determination of cellular energetic health, mitochondrial cellular injury, or pharmacological cellular toxicity, which makes it invaluable when the aim of the study is to determine cellular survival and the pathophysiology of disease. There is a routine use of cationic fluorescent probes, such as tetramethylrhodamine methyl ester (TMRM) and Mitotrackers Deep Red, to measure MMP [63,64].

Mdivi-1 (25 μM) and gemcitabine (1 μM) were combined to investigate the effects of the two agents on mitochondrial membrane potential (MMP) after 48 h and 72 h, respectively because both agents have been reported to disrupt MMP. MMP was estimated by taking the ratio with mean fluorescence intensity of TMRM and Mitotraker Deep Red, which were two fluorogenic dyes that were used to measure the integrity and functional capability of the mitochondrial membrane. Concomitant use of Mdivi-1 and gemcitabine resulted in statistically significant reduction of MMP at 48 and 72 hrs compared to controls. This severe deterioration of MMP is a sign of a synergistic impairment of mitochondrial activity by Mdivi-1, a mediator of mitochondrial fission inhibition, and gemcitabine, a cytotoxic chemotherapeutic compound, presumably via the amplification of mitochondrial stress and the reduction of bioenergetic capacity. These observations imply that the dual therapy enhances mitochondrial dysfunction in a time-dependent fashion hence contributing to the cytotoxic and apoptotic results of HCT116 cells at the tested time points.

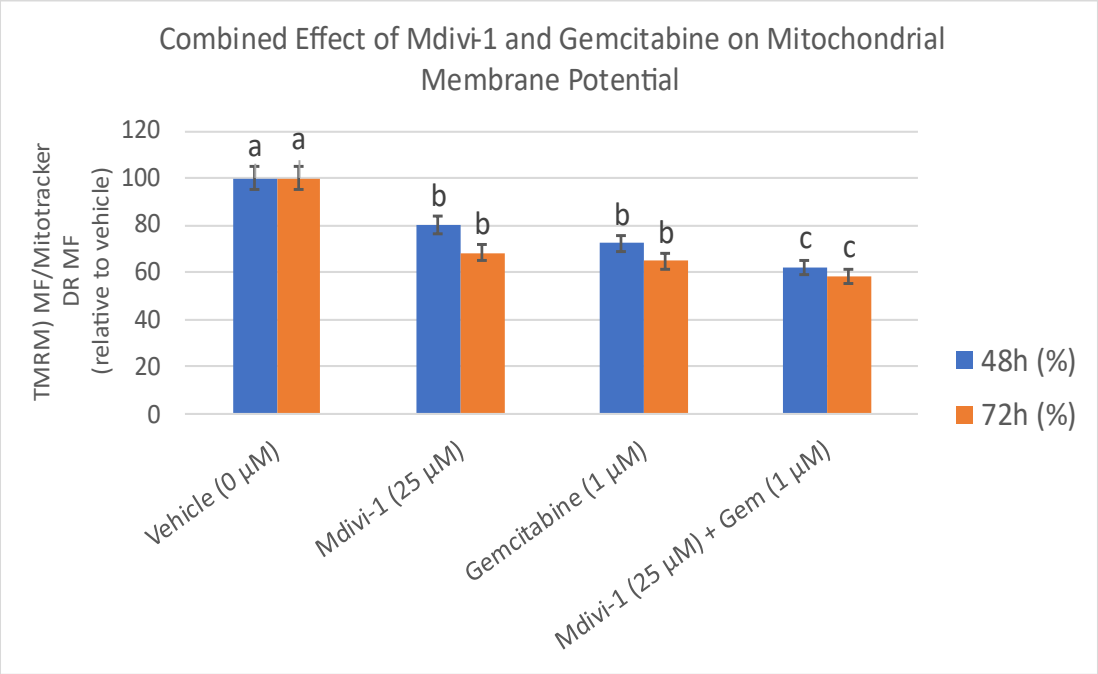


Figure 4.3: **Effect of Mdivi-1 and Gemcitabine Combination on Mitochondrial Membrane Potential** The mitochondrial membrane potential (ΔΨm) in HCT116 cells was evaluated following exposure to a control, Mdivi-1 (25 µM), gemcitabine (1 µM), or their combination for 48 and 72 hours. The combined treatment significantly lowered ΔΨm compared to either drug alone. Groups marked with distinct superscript letters (a, b, c) showed significant differences from one another, as confirmed by Tukey's post hoc test ($p < 0.05$).

4.4 THE COMBINED EFFECT OF MDIVI-1 AND GEMCITABINE ON REACTIVE OXYGEN SPECIE OF HCT116 CELLS

Reactive oxygen species (ROS) include highly reactive species, including superoxide anion and hydrogen peroxide, which are byproducts of electron transport chain in oxidative phosphorylation. ROS in the mitochondrial environment serve two roles: they play a role as signalling molecules occurring at low concentrations, modulate cellular processes, but at high concentrations cause oxidative stress leading to the destruction of proteins, lipids, and nucleic acids. As a result, the measurement of mitochondrial ROS is necessary to determine the oxidative stress condition, mitochondrial dysfunction, and to clarify the role of ROS in cellular functions including apoptosis, cellular ageing, or disease progression, and, therefore, essential information on cellular health and adaptation to environmental or therapeutic changes is obtained [66].

The confounding effect of Mdivi-1 (25 μM) and gemcitabine (1 μM) on intracellular ROS levels in HCT116 cells was questioned at 48 and 72 hours after incubation. The measurements of ROS were done through the DCF diacetate oxidative stress fluorescence emitting the oxidised product of 2',7'-dichlorofluorescin through DCF-DA assay. The findings showed that the combination of Mdivi-1 and gemcitabine significantly increased intracellular ROS levels at 48 h, which supports the concept that the combination increased oxidative stress, possibly by disrupting the mitochondrial dynamics by Mdivi-1 and DNA damage by gemcitabine, which compounded ROS generation. However, at 72 h, there was a decrease in level of ROS compared to 48 h. This attenuation could be due to some adaptive cellular mechanisms, including the recruitment of antioxidant defence mechanisms, or an over time reduction in the number of metabolically active cells due to persistent cytotoxicity, and hence reduces the production of ROS over time. These results show that there is a time-dependent variation in the modulation of the oxidative stress of the combination therapy, with a peak in ROS formation at 48 h and a decrease after 72 h in HCT116 cells.

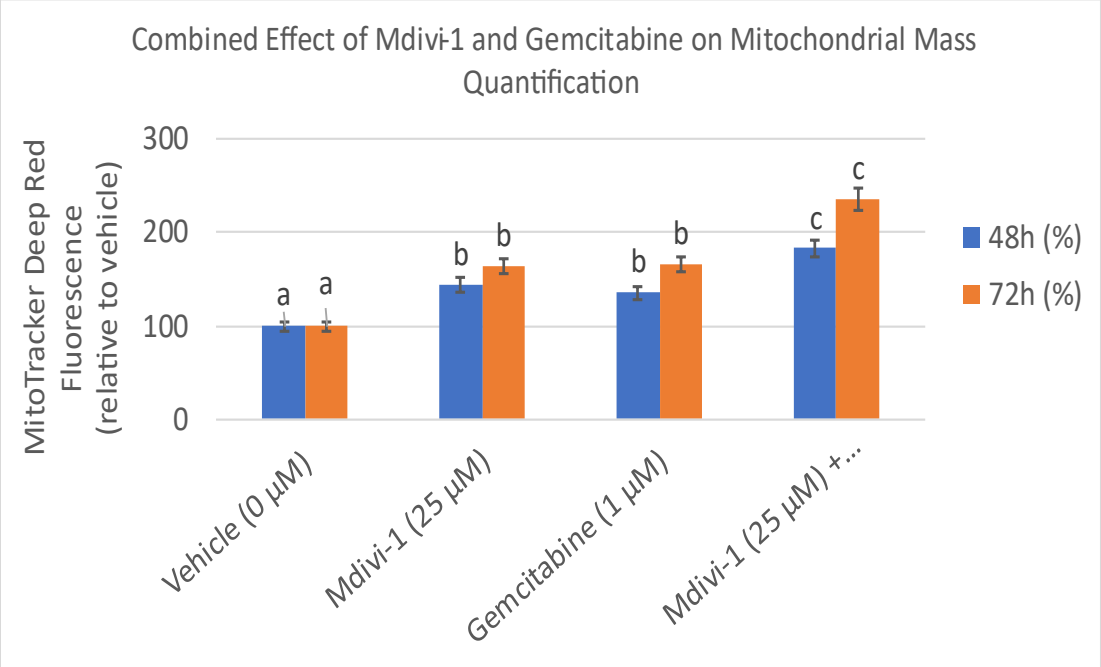


Figure 4.4: **Effect of Mdivi-1 and Gemcitabine on ROS Levels in HCT116 Cells:** Intracellular reactive oxygen species (ROS) levels were assessed in HCT116 cells exposed to a control, Mdivi-1 (25 µM), gemcitabine (1 µM), or their combination for 48 and 72 hours using the DCF-DA assay. The combined treatment markedly increased ROS at 48 hours, though levels declined by 72 hours compared to the earlier time point. One-way ANOVA revealed highly significant results ($P < 0.0001$). Groups marked with distinct superscript letters (a, b, c) showed significant differences from one another, as confirmed by Tukey's post hoc test ($p < 0.05$).

4.5 MIDIVI-1 AUGMENTS ANTI METASTATIC ACTIVITY OF GEMCITABIN IN HCT116 CELLS.

Wound-healing assays and Transwell chamber assays were performed to compare the anti-migratory activity of Mdivi-1, gemcitabine and a combination of the two in the HCT116 cells. Migratory cells in the wound healing test treated with vehicles effectively closed the scratched region in 24 and 48 hours. Wound healing was significantly decreased in both Mdivi-1 (10-50 μM) and gemcitabine (50 100 nM) in dose-dependent fashion compared with controls ($p < 0.05$). It is noteworthy that Mdivi-1 combined with gemcitabine caused a stronger inhibitory effect as compared to each agent alone, and the greatest residual wound area was recorded at 48 hours ($p < 0.01$ compared to each agent) (Figure 4.5.A -B). Likewise, Transwell migration experiments revealed that Mdivi-1 and gemcitabine reduced the migrated amount of HCT116 cells in isolation of each other ($p <0.05$). Notably, the migration reduction was the greatest with the combination treatment ($p < 0.001$ compared with control; $p < 0.01$ compared with single agents), which was indicative of a synergistic effect on migration (Figure 4.5.B). Taken together, these findings indicate that Mdivi-1 was able to increase the anti-migratory effect of gemcitabine, hence supporting its promise to complement the anti-metastatic effect of chemotherapy in colorectal cancer cells.

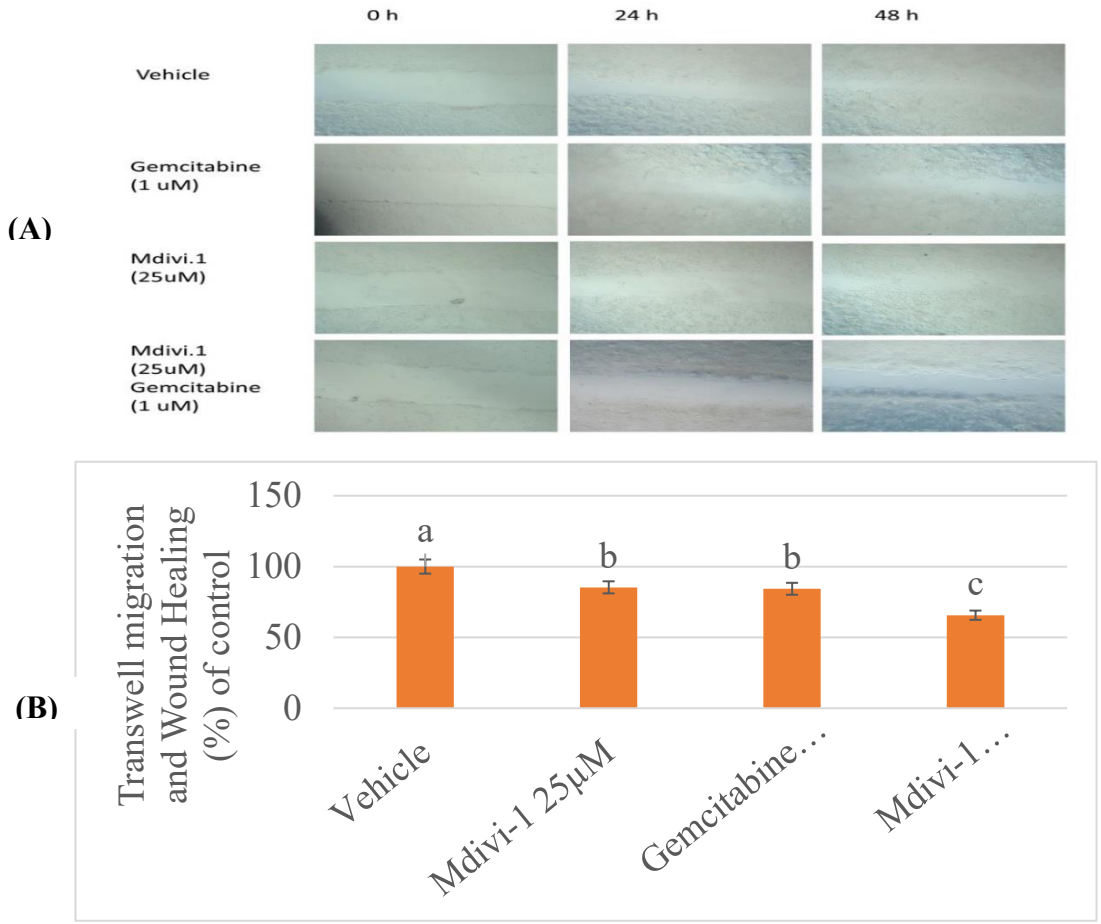


Figure 4.5: **Effect of Mdivi-1 and Gemcitabine on Cell Migration in HCT116 Cells:** **(A)** Wound healing assay showing migration of HCT116 cells treated with vehicle, Mdivi-1 (25 µM), gemcitabine (1 µM), or their combination at 0, 24, and 48 hours. Vehicle-treated cells showed efficient wound closure over time, while Mdivi-1 or gemcitabine alone reduced migration. The combination treatment resulted in the largest residual wound area, indicating a synergistic inhibition of cell migration. **(B)** Transwell migration assay quantifying the percentage of migrated HCT116 cells relative to controls after treatment with vehicle, Mdivi-1, gemcitabine, or their combination. Both agents individually reduced cell migration compared with control ($p < 0.05$), whereas the combination significantly suppressed migration to the greatest extent ($p < 0.001$ vs control; $p < 0.01$ vs single agents). Statistical analysis by one-way ANOVA followed by Tukey's post hoc test confirmed significant differences between groups marked with distinct superscript letters(a, b, c) ($p < 0.05$).

4.6 EFFECT OF MDIVI- 1 AND GEMCITABINE ON THE MITOCHONDRIAL DYNAMIC REGULATING MARKERS IN HCT116 CELLS

Mitochondrial dynamic regulating markers are proteins or genes, such as MFN1, MFN2, OPA1 (for fusion), and DRP1, FIS1 (for fission), that control mitochondrial dynamics, the processes of mitochondrial fusion and fission that regulate their shape, number, and distribution. The dynamics described are needed to sustain the functioning of the mitochondrion, to have quality control, and to spend the cellular energy homeostasis. Experimental studies have found these markers to be the key indicators used to assess mitochondrial adaptability, biogenesis and dysfunction in relation to different types of stresses, pathologic conditions or therapeutic treatment. The aberrant expression or activity of these markers can indicate an abnormal mitochondrial dynamics and has been linked to neurodegenerative disease, malignancies and metabolic diseases [67].

The effect of single agent Mdivi-1 (25 μM), gemcitabine (1 μM), and the combination of both on the pivotal proteins that regulate mitochondrial activities in HCT116 cells was examined at 48 and 72 hours post incubation. Mdivi 1 treatment per se led to a decrease in dynamin related protein 1 phosphorylation (pDRP1) both at 48 and 72 h, which supports its role in preventing mitochondrial division by inhibiting the activity of DRP1. On the same note, the expression of DRP1 protein was higher at 48 h after each separate treatment but the expression was suppressed when Mdivi-1 and gemcitabine were administered concomitantly suggesting a modulatory interaction on the expression of DRP1 protein. It is important to note that the combined treatment did not reduce the expression of DRP1 at a time of 72 h, which suggests that its regulation effect changed with time. With regard to mitofusin-2 (Mfn2), a central protein in the fusion of mitochondria, both Mdivi-1 and gemcitabine alone enhanced the expression of Mfn2 at 48 and 72 h which is likely to mediate mitochondrial lengthening. The combination treatment, on the other hand, induced a decrease in the level of Mfn2 at 48, which is probably due to a synergistic cytotoxic stress condition that disrupts the fusion dynamics; the increase in the level of Mfn2 at 72 is likely a compensatory enhancement of mitochondrial fusion to further treatment. All these findings highlight the complex, time-dependent interplay between Mdivi-1, and gemcitabine in regulating mitochondrial dynamics, which are reflected by differentially changing effects on

DRP1 phosphorylation, DRP1 expression and Mfn2 expression over the 48 and 72 h time range in HCT116 cells.

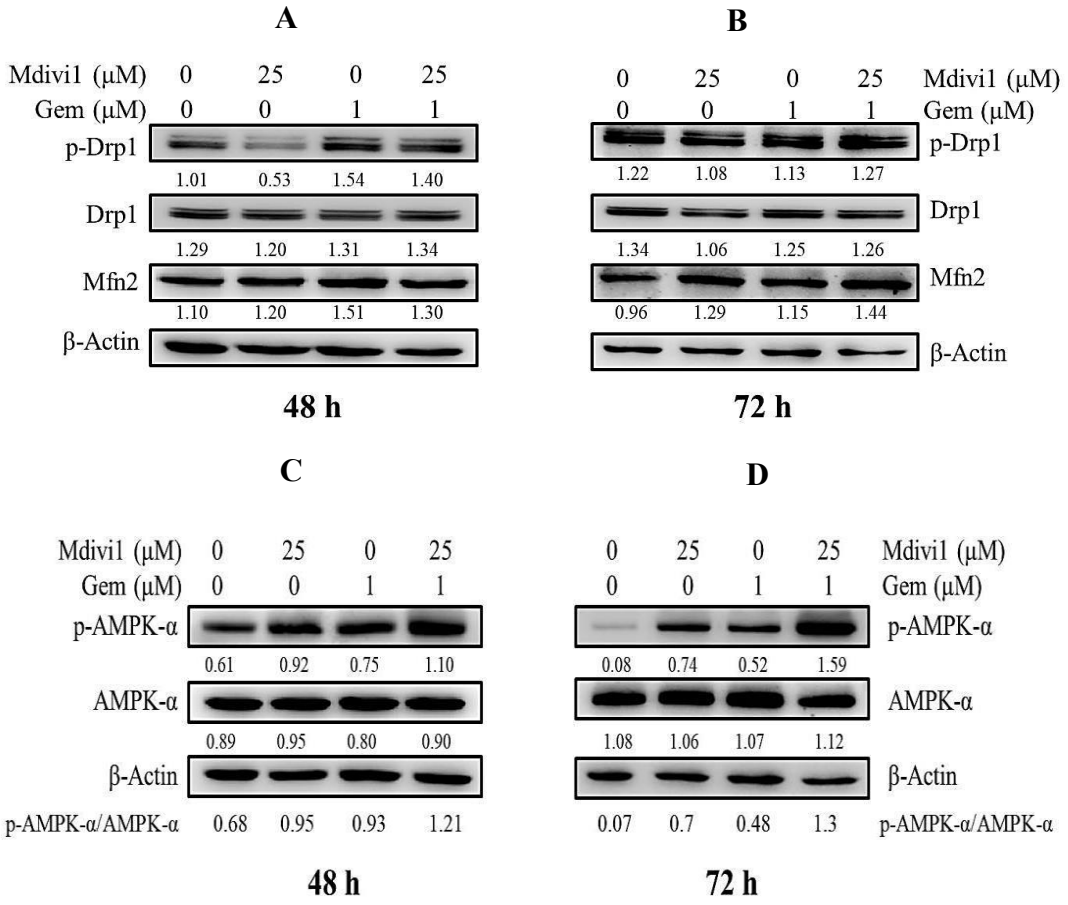


Figure 4.6: Figure 4.6: (A-B) Influence of Mdivi-1 and Gemcitabine on Mitochondrial Dynamic Markers in HCT116 Cells: Western blot analyses of phosphorylated Drp1 (p-Drp1), total Drp1, mitofusin-2 (Mfn2), and β-Actin (loading control) were performed on HCT116 cells treated with Mdivi-1 (0 or 25 μM) with or without gemcitabine (Gem; 0 or 1 μM) for 48 hours (A) and 72 hours (B). Mdivi-1 lowered p-Drp1 levels (0.53 at 48 h, 1.08 at 72 h); gemcitabine raised p-Drp1 (1.54 at 48 h, 1.13 at 72 h) and Mfn2 (1.51 at 48 h, 1.15 at 72 h); the combination adjusted both markers accordingly. **(C-D) Influence of Mdivi-1 and Gemcitabine on AMPK Expression in HCT116 Cells:** Western blot assessments of phosphorylated AMPK-α (p-AMPK-α), total AMPK-α, β-Actin (loading control), and the p-AMPK-α/AMPK-α ratio were conducted on HCT116 cells treated with Mdivi-1 (0 or 25 μM) with or without gemcitabine (0 or 1 μM) for 48 hours (C) and 72 hours (D). The combined treatment elevated the p-AMPK-α/AMPK-α ratio (1.21 at 48 h, 1.3 at 72 h) compared to individual treatments, suggesting greater AMPK activation.

4.7 EFFECT OF MDIVI-1 AND GEMCITABINE ON AMPK EXPRESSION IN HCT116 CELLS

AMPK (AMP -activated protein kinase) is a key cellular energy sensor that is activated in response to an increase in the AMP/ATP ratio, which is an indication of reduced cellular energetic status. Mitochondrial AMPK, which is characterized by localization to or action on mitochondria, is controlled in low ATP environments and coordinates mitochondrial biogenesis, activity and general energy metabolism through the phosphorylation of substrates that stimulate ATP production and mitochondrial survival. The analysis of mitochondrial expression of AMPK is an essential measure of cell adaptive responses to energetic stress, metabolic regulation and disease, including diabetes, oncogenesis, or neurodegeneration, because it indicates the degree of cellular energetic balance or mitochondrial pathology repair [68].

The effects of concurrent treatment with Mdivi-1 (25 μM) and gemcitabine (1 μM) on AMPK signalling were analyzed at 48 and 72 hrs after incubation in HCT116 colorectal carcinoma cell line. Quantitative determination showed that the overall levels of AMPK protein were statistically equivalent between the co-treated group and controls (vehicle) which means that the general abundance of this energy-sensing protein was not disturbed. Conversely, the enzyme activation signal (phosphorylation of AMPK (p -AMPK)) was significantly increased, at 48 and 72 hours, when it was co-incubated. In turn, it was observed that the p-AMPK/ total AMPK protein ratio has been greatly elevated, which leads to the conclusion that the combinatorial regimen of Mdivi-1 and gemcitabine enhances the activation of the AMPK pathways. This enhancement of the ATP synthesis cascade is probably a cellular compensatory mechanism to this energy catabolism impairment which is caused by mitochondrial dysfunction and cytotoxic stress caused by the therapeutic agents.

CHAPTER NO 5

DISCUSSION

This study looked at the synergistic effects of Mdivi-1, a mitochondrial fission inhibitor, and gemcitabine, a nucleoside analog that is applied on chemotherapy, on the colorectal adenocarcinoma cancer cell line HCT116. Specific attention was given to how the Mdivi-1-induced changes in the mitochondrial dynamics contribute to the enhancement of the anti-metastatic properties of gemcitabine. In accordance with this hypothesis, co-treatment of 25 μM Mdivi-1 and 1 μM gemcitabine resulted in a significant and pronounced cytotoxicity, anti-metastatic effect, disruption of mitochondrial membrane potential (MMP), mitochondrial mass, reactive oxygen species (ROS) production, mitochondrial dynamics protein expression, (DRP1 and Mfn2) and activation of AMP-activated protein kinase (AMPK) signal transduction. These were noted at 48 and 72 hours indicating a time dependent pattern of synergistic and additive interactions between the two agents. By placing the findings in the context of the large literature, the study emphasizes their clinical applicability, their importance, and outlines future research that can help to enhance treatment options in addressing colorectal cancer. It was demonstrated by the MTT assay that in a dose- and time-dependent manner, Mdivi 1 and gemcitabine, when used separately, decreased the viability of HCT116 and caused the apoptosis. Co-treatment resulted in a synergistic augmentation in gemcitabine induced cytotoxicity at 24 and 48 hours and additive effect at 72 hours, which can be supported with previous findings that Mdivi-1 augments the efficacy of chemotherapy by suppressing mitochondrial fission. Notably, mitochondrial fission inhibited with Mdivi 1 is not only important to decrease the migration of colorectal cancer cells, but also to enhance the anti-metastatic effect of gemcitabine. Both wound-healing and Transwell migration experiments revealed that either of the two agents retarded HCT116 motility significantly, but the combination had the greatest effect of all (p 0.01 compared to single agents). The observations contribute to the hypothesis that mitochondrial dynamics promote the development of metastases by regulating cellular energetics and production of ROS. The reported synergy of Mdivi-1 and gemcitabine suggests that mitochondrial dynamics alteration can be used to prime colorectal cancer cells to chemotherapy by inhibiting metabolic versatility and raising the rate of apoptosis. As a result, the synergy between Mdivi-1 and gemcitabine becomes an

effective therapeutic option in order to achieve improved outcomes and limit metastatic potentials of colorectal cancer. Indicatively, Qian et al. (2014) found that Mdivi-1 induces breast cancer cells to be sensitized to cisplatin through mitochondrial dynamics alteration, which leads to an increased rate of apoptosis [22]. On the same note, when Wang et al. (2015) used Mdivi-1 to increase doxorubicin cytotoxicity in colorectal cancer cells, they demonstrated that the drug inhibited DRP1-mediated fission and consequently decreased migration and metastatic potential. The current research builds upon these findings by showing a time-dependent synergy of Mdivi-1 and gemcitabine on the cells of HCT116 with a change to additivity at 72 hours which could be due to adaptive induction of antioxidant defenses or loss of viable cells. This is contrasted with the study of Qian et al. who only examined breast cancer, but in this case, the authors support the importance of mitochondrial-targeted therapy in colorectal cancer, a cancer with specific metabolic and metastatic properties [22]. The MMP determined through TMRM/mitotracker Deep Red fluorescence ratio was lower than in the co-treatment at 48 and 72 hours which is an indicator of mitochondrial dysfunction. This observation is in line with reports that have indicated that Mdivi-1 induces mitochondrial stress by blocking fission resulting in dysfunctional and long mitochondria [71], and gemcitabine disrupts MMP by damaging DNA and causing cellular stress [72]. The stepwise increase in MMP was consistent with the findings of Zhang et al. (2016), which also concluded that Mdivi 1 increased the mitochondrial dysfunction and apoptosis when combined with cisplatin in lung cancer cells [21]. This effect was still observed at 72hours in colorectal cancer cells indicating that mitochondrial integrity was disrupted on a sustained basis and may have blocked energy-intensive events during the process of metastasis, including migration and invasion [70]. Mitochondrial mass, as measured using Mitotracker Deep Red, was significantly increased during co-treatment at 48 and 72hours, which is indicative of mitochondrial fission inhibition and unopposed fusion caused by Mdivi-1 (Cassidy-Stone et al., 2008). This result is in line with Bordt et al. (2017), who observed an increase in the mass of neuronal cells after treatment with Mdivi-1 because the inhibition of DRP1-mediated fission occurred [16]. Interestingly, gemcitabine enhanced this effect implying a new interaction which may be due to gemcitabine induced stress inducing mitochondrial biogenesis as a negative feedback mechanism. Conversely, Wang et al. (2015) found that the Mdivi-1 increased the mitochondrial mass of colorectal cancer cells, but failed to evaluate it jointly with gemcitabine. The simultaneous increase of mitochondrial mass and decrease in MMP

suggests accumulation of dysfunctional mitochondria, which is likely one of the causes of the anti-metastatic effects observed, by restricting the bioenergetic ability required to invade cells. Production of ROS measured using the DCFDA assay was highly increased at 48 hours after co-treatment and reduced at 72 hours. This shows a similarity to previous reports that show both Mdivi 1 and gemcitabine induce oxidative stress each on their own [19]. As an example, Donadelli et al. (2007) showed that gemcitabine raises the levels of ROS in pancreatic cancer cells, which stimulates apoptosis [72], and Bordt et al. (2017) found that Mdivi-1 raises the levels of ROS through decreasing the activity of mitochondrial dynamics [16]. The presently observed synergistic ROS surge at 48 hours is in line with the results reported by Zhang et al. (2016) who reported increased ROS generation when Mdivi 1 was co-treated with cisplatin in lung cancer cells [21]. The attendant ROS reduction at 72 hours is a new discovery that could potentially be due to an increased antioxidant defense mechanism like superoxide dismutase or glutathione in response to chronic oxidative stress like that observed by Han et al. (2019) [49]. The temporal dynamics of the ROS modulation indicates that the anti-metastatic effect of the combined therapy is the strongest at 48 □ 1h when oxidative stress probably interferes with metastasis-promoting signalling pathways, including integrin-mediated adhesion (Ishikawa et al., 2008). The involvement of the anti-metastatic effect of the combination therapy is further supported by the regulation of mitochondrial dynamics proteins. Mdivi-1 also inhibited phosphorylation(pDRP1) of DRP1 at 48 h and 72 h, which were in line with its action of a DRP1 inhibitor (Cassidy-Stone et al., 2008). The expression of DRP1 peaked at 48h after individual treatments but was inhibited when co-treated, which is not similar to the results of Qian et al. (2014), who showed sustained DRP1 up-regulation with co-treatment of Mdivi-1 only [22]. The fact that the effect of DRP1 suppression was not observed at 72 h, in the presence of co-treatment, indicates that there was a compensatory effect, which was likely initiated by gemcitabine stress. Individual treatments up-regulated Mfn2 at the two time points, but co-treatment down-regulated Mfn2 at 48^{-} 1 time and up-regulated Mfn2 at 72 time. This does not align with Wang et al. (2015), who have reported constant up-regulation of Mfn2 in Mdivi-1 in colorectal cancer cells [19]. The initial inhibition of Mfn2 can be associated with acute stress, and the following elevation can be regarded as the compensatory fusion reaction, which underlines the active interaction of the process in the mitochondrial remodelling that probably hinders metastatic events. The evidence of energy stress caused by co-treatment is the AMPK

activation, which is reported in the form of high pAMPK/AMPK ratios at 48 and 72 h of co-treatment without alterations in the overall level of AMPK expression. This fact corresponds to the results obtained by Hardie et al. (2012), as the authors observed that AMPK activation upon mitochondrial dysfunction facilitates apoptosis in energy-stressed cancer cells [69]. In contrast to Zhang et al. (2016), who also cited AMPK activation in case of cisplatin, the present study shows that Mdivi-1 and gemcitabine collaboratively increase the activation of AMPK, which is likely due to the integration of the effect on the mitochondrial membrane potential and the generation of ROS [21]. This activation can decrease the energetic potential needed to support metastatic behaviours, such as cell motility, which will enhance the anti-metastatic effect of gemcitabine.

CONCLUSION:

The HCT116 colorectal cancer cells indicated that anti-metastatic effects by co-treating Mdivi-1 (25 μM) with gemcitabine (1 μM) would help in the mitochondrial dynamics and energy homeostasis in cells. The combination resulted in significant cytotoxicity and apoptosis, destabilisation of mitochondrial membrane potential, and an increase in mitochondrial mass, and became synergistic with effects becoming additive probably because of cellular adaptations including an upregulation of antioxidants. Co-treatment repressed DRP1 phosphorylation and expression, mitochondrial fission, and dynamically regulated Mfn2 expression, indicating that mitochondrial remodelling is a complicated process. Additionally, the mixture triggered the AMPK signalling pathway, which is a sign of energy stress that restricts the metastatic potential by restricting the bioenergetic capacity. These results, as well as earlier research that has shown Mdivi-1 to increase chemotherapeutic response, are indicative of its role as a mitochondrial-targeted adjuvant to gemcitabine in colorectal cancer, which requires further studies to streamline treatment regimes and potential clinical implementation.

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