THE EFFECT OF RAN INHIBITION ON HUMAN COLORECTAL CANCER CELLS (CRC)

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The effect of RAN inhibition on human colorectal cancer cells (CRC)

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Abstract

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Keywords: Human colorectal cancer, Metastasis, *K-Ras* mutation, *Pten* deletion, Repurposing mebendazole (MBZ), Ran inhibition, western blot, Polymerase chain reaction, Colony formation, Invasion, Migration.

Colorectal cancer (CRC) is the third most widespread and fourth most fatal malignancy disease. The CRC from a primary site can spread to other tissues, forming secondary tumours. CRC can metastasise to the liver through the effect of *K-Ras* and *Pten* mutation (Mt.) (Abbas et al. 2020). This study aimed to assess the hypothesis that the Ran inhibitor mebendazole MBZ reduces cell invasion and metastasis of CRC. I have investigated MBZ effect on the CRC isogenic human cell lines with specific mutations (HCT-116 *K-Ras*, DLD-1 *K-Ras* and *Pten* deletion and wild type HCT-116 and DKO-3. I used qRT-PCR and western blotting to identify expression levels of various genes and signalling molecules after treatment with 0.5 mM MBZ. In addition, several assays were performed to investigate MBZ effect on biological properties of the cells such as proliferation, migration, invasion, and colony formation. MBZ downregulated Ran and induced apoptosis through inhibition of Bcl-2 expression as well as inducing caspase -3, -7, -9 and PARP cleavage. Moreover, MBZ showed an effect on immune response by down regulating C5a, IL-1ß and IL-1α analysed at mRNA level. When treated with MBZ, the migration, invasion and colony formation abilities of HCT-116 *K-Ras* Mt., DLD-1 *K-Ras* Mt. and HCT-116 *Pten*$^{-/-}$ were significantly reduced compared to a control treated cell line. This was also the case with wild-type cell lines such as HCT-116 and DKO-3. Furthermore, signalling molecules such as p- Erk 1/2 and p- Akt were upregulated after MBZ treatment and exert inhibition on Akt 1/2/3 and VEGFR1/2 mRNA levels. In conclusion; MBZ which is a Ran inhibitor, has significantly reduced proliferation, colony formation, and migration in colorectal cell lines with *K-Ras* and *Pten* gene deletion compared to wild type cells in a dose-dependent manner. This work paves the way to clinical validation of MBZ as a combination therapy for reducing the invasion of CRC cells.

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Publication and conferences abstract

Publication

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Abbreviation	Definition
Akt	A serine-threonine protein kinase called protein kinase B
Apaf1	Apoptosis protease activating factor 1
Apc gene	Adenomatous polyposis coli
ATF	Activating transcription factor
Bad	Bcl-2-Associated-death promoter
Bak	Bcl-2 homologous-antagonist- killer
Bax	Bcl-2 Associated protein
Bcl-2	B-cell lymphocytic-leukemia proto-oncogene 2
Bid	BH3 interacting domain death agonist
Bik/Nbk	Bcl-2-interacting killer/natural born killer
BimL	Long version of the Bim protein
BimS	Short splicing variant, which is localized in the cytosol
B-Raf	*B-Raf* proto-oncogene serine/threonine kinase
B2M	Beta-2 micro globulin tumour marker
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Caspase	Cysteine-Aspartic acid Protease
CBP	CREB-binding protein
CEBPA	CCAAT/enhancer binding protein alpha
CIMP	Cpg island methylator phenotype
CIN	Chromosomal instability
UC	ulcerative colitis
CD	Crohn's disease
Cleavage-PARP	Cleavage of poly-ADP ribose polymerase
CMS	Consensus molecular subtype

List of abbreviation

CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CRE	cAMP response element
CREB	cAMP response element binding protein
Cyclin	Cell cycle regulator
DAPI	4,6-diamidino-2-phenylindole (DAPI)
DCC	Deleted in colorectal carcinoma
DMEM (1X)	Dulbecco's modified eagle medium (DMEM (1X) **+**Glutamine MAX)
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptors belonging to the family of ErbB tyrosine kinase receptors
HGFR	Hepatocyte growth factor receptor
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion/activating molecule
Erk	Extracellular signal-regulated kinase
EU	European Union
FCS	Foetal calf serum
FOBT	Faecal occult blood testing
GDP	Guanine diphosphate
GTP	Guanine triphosphate
HNPCC	Hereditary nonpolyposis colon cancer
HPV	human papilloma virus
IL	Interleukin
JNK	c-Jun N-terminal kinase pathway
KDa	Kilodalton

List of abbreviation

***K-Ras* gene**	Kirsten's rat sarcoma viral oncogene homolog
MAPKAP-K1 1; also known as p90rsk	Isoforms of mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1)
Mcl-1	Myeloid cell leukemia 1
MDa	Mega dalton
Mek	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
MiR	MicroRNA
MMPs	Matrix metalloproteinases.
MMR gene	MLH1, MSH2, MSH6 or PMS2
mRNA	Messenger RNA
MCC	Mutated colorectal cancer
MSCs	Metastatic stem cells
MSH2	Monoallelic methylation
MSI	Microsatellite instability
Mt.	Mutant-type
MTT assay	Microculture tetrazolium assay
NE	Nuclear envelope
NF-κB	Nuclear factor (NF)-κB
NOXA	PMAIP1
NPC	Nuclear protein complex
Nups	Nucleoporins
P	*P Value* (*Student –t* test)
p53	Protein acting as tumour suppressor
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline

List of abbreviation

PCR	Polymerase chain reaction
Phospho-Bcl-2	Phospho-B-cell lymphocytic-leukemia proto-oncogene 2
PI3K	phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
pp90RSK	Ribosomal protein S6 kinase
PAH	polycyclic aromatic hydrocarbons
Pten	Phosphatase and tensin homologue
Puma	p53 upregulated modulator of apoptosis
Ran-BPs	Ran-binding proteins
Ran-GAP	Ran-GTPase-A-protein
Ran-GDP	Ran-Guanine diphosphate
Ran-GEF enzyme	Ran-Guanine nucleotide exchange factor
Ran-GTP-importin	Ran-Guanine triphosphate-importin
Ran-BP1	Ran-binding protein 1
Ras	Rat Sarcoma, an oncogene
Ras/Mek/Erk	Mitogen-activated protein/extracellular signal-regulated kinase. (Erk; Mek)] phosphoinositide 3-kinase (PI3K)/Akt/mTORC1
RCC	Regulator of chromosome condensation
RNAi	RNA interference
RPMI-1640	Roswell Park memorial institute -1640
S	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Ser-133	Serine 133 amino acid
shRNA	Small hairpin RNA
SiRNA	Small interfering RNA

List of abbreviation

TATI	Tumour-associated trypsin inhibitor
T/EDTA	Trypsin /ethylenediaminetetraacetic acid
Tcf4	T-cell factor
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor-β
TME	Tumour microenvironment
TORC	Transducer of regulated CREB activity coactivator
TPX-2	Targeting protein for Xklp2
Tris	Tris-(hydroxymethyl)-aminomethane
USA	United States of America
V	Volt
V/V	Volume per volume
VEGF	Vascular endothelial growth factor
W/V	Weight per volume
Wt.	Wild type
XTP	Xanthosine 5’-triphosphate
µL	Microliter
µM	Micromolar

Chapter 1
Introduction

1. Introduction

1.1 Cancer

Uncontrollable growth of cells in a specific part of the body under a particular condition is called cancer. Cancer is a result of a multistep mechanism called carcinogenesis (Nguyen and Duong 2018), where a single cell multiplies and accumulates changes and then passes survival advantages to neighboring cells (Lopez et al. 2020). There are different characteristics between cancer cells and healthy cells as they do not need growth signals to stimulate proliferation, they resist cell death and develop migratory potential (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011; Nguyen and Duong 2018).

Cancerous cells invade the surrounding healthy tissue and do not respond to checkpoint control mechanisms (Barrueto et al. 2020). The leading cause of carcinogenesis is environmental carcinogens, including different chemicals, viral (Sher et al. 2020) and radiation. Chemical carcinogens include genotoxic and non-genotoxic carcinogens. Genotoxic carcinogens damage the deoxyribonucleic acid (DNA) by direct interaction or after metabolic activation. These include polycyclic aromatic hydrocarbons (PAH), amines and free radicals such as reactive oxygen species. Non-genotoxic carcinogens do not react directly with DNA. Hormones, esters, asbestos are common non-genotoxic carcinogens and initiators of cancer (Tannock 1970; Weisburger and Williams 2020). Viral causes of cancer include the human papilloma virus (HPV) (Haghshenas et al. 2013). All cancers share a frequent fundamental basis; they grow and divide abnormally, in the end growing large enough to hinder the normal functioning of the organs and tissues due to physical compression or direct invasion (Hernández et al. 2009). Tumours can also undergo metastasis; a process in which the disease is transferred to another location. Metastasis deposits can block vital organ function, e.g., liver, bone marrow and lung tissue (Obenauf and Massagué 2015). In either case, the subsequent progression of the disease will lead to the death of the host (Tannock 1970; Roberts 1984; Videnros 2019). In cancer cells, the genes that are responsible for controlling differentiation and cell growth are mutated and the cell left uncontrolled; in contrast to the healthy cells, the cell growth and differentiation are completely under control (Croce 2008; Kasi et al. 2020). The mutated genes that are found in cancer patients are divided into

two broad categories: Oncogenes and tumour suppressor genes (Kasi et al. 2020).

1.1.1 Cancer hallmarks

Douglas Hanahan and Robert Weinberg proposed that cancer can be summarized by six fundamental common traits (Hanahan and Weinberg 2011; Santibáñez-Andrade et al. 2020). These behaviors or shared features include (I) self-sufficiency in growth signals (cancer cells excite own growth), (II) unresponsiveness to anti-growth signals, (III) evasion of apoptosis (they attack their programmed cell death), (IV) uncontrolled replicative potential (they can reproduce forever), (V) angiogenesis (they excite the growth of new vasculature to provide tumours with essential nourishment), (VI) tissue invasion and metastasis. The hallmarks can be developed at several time points through different mechanisms in many cancer types, but they can eventually lead cancer cells to start proliferation, survival, to then invade and form colonies in other tissues (Mathonnet et al. 2014; Bakir et al. 2020). They comprise “enabling” hallmarks that were published in an update paper (Hanahan and Weinberg 2011) which are compulsory to allow the gaining of all the earlier stated features resulting from the genomic instability and the pro-inflammatory state of cancer cells. Also, two emerging hallmarks include evading the immune response and reprogramming of metabolism to supply energy to sustain the cancer cells to endless growth and survival (Figure 1.1).

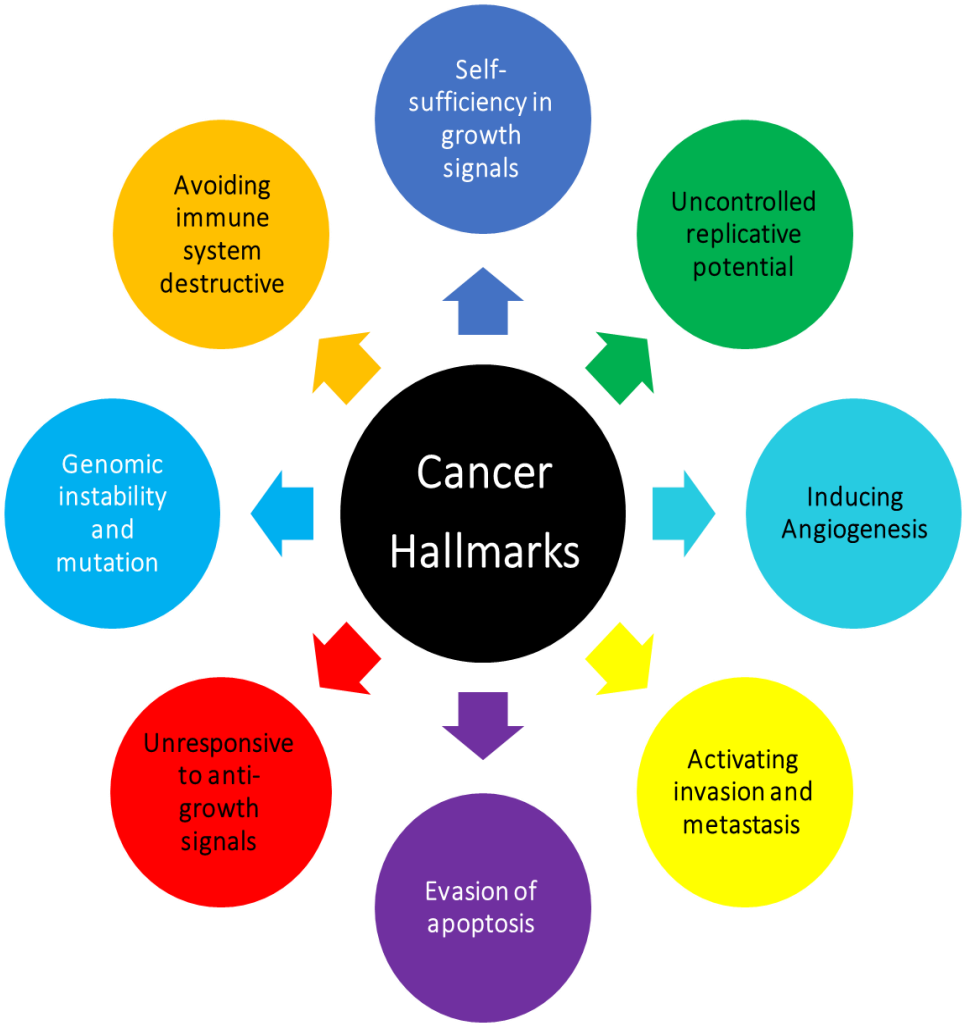

Figure 1.1: The cancer hallmark.

This figure exhibits the hallmark structures developed by most cancers. These various features contribute to the pathogenesis of cancer such as avoiding the immune system, leading to ignore anti-tumours pathway of tumour suppressive genes, then activating cell proliferative and activated invasionand finally encouraging metastasis. photo is adapted from (El-Tanani et al. 2016).

1.1.2 Oncogenes

An oncogene is a gene that gains oncogenic or transforming potential as a consequence of genetic changes in either regulatory sequences or coding region (Song et al. 2012; Martínez-Jiménez et al. 2020). Healthy cells experience apoptosis (a programmed form of death), after accomplishing a certain age and reaching a particular stage of division (Kasi et al. 2020). They do not have a valuable function for the organism anymore. In contrast, when oncogenes are activated, cells avoid a programmed death signal and continue to proliferate (Figure 1.2). Several oncogenes have been identified in human cancers such as Ras proteins (Boccaccio and Comoglio 2013; Orgován and Keserű 2020), vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR) and Src-family proteins (Boccaccio and Comoglio 2013). There are many drugs for cancer therapy which impact oncoproteins that are proteins encoded by oncogenes. These drugs include perylene compounds which inhibit the c-Myc (Chen et al. 2014).

1.1.3 Tumour suppressor genes

Tumour suppressor genes are genes that regularly protect the cells against cancer (Kasi et al. 2020). Growth-inhibitory proteins encoded by these genes prevent healthy cells from becoming cancer cells. Mutations in tumour suppressor genes (Figure 1.2), cause cancer due to defects in the response of cells to carcinogens (Vidotto et al. 2020). Tumour-suppressor genes produce proteins which regulate the cell cycle, provide a programmed cell death and DNA injury repair (Lubecka-Pietruszewska et al. 2013). Examples of tumour suppressor genes include *Pten* (Phosphatase and tensin homolog), p*53* (protein 53 or tumour suppressor protein 53) (Moxley and Reisman 2020), *BRCA2* (Breast cancer susceptibility gene 2), *BRCA1* (Breast cancer susceptibility gene 1) (Hatano et al. 2020), *Apc* (Adenomatous polyposis coli) (Kariv et al. 2020) and *CD95* (Cluster of differentiation 95) (Song et al. 2012). Suppressors have more clinical importance in human carcinogenesis. For example, tumour suppressor *p53* is defined as the protector of the genome (Lane 1992; Moxley and Reisman 2020), because it defends the cells by regulating the main cellular functions such as the cell cycle, apoptosis (Abbas and Larisch 2020), differentiation and DNA repair

Cells expressing mutated or non-functional *p53* do not respond properly to stress signals and finally become cancerous (Muller and Vousden 2014). *Pten* regulates the PI3K/Akt and Ras/MAPK/AP-1 pathways (Jia et al. 2016), whereas *Apc* controls Wnt-1/beta-catenin cascade pathway (Lubecka-Pietruszewska et al. 2013; Nguyen and Duong 2018; Kasi et al. 2020).

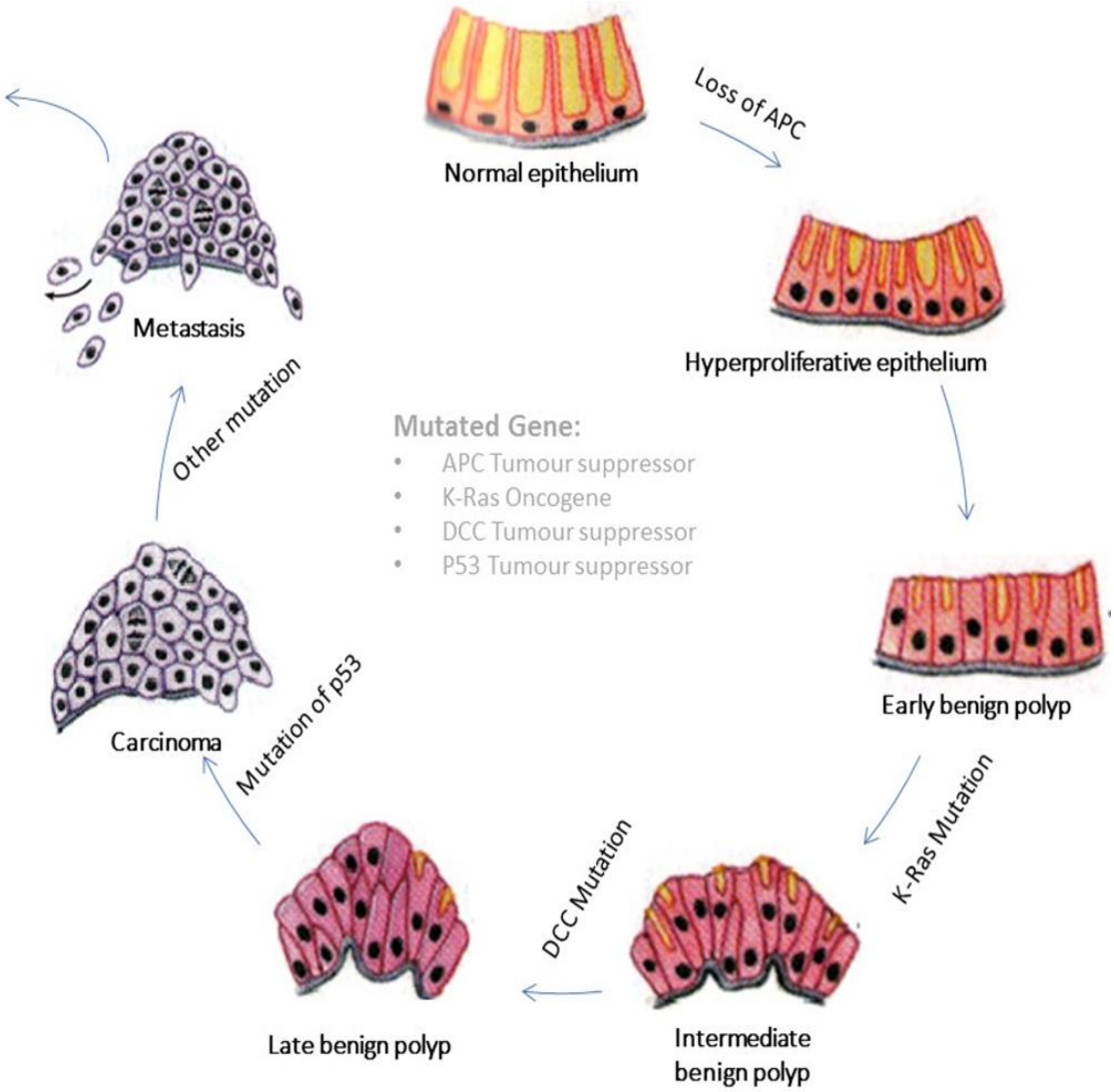

Figure 1.2: Illustration of multistep of cancer formation.

Somatic mutations: The first mutation occurs early in the adenoma polyposis coli (*Apc*) gene which is considered as the gatekeeper gene. This mutation permits the cell to proliferate and decreases intercellular contact through beta-catenin. Consequently, this permits familiar adenoma polyposis (FAP) (cf HNPCC: caretaker DNA repair genes defective). Then a second mutation in oncogene such as *K-Ras* which represent 50% of what in CRC (Liu et al. 2011). The third mutation or loss of a tumor suppressor gene such as p53 which represent 80% in CRC (Nakayama and Oshima 2019). Furthermore, the mutation that will happen is deleted colorectal carcinoma (DCC) as well-known as (cell adhesion

molecule). It has been estimated that 90% initiate from an adenoma (adapted from (Goyal et al. 2016).

1.2 Cancer history

It is a common misconception that cancer is a modern-day disease. It may be more prevalent today, but cancer was a recognised disease in ancient times. The word cancer comes from the ancient Greek word for crab (Kaur et al. 2020). During the middle ages, references to cancer families or cancer villages suggested that it might not be a randomly occurring phenomenon (Merrill 1978). In 1556, Gregorius Agrcola published his observation of a disease known as mala metallorum, a bronchogenic lung carcinoma plaguing miners in the Black Forest (Whysner 2020). In medical history, cancer appears in early 1600 BC in the Edwin Smith papyrus, where the oldest description of the illness exists. However, the origin of the word “cancer” is credited to the Hippocratic physicians, who used the terms karkinos and karkinoma in order to describe tumours (Kaur et al. 2020). Karkinos was used for any nonhealing swelling or ulcerous formation, even hemorrhoids, whereas karkinoma was reserved for non-healing cancer (Papavramidou et al. 2010; Villagomez et al. 2020). In 1700, Ramazzini recorded a high incidence of breast cancer among Italian nuns. Both these observations suggested an environment or lifestyle cause as being responsible. However, a direct causative link between an environmental factor and cancer was not made until in 1775, Percivall Pott discovered cancer of the scrotum (Poirier 2016). A rare form of cancer was commonly found among young British chimney sweepers (Benmoussa et al. 2019). He demonstrated that the soot, collecting on the skin due to poor hygiene standards, was the agent responsible although now we know it to be the tar constituent (Benmoussa et al. 2019). Pott also found that exposure to the carcinogen may also result in cancer many years later (Poirier 2016), indicating that cancer may have a latency period (Merrill 1978; Falzone et al. 2016).

From the middle of the 19th century onwards, cancers progression comes late in life so that l life expectancy increased. Relatively few people survived to develop cancer. Nowadays, the key causes of premature death and disease have been eliminated so the proportion of older people at risk has increased dramatically.

In the present day, cancers signify one of the principal causes of mortality, following ischaemic heart diseases (Laukkanen et al. 2020). It is thought that in

the next ten years, cancer will overtake heart disease and become the most common cause of death in the UK mortality statistics (Laukkanen et al. 2020). One reason that makes cancer so fatal is that it is a very complicated disease governed by a plethora of genetically determined factors. Treatment is additionally problematic as healthy cells and cancer cells are not dissimilar and acceptable therapeutics that can differentially target and destroy cancer cells without causing harm to the host remains challenging.

1.3 Colorectal cancer

Cancers of colon and rectum together are stated as colorectal cancer (CRC) (Kasi et al. 2020). Cancers arise from the epithelial lining of the colon or the rectum. The large intestines comprise the last part of the digestive tract and measure one and half meters in an adult human being. The key functions of the large intestines are to absorb H_2O and vitamins from the food prior to excreting the waste as stool (Tomasetti and Vogelstein 2015).

At the global level, CRC is the third most prevalent and the fourth deadliest malignancy (Chisanga et al. 2015). CRC is a potentially lethal disease in the UK. When cells proliferate excessively and unregulated by control mechanisms, they give rise to cancer. In addition to forming a tumour at the primary site, the cancer cells can disseminate to other organs and tissues, forming secondary tumours (Li and Wang 2020). During oncogenesis (Hanahan and Weinberg 2011), specific properties are known as the ‘hallmarks of cancer’ are sequentially acquired by normal cells leading to their transformation to malignant cells. These include avoidance of growth suppression and apoptosis and loss of their dependence on mitogenic stimulation and angiogenesis (Mathonnet et al. 2014; Santibáñez-Andrade et al. 2020).

CRC tumorigenesis in humans is associated with multiple genetic alterations, including activation of the *K-Ras* proto-oncogene (Nguyen and Duong 2018) and inactivation of the tumour suppressor genes *p53* (Duffy et al. 2020). Deleted in colorectal cancer (DCC), the mutated colorectal cancer (MCC) gene, and *Apc* (Nguyen and Duong 2018).

CRC is a multi-factorial process, and the formation is a multistage process. CRC development includes an alternative pathway from the normal epithelium to adenomas, and then a gradual lesion size increase and invasiveness that

ultimately affect the development of cancer (Testa et al. 2018). Molecular processes of adenoma to carcinoma are initiated from DNA repair errors, genetic instability, deviations, and mutations that induce the suppression of inhibitory factors (e.g. *Apc, p53,* TGF-$^{\alpha}$) or activation of proto-oncogenes, including *Ras, B-Raf* and *Pten.* Genetic processes are also attributed to the effect on tumour formation (Copija et al. 2016).

1.3.1 Isogenic human cancer cell lines

In vitro modelling of the genetics of a particular group of patients can be effectively undertaken through purposefully designed cells known as isogenic human disease models. To enable investigation of disease biology and new treatment agents, these cells create an isogenic system alongside a genetically compatible 'normal cell' (Vizeacoumar et al. 2013; DeWeirdt et al. 2020).

The unavailability of suitable control cells has hindered cell-based screening for new pharmaceutical agents with tumour specificity. Application of targeted homologous recombination for deletion of important tumorigenic genes was the basis for the proposed approach to drug screening involving the use of isogenic human cancer cell lines. The test case entailed insertion of a yellow fluorescent protein (YFP) expression vector and a blue fluorescent protein (BFP) expression vector in the colon cancer cell line DLD-1 and an isogenic derivative with deletion of the mutant *K-Ras* allele, respectively. Compounds that were selectively toxic towards the mutant Ras genotype could be easily screened by culturing the two cell lines together (Torrance et al. 2003; Verissimo et al. 2016).

The concept has also led to the development of a technique for detecting whether a test compound has targeted action on a Ras gene, Ras protein or downstream gene or protein in its pathway. A test compound is used to contact first and second cells of an isogenic nature apart from their Ras gene and a fluorescent protein-encoding gene (Torrance et al. 2003; Mason et al. 2004). The first cell has the Ras genotype c-Ki-Ras'Y' and additionally includes a first gene that encodes a first fluorescent protein with a first absorption spectrum and first emission spectrum. Similarly, the second cell has the Ras genotype c-Ki-Ras and additionally includes a second gene that encodes a second fluorescent protein with a second absorption spectrum and second emission spectrum. If the first cell exhibits a growth rate different from that of the second cell, then a test compound

is confirmed to have targeted action on a Ras gene, Ras protein or downstream gene or protein in its pathway (Torrance et al. 2003; Verissimo et al. 2016). Around half of all colorectal tumours affecting humans exhibit point mutations triggering the *K-Ras* proto-oncogene. In order to determine how functionally important such mutations were, homologous recombination was performed to disrupt the activated *K-Ras* gene in the cell lines of human colon carcinoma DLD-1 and HCT-116. Morphological changes and loss of the ability for anchorage independent growth were exhibited by the cells suffering disruption at the activated *K-Ras* gene in contrast to parent cells (Torrance et al. 2003; Bentley et al. 2013a). As predicted, deleting the Mt. allele of *K-Ras* eradicated tumour growth, however deletion of the remaining Wt. copy of *K-Ras* expanded the tumorigenic properties of these type of cell (Bentley et al. 2013b)

1.4 Epidemiology of colorectal cancer

1.4.1 Incidence and mortality

CRC in the UK (White et al. 2018) regarded as the third most common type of cancer in men after prostate and pulmonary cancer and women after breast and pulmonary cancer. It is the second most common cause of cancer death and costs around £1.6bn (Logan et al. 2012; White et al. 2018; Almasaudi et al. 2019). CRC develops more in men, with age-standardised rates (ASRs) at 86.1/100,000 men versus to 56.9/100,000 women in the United Kingdom in 2014 (which produces 22,844 cases in men and 18,421 cases in women every year).

CRC death rates are similarly greater in males (ASRs of 33.9/100,000 men compared to 21.8/100,000 women). Death rates are substantially greater for men than for women in all age groups from 45 to 49 and over, and the difference is broadest at the ages of 70–74, while the men: women age-specific death rate ratio is about 1.7:1. Furthermore, there is an international tendency for men to have equally higher prevalence (746,298 vs 614,304 cases [20.6 vs14.3 ASR]) and deaths (373,639 vs 320,294 [10 vs 6.9 ASR]) for CRC (White et al. 2018).

In 1979-1999, CRC rates increased by an average of 1 per cent per year on average although there has been a slight decrease since then (Logan et al. 2012). At the same time, the proportion of cases in women has altered slightly. On the other hand, in the UK, from 1997 to 2006, the incidence of CRC deaths decreased

by 17%. This reduction in mortality has appeared in all age groups with a significant decline in men between the ages of 40 to 69 years and in women between the ages of 55 to 79 years (Parkin 2008). Despite the gradual improvement in treatment over the past 30 years, the 5-year survival rate in the UK is still only 50%, with less survival in similar countries (Coleman et al. 2011; Morris et al. 2012)**.**

CRC has the third highest mortality rate in the United States. The number of CRC diagnoses in 2016 was 134,490, with more men developing it than women (70,820 vs 63,670). Of these, 26,020 men and 23,170 women died of the disease. In both men and women, CRC accounts for 8% of the total number of new cancer cases, being the third most prevalent after prostate and lung cancer in men and after breast and lung cancer in women. Likewise, in 2016, CRC had the third highest mortality rate after lung and prostate cancer in men, and after lung and breast cancer in women in the US, accounting for 8% of the overall number of deaths due to cancer for both men and women (Marley and Nan 2016). Estimates for 2013 indicated that 1,177,556 US residents were living with CRC, which is thus a disease that is highly burdensome on the American population (Valastyan and Weinberg 2011).

Despite the above figures, there has been a reduction in the CRC burden in the US in recent times. According to national statistics, incidence and mortality rates have both decreased, and there has been a gradual improvement in 5-year survival rates as well. Overall, there has been a decline in age-adjusted incidence rates from the low-to-mid 60s per 100,000 during the 1970s and 1980s to 37.20 new cases per 100,000 in 2013 (DeSantis et al. 2016). Furthermore, the 5-year survival rate increased by 16.4%, from 49.8% to 66.2% during the period 1975-2011.

The attenuation of the CRC burden since the 1970s is well-illustrated in the US public federal databases. However, on a broader geographical scale, data reveal significant discrepancies in this trend, especially in Eastern countries. North America, Europe and Australia/ New Zealand continue (Shadmani et al. 2017) to be the regions with the highest incidence rates of CRC, but there has begun to be a growing risk in other countries that had previously had low rates (Shadmani et al. 2017). For instance, CRC incidence is fast increasing in Japan (Hashiguchi et al. 2020) and Thailand (Onyoh et al. 2019), while in Iran, it has been increasing

steadily over the last three decades,where CRC in Iran has represented the fourth greatest type of cancer that was the third and the fifth most common in females and males respectively. The occurrence of the illness has experienced the same upsurge in persons of other Asian countries, with CRC representing 8.4% of all types of cancers in Iran (Shadmani et al. 2017). In Saudi Arabia, there has been a more than two-fold increase in rate since 1994 (Torre et al. 2015), and around that time, the Philippines also began to experience a rise in mortality rates (Arnold et al. 2017; Siegel et al. 2017) Similarly, in other Eastern countries, such as Jordan (Dolatkhah et al. 2015), China, South Korea and Singapore and all areas where stomach and liver cancers have usually constituted more significant burdens (Ibrahim et al. 2008; Onyoh et al. 2019), the incidence rates of CRC have also been gradually increasing (Fidler et al. 2017). The cases of this illness, though, has been on the rise in Asia as well, particularly in China, Japan, South Korea, Singapore, and Taiwan with a 2-4-fold rise in prevalence throughout the past few years. South Korea showed the greatest occurrence (Onyoh et al. 2019). The average age when CRC is diagnosed also reflects discrepancies in CRC incidence between east and west. While CRC is diagnosed in 2-8% of people younger than 40 years of age in the US and EU (Al-Jaberi et al. 1997; Kaw et al. 2002), it is diagnosed in 38% of this age group in Egypt, 21% in Saudi Arabia, 17% in the Philippines, and 15-35% in Iran (Sung et al. 2005; Khuhaprema and Srivatanakul 2008; Stigliano et al. 2014). Since 1996 to 2012, CRC occurrence in Oman has risen by 282% in women and 386% in men (Al Bahrani et al. 2021). From 1989–2013, CRC prevalence rates in adults age 50 - 54 years progressed from half of that in adults age 55 - 59 equal to (24.7 vs 24.5 per 100 000 people and the percentage of CRC identified in adults younger than age 55 years has risen from 14.6% to 29.2% (Siegel et al. 2017).

1.4.2 Genetic predisposition

Human CRC aetiology (Jones et al. 2018) can be divided into three causes: the first type is inherited (Basso et al. 2017), which divided further into two types: hereditary non-polyposis CRC (Basso et al. 2017) (Lynch Syndrome/HNPCC) - caused by genetic instability, and familial adenomatous polyposis coli (FAP) caused by a mutation in the adenomatous polyposis coli gene *Apc* (Kasi et al. 2020). The second type is inflammatory, which is divided into ulcerative colitis

(UC) and Crohn's disease (CD). The third type is sporadic, accounting for 80% of human CRC but the aetiology of this type is poorly defined (Kraus and Arber 2009; Dwyer et al. 2019)**.** Most CRC cases are not familial but sporadic (De la Chapelle 2004; Rosa et al. 2016).

1.4.2.1 Hereditary syndromes

Approximately 5-7% of cases are due to the fact that known genes undergo high penetrance germline mutations (Stoffel et al. 2018). The mutations of greatest relevance are discussed below.

1.4.2.2 Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC)

CRC develops because of the autosomal dominant syndrome HNPCC. In general, families that receive a Lynch syndrome diagnosis present a germline mutation (Perrott et al. 2020) in an allele of a mismatch repair (MMR) gene, such as MLH1, MSH2, MSH6 or PMS2 (Ligtenberg et al. 2009b; Ligtenberg et al. 2009a; Haanstra et al. 2013; Connell et al. 2017) MSH2 monoallelic methylation may be triggered by the epithelial cell adhesion/activating molecule EpCAM mutations. From a perspective of phenotype, these mutations cause early right-sided CRC diagnosed on average at 48 years of age (Lynch and De la Chapelle 2003; Hendriks et al. 2006; Koornstra et al. 2009; Engel et al. 2020).

1.4.2.3 Familial adenomatous polyposis (FAP)

CRC develops due to FAP in less than 1% of patients, which in turn arises from germline mutations in the adenomatous polyposis coli (*Apc*) gene (Kasi et al. 2020). The implications for phenotype differ depending on where in the *Apc* gene the mutations occur. In 90% of cases, patients exhibit more than one polyp in the colon and develop CRC by 45 years of age (Galiatsatos and Foulkes 2006; Perrott et al. 2020).

1.4.2.4 MUTYH-associated polyposis

The base excision repair system repairs mutations caused by oxidative DNA damage (Kasi et al. 2020). Biallelic germline mutations in the base excision repair gene *mutY* homolog (*MUTYH*) are the reason why individuals suffering from this condition imitate the FAP phenotype (Dunlop and Farrington 2009; Perrott et al. 2020).

1.5 The biology of CRC tumour progression

The transformation of normal colorectal mucosa into adenoma and CRC has been extensively studied and is known to involve some different pathways. These include changes in Wingless-related integration site (Wnt), the Wnt pathway through *Apc* loss and nuclear β-catenin accumulation, mutations in the *K-Ras*, *B-Raf*, and *PI3K* pathway genes, 18q chromosome loss, SMAD4, *TP*53 inactivation and *Pten* deletion (Nguyen and Duong 2018; Kasi et al. 2020).

1.5.1 *Apc* gene and Wnt signaling pathway

Apc is a gene that inhibits tumour development. Somatic mutations in both alleles accompany a significant proportion of sporadic CRCs. FAP syndrome is due to one germline mutation in *Apc (*Kasi et al. 2020). In the development of CRC, the loss of *Apc* is one of the first events to occur (Nguyen and Duong 2018). The regeneration of intestinal epithelium depends greatly on the Wnt pathway, which represents an evolutionarily conserved signal transduction pathway (Bienz and Clevers 2000; Ghosh et al. 2019). Disruption of the free β-catenin is the primary role of *Apc* as a tumour inhibitor, which is why nuclear β-catenin accumulates when *Apc* is lost. The Wnt pathway is activated (e.g., via T-cell factor Tcf4 activation) and the proliferating crypt compartment in the colon mucosa becomes enlarged because of free β-catenin derived from *Apc* loss or β-catenin mutations that stabilise it and prevent its degradation (Fodde 2002; Henderson and Fagotto 2002; Jiang et al. 2015).

1.5.2 Epidermal growth factor receptor (EGFR)

A transmembrane glycoprotein, EGFR belongs to the family of ErbB tyrosine kinase receptors (Kasi et al. 2020). Tyrosine autophosphorylation occurs when the ligand and receptor interact, and the receptor dimerises, triggering the activation of intracellular signal pathways, such as the PI3K pathway, Ras-MAPK pathway, Janus kinase pathway and signal transducer and activator of transcription (STAT) pathway that encourages cancer cells to divide and migrate, hinders apoptosis, and promotes angiogenesis (Mendelsohn and Baselga 2003; Kasi et al. 2020).

No consensus exists about EGFR expression and CRC prognosis. Therapy of Dukes D or recurrent CRC targets EGFR. Tyrosine-kinase activation and

downstream signalling pathways are deactivated by the monoclonal antibodies cetuximab and panitumumab by binding to EGFR (Bentley et al. 2013a; Neitzel et al. 2020). Therefore, current CRC treatment often involves administration of these antibodies (Folprecht et al. 2010; Van Cutsem et al. 2011). Carcinogenesis and resistance to targeted treatment may arise if mutations occur in genes that encode molecules in an EGFR pathway.

A single clinically applicable test exists, namely, *K-Ras* mutation status and therapies targeting EGFR are restricted to *K-Ras* Wt. tumours. The intracellular domain is usually activated when EGFR is activated, triggering the *K-Ras* signalling cascade. Activated *K-Ras* accumulates and targeted anti-EGFR treatment loses efficiency when GTPase activity is disabled by mutations (Bokemeyer et al. 2012).

1.5.3 Transforming growth factor-β (TGF-β)

TGF-β signalling pathway is involved in regulating the proliferation, migration, differentiation and apoptosis of CRC cells (Zhao et al. 2020). This pathway primarily consists of three TGF-β isoforms (TGF-β1-3 is the TGF-β1 isoform expressed most frequently), the TGF-β receptors TGF-β R1 and TGF-β R2 (Tosi et al. 2018), and the downstream transcription factors (small mothers against decapentaplegic homolog 2, 3 and 4), SMAD2, SMAD3 and SMAD4 (Blobe et al. 2000; Moez et al. 2019). When the pathway becomes activated, the SMAD factors move to the nucleus where they undertake the regulation of a number of TGF-β-responsive genes, some of which serve as key cell cycle checkpoint genes (Kasi et al. 2020). TGF-β inhibits tumour development in normal epithelium by inhibited cell proliferation, but when cells acquire resistance to proliferative suppression mediated by TGF-β, as in established tumours, TGF-β intensifies angiogenesis and disturbance of extracellular matrix and suppresses infiltrating tumour immune cells, thus encouraging metastasis (Oft et al. 1998; Blobe et al. 2000; Massagué 2008; Lodyga and Hinz 2020). SMAD7 plays the role of a downstream suppressor of TGF-β. Apart from SMADs, additional pathways can also be activated by ligands associated with TGF-β, including the MAPK pathway, JNK pathway, and phosphatidylinositol 3-kinases/Akt (PI3K-Akt) pathway (Elliott and Blobe 2005; Lodyga and Hinz 2020).

Inhibition role of TGF-β in highly metastatic colon carcinoma and TGF-β R signalling in Ha-Ras-transformed mammary epithelial cells (EpRas cells) was performed by a dominant-negative type II TGF-βR (TGF-βRII-dn) that show the phenotype of mesenchymal-like spindle and has the ability to inhibit to resistance to TGF-β-mediated growth. Epithelial EpRas cells retarded tumour formation through TGF-βRII-dn as well as preventing mesenchymal-epithelial transition (MET) *in vivo* but induced MET or epithelial- mesenchymal transition (EMT) in the mesenchymal colon carcinoma cells and *in vitro* eradicated invasiveness (Oft et al. 1998). EMT was prevented in epithelial cells leading to EMT in mesenchymal spindle tumour cells due to TGF-β signalling inhibition (Oft et al. 1998; Jonckheere et al. 2021).

1.5.4 RAS

The *Ras* oncogene has a range of variants (i.e., *H-Ras, K-Ras, N-Ras*) (Lim and Leprivier 2019) that encode the GTPase Ras proteins (Porru et al. 2018). Various signalling pathways that regulate cell processes, including proliferation, differentiation, migration, survival and apoptosis, are controlled by the Ras proteins (Giehl 2005; Lim and Leprivier 2019). Raf or Ras kinases and phosphatidylinositol 3-kinases are the most common Ras GTPase effectors. Approximately half of sporadic CRC cases manifest Ras mutations (Miranda et al. 2006; Kasi et al. 2020). CRC invasion and metastasis are believed to involve *K-Ras* mutations (Porru et al. 2018). The occurrence of *Ras* mutations hinders monoclonal antibodies from responding to epidermal growth factor receptors (EGFRs) (Karapetis et al. 2008; Bentley et al. 2013a; Neitzel et al. 2020), such as cetuximab and panitumumab, thus they are clinically important (Neitzel et al. 2020).

Transduction signals through the nucleus passing from the surface by the activation signalling that initiate from the front-line sensors by signalling molecules which controlled by *K-Ras* therefore affect cell growth, chemotaxis, differentiation and finally apoptosis. This subsequently affects cell adhesion, shape, and migration as outcomes of cytoskeleton changes (Esser et al. 1998; Zuber et al. 2000; Jančík et al. 2010).

Progression of the cell cycle which usually happens by wild-type *K-Ras* in addition to encouraging apoptosis, replicative senescence, and cell arrest. This might

happen by heat shock, some cytokines, cellular stress, ionising or ultraviolet radiation. The antagonist mechanism of growth arrest was the first line of defence in the case of *Ras* activation. It can be established that the Wt. *K-Ras* gene is working as a tumour suppressive and commonly nowhere to be found in several types of cancer progression (Chen et al. 2009a; László et al. 2021). Therefore, the oncogenic possessions were obtained when its Wt. *K-Ras* mutates then produces cancer development (McCoy et al. 1983; Kranenburg 2005; Kumagai et al. 2020). Alteration of the oncogenic *K-Ras* allele occurs when the wild type *K-Ras* allele is lost (László et al. 2021). Finally, the permanent activation in *K-Ras* stops GTP hydrolysis due to a mutation in the *K-Ras* gene.

K-Ras is an oncogene that is encoded by a small GTPase transductor protein (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) (László et al. 2021). *K-Ras* can pass on external signals to the cell nucleus, and thus takes part in regulating cell division. *K-Ras* can no longer shift between active and inactive modes if the *K-Ras* gene undergoes activating mutations; consequently, cells begin to transform and become unresponsive to chemotherapy and biological therapies targeting epidermal growth factor receptors (EGFR) (Jančík et al. 2010; László et al. 2021).

In tissues, *Pten* is a tumour suppressor gene (Song et al. 2012) which blocks cell proliferation and survival, so its loss leaves these processes uncontrolled. However, compared to heterozygous *Pten* loss, complete *Pten* loss (Abbas et al. 2020) is not as tumorigenic so that it could inhibit tumours (Kasi et al. 2020). Among tumour suppressor genes, *Pten* undergoes mutations particularly often, and it is inhibited or down-regulated when not undergoing mutation (Song et al. 2012).

1.6 CRC sub-typing

Cancer is a heterogeneous disease. This means that numerous types of cancers consist of subtypes of biological and clinical diversity. Prognosis and treatment response both depend on the subtype. A general molecular classification system for CRC was recently proposed by a multicentre CRC subtyping consortium, which allows CRC specimens to be homogeneously classified across various institutes and studies to aid basic and clinical research in the future (Kasi et al. 2020). Furthermore, the creation of therapies tailored to each subtype may be

achieved based on the biological consistency noted in every consensus molecular subtype (CMS). The CMSs of CRC are explored here, emphasising the impact of different variables, including origin, route of development, and microenvironmental regulation (Linnekamp et al. 2018).

Molecular classification divided CRCs into genomic/epigenomic abnormalities accumulating serially. Three pathogenetic pathways have been discovered in the development of these tumours such as chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) which give one framework for CRC classification (Kasi et al. 2020). However, due to the heterogeneity of the methods/markers and criteria used in CIN analysis, the use of CIN to classify CRCs is challenging (De Palma et al. 2019). The CIN pathway is associated with oncogenes such as *Apc*, *K-Ras*, DCC/SMAD4, and *TP53* and the consecutive deregulation of tumour suppressor genes (TSGs). It has also been associated widely with sporadic CRCs. But it mostly happens within inherited tumours, such as familial adenomatous polyposis (FAP), (Pancione et al. 2012; Kasi et al. 2020).

Microsatellite instability MSI is responsible for sporadic tumours and Lynch syndrome and is mostly produced through inactivation of the DNA mismatch repair genes such as (hMLH1, hMSH2, hMSH6, and hPMS2).CRCs exhibiting MSI tend to be right-sided, as well as histologically high graded, however a mucinous phenotype is diagnosed pathologically at lower stages than CIN cancers (Pancione et al. 2012).

The CpG island methylator phenotype (CIMP) arises due to extensive hypermethylation of CpG islands at several loci (Kasi et al. 2020). These pathways rarely overlap as, one type of molecular signature predominates. A complex interaction arises in some tumours although one pathway is a consequence of another (i.e., MSI and CIMP arise due to hMLH1 promoter hypermethylation), (Pancione et al. 2012).

Unlike CIN, a better definition of MSI and CIMP exists, and these two abnormalities permit classification of CRCs into four molecular subtypes. These are CIMP+/MSI- (5%–10%), CIMP-/MSI- (75%–80%), CIMP+/MSI+ (10%), and CIMP-/MSI+ (5%). The frequency of each of these subtypes represents an estimated value derived from CRCs from individuals in the US. A comparable distribution has also been established for a Korean population, namely,

CIMP+/MSI- (8%), CIMP-/MSI- (79%), CIMP+/MSI+ (5%), and CIMP-/MSI+ (8%) (Kim et al. 2009). However, American and Korean populations exhibit a significant difference in terms of the ratio of the CIMP+/MSI+ subtype to the CIMP2/MSI+ subtype (2:1 and 1:1.6-1:2.3, respectively) (Ogino and Goel 2008; Poynter et al. 2008).

1.7 CRC progression and metastasis mechanisms

CRC is a multi-factorial process, and the formation is a multistage process (Copija et al. 2016). CRC development includes an alternative pathway from the normal epithelium to adenomas, and then a gradual increase of the lesion size and invasiveness that ultimately bring about the development of cancer and are supported by the tumour microenvironment (Fearon and Vogelstein 1990; Peddareddigari et al. 2010; Cui 2020). Molecular processes are from adenoma to carcinoma (Cui 2020) initiated from DNA repair errors, genetic instability, deviations, and mutations that induce the suppression of inhibitory factors (e.g., *Apc, p53,* TGF-$^{\alpha}$) or activation of proto-oncogenes, including Ras*, B-Raf* and *Pten*. Genetic processes are also attributed to tumour formation (Copija et al. 2016).

The first mutation that leads to CRC onset is the inactivation of the *Apc* gene that activates the Wnt signaling pathway (Moez et al. 2019; László et al. 2021), followed by the accumulation of β-catenin into the nucleus (Hankey et al. 2018). The second step is the inactivation of the *p53* pathway that inhibits cell cycle arrest and allows the transition of adenomas into invasive carcinomas (Li et al. 2019). The third step is the inactivation of TGF-$^{\beta}$ (Biswas 2004; Morris et al. 2017). This anti-inflammatory cytokine exerts an anti-tumorigenic effect by promoting apoptosis and inhibiting cell proliferation and pro-tumorigenic cytokine expression. Epithelial cell mutations in this pathway can sustain tumour growth in the colon. Oncogenic mutations of Ras and *B-Raf* genes (László et al. 2021). Can lead to the activation of the mitogen-activated protein kinase (MAPK) signalling cascade (Blaj et al. 2017). That in turn induces cell proliferation, angiogenesis, cell motility and metastasis. The Ras mutations can lead to the production of a permanently activated protein with GTPase activity (Rajagopalan et al. 2002; Buscail et al. 2020). Genomic instability also plays a pivotal role in CRC. Chromosomal instability describes changes in the chromosome copy number and

structure or the loss of the wild-type copy of tumour suppressor genes, such as *Apc* and *TP53* (Mendelsohn et al. 2015).

A particular metastatic pattern and latency define every type of tumour. CRC first metastasises remotely in the liver (Figure 1.3), followed by the lung and bones (László et al. 2021). Significant light has been shed on metastasis in tumours such as breast cancer (Chiang and Massagué 2008; Fares et al. 2020), but this knowledge is not directly applicable to CRC because it metastasises differently.

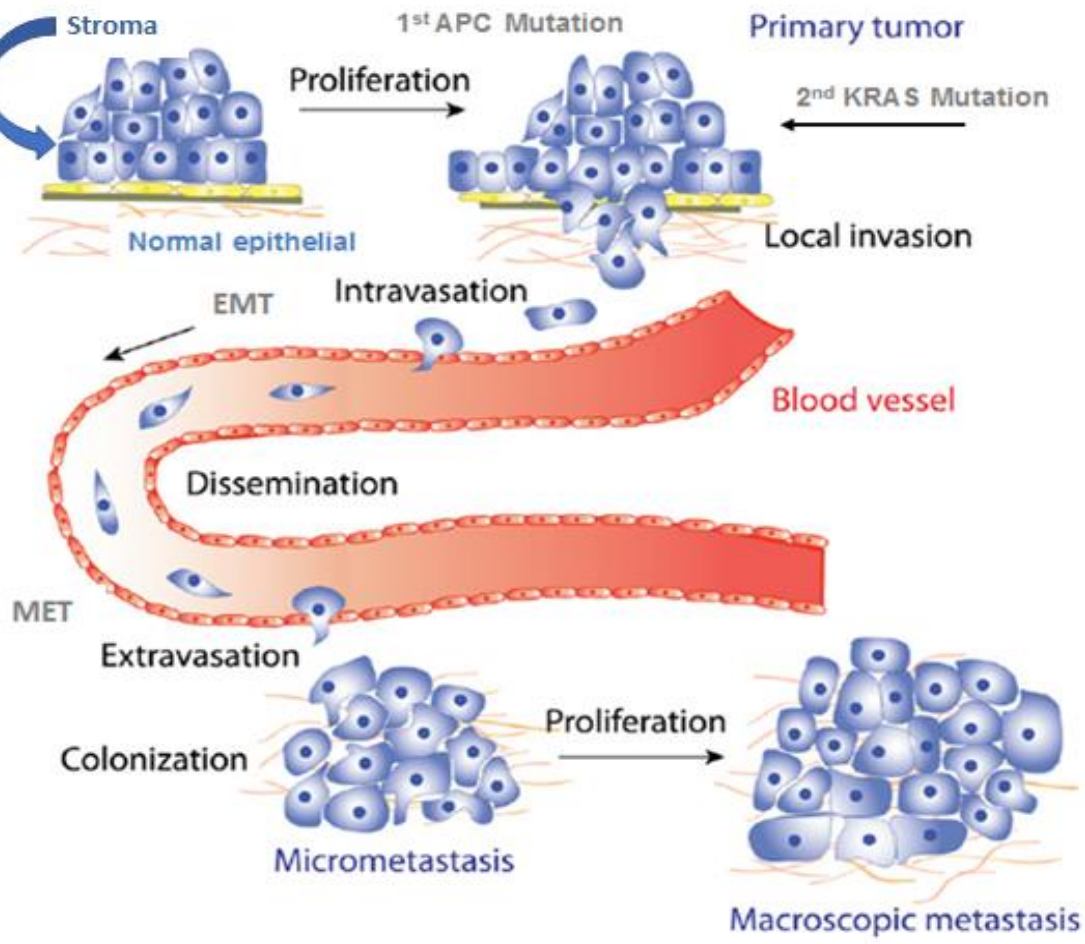

Figure 1.3: Simplified illustration of the different phases of cancer cell metastases.

The figure is adapted from (Chiang and Massagué 2008). Cancer cells detach from a primary tumour and intravasate into the bloodstream, after which the cells extravasate at a remote secondary site, where they initiate micrometastasis to colonise the organ/tissue and eventually become visible (Bakir et al. 2020).

Due to drainage of mesenteric circulation into the liver (László et al. 2021) (Figure 1.4), CRC metastasis may depend greatly on colon vascular drainage (Nguyen et al. 2009; Kuo et al. 2021). This facilitates CRC cell invasion of the hepatic capillary network. However, the formation of macroscopic metastases (Figure 1.3), requires metastatic cells to acquire extra function, besides relying on circulation. Cancer evolution is reflected in how metastasis develops, with metastasis facilitators gradually enhancing the likelihood of successful process completion (László et al. 2021).

The cancer cells require many steps to metastasis and each step can be considered as potential for new therapies. The normal epithelial cell stroma exposed to the first mutation in *Apc* start to proliferate, subsequently due to a second mutation in the *K-Ras* gene the primary tumor is formed (László et al. 2021). The cancer cells then detach from a primary tumour. To complete the metastasis, cancer cells must decrease adhesion to their neighboring cells to migrate into the vasculature-rich stroma (Cavallaro and Christofori 2004; Bakir et al. 2020). When they reach the vasculature, detached cancer cells can without restrictions go in the circulation to certain areas of the kidneys, liver, and bone marrow, if the vasculature is discontinuous. If the vasculature is continuous then intravasation is required; cells either cause endothelial cell retraction by liberating compounds such as vascular endothelial growth factor (VEGF) (László et al. 2021) or endothelial cell death by releasing reactive oxygen species and factors including matrix metalloproteinases (MMPs) (Lin et al. 2011; Fares et al. 2020). In the circulation, cancer cell spreading is determined through blood flow and interactions between cancer cells and the secondary organs that they colonize (Bakir et al. 2020). The cancer cell can express receptors that bind to metastasis-supporting sites when they are trapped in narrow vessel, such as that found in the lung and liver or to platelets, which plays the major role to spare the cancer cells from targeting by the immune system (Gay and Felding-Habermann 2011). Cancer cells can stay for more than 2 h, providing that they do not permanently become blocked into the first vessel beds at which they arrive (Schroeder et al. 2012). After reaching the secondary site, cancer cells can exit the bloodstream by encouraging endothelial cell refutation or death (Gupta et al. 2007; Naderi-Meshkin and Ahmadiankia 2018). To grow in the secondary organ, the cancer cells a the local environment by secretion of pro-inflammatory chemokines and

proteinases that encourage their adjacent cells to liberate growth factors (Psaila and Lyden 2009; Schroeder et al. 2012; Liu et al. 2017).

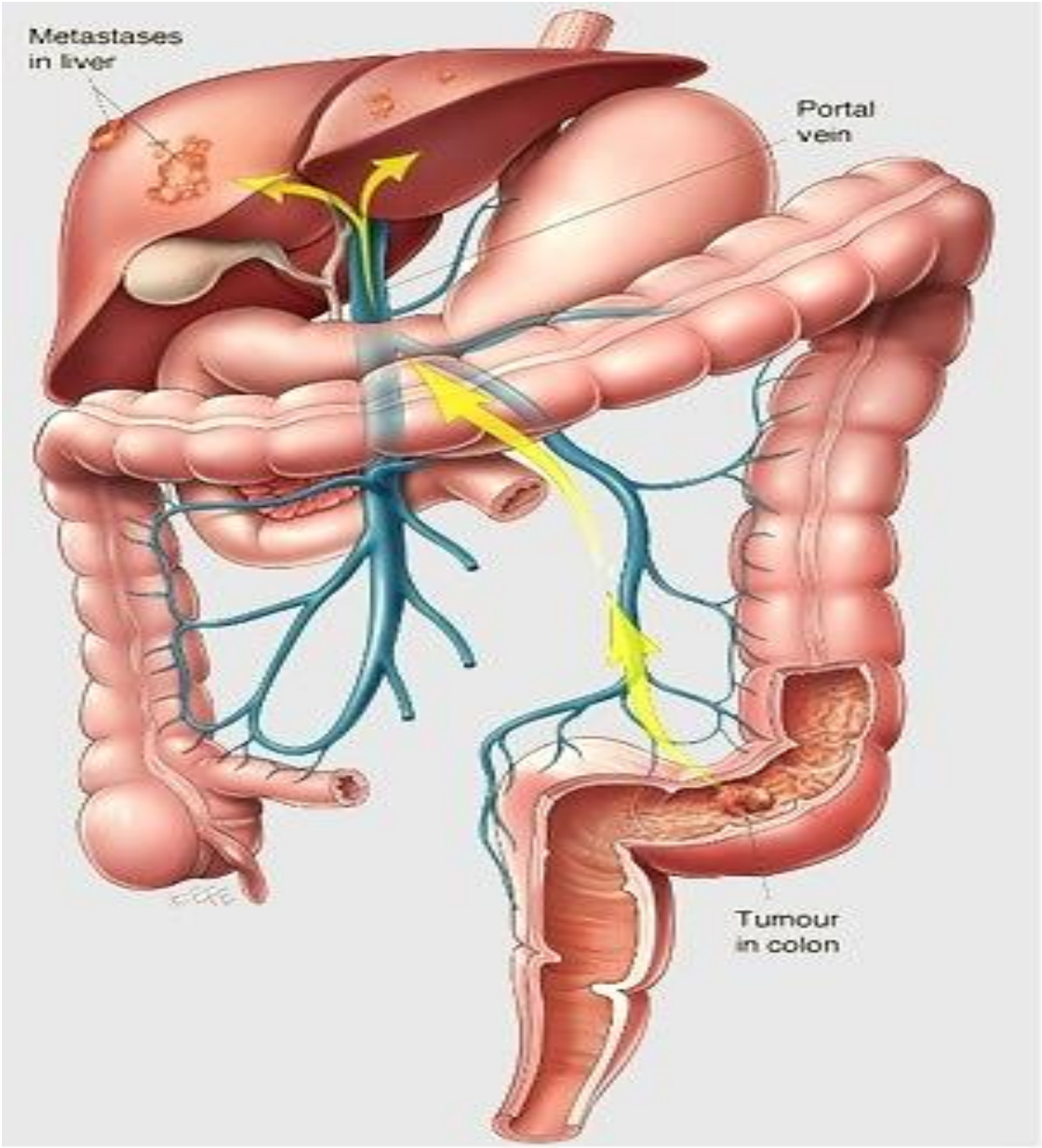

Figure 1.4: Diagram of CRC metastasis to the liver.

The figure adapted online from (Sirtex 2018).
Blood from the bowel flows directly to the liver through the superior, inferior mesenteric veins and the portal vein (which transport blood from the bowels back to the liver to retrieve nutrients). This means the liver becomes the first site to experience metastasis as it receives returned blood from the intestine (Sirtex 2018).

1.7.1 The tumour microenvironment (TME)

The tumour microenvironment (TME) represents a dynamic network that plays an important role in tumour initiation (Hamilton and Rath 2018; Fares et al. 2020), proliferation, growth, and metastasis (Bakir et al. 2020). This network is a highly dynamic system, the regulation of which depends on the interaction between tumour cells, stromal matrix and extracellular matrix (ECM) (Colangelo et al. 2017).

1.7.2 Angiogenesis

Data show that the mutation in *K-Ras* activation has an essential role in angiogenesis (László et al. 2021) due to activated vascular endothelial growth factor (VEGF), a crucial mediator of angiogenesis (László et al. 2021). Oxygen and nutrient unavailability are a major stressor affecting tumour cells migrating to a remote organ. Hence, a change in the normal angiogenesis balance is necessary for them to survive (Fares et al. 2020). VEGF is a key angiogenesis factor (Melincovici et al. 2018; Fares et al. 2020). The angiogenic potential of invading cells is enhanced by certain genes related to higher metastatic capacity. For instance, breast cancer neo-angiogenesis is permitted by Epiregulin *EREG* gene (HGNC Symbol), cyclooxygenase-2 (COX-2) and MMP-1, contributing to vasculature restructuring (Gupta et al. 2007; Thakur et al. 2020). Furthermore, angiogenesis facilitators are activated by the hypoxia-inducible factor (HIF), which is stabilised by oxygen unavailability (Pouysségur et al. 2006; Hamblin 2018).

1.7.3 Stroma activation

To access the bloodstream and migrate to remote organs (Fares et al. 2020), tumour cells depend on stroma interaction (Figure 1.3) (Roussos et al. 2011). To survive, invade, self-regenerate, and migrate, they use signals issued by different types of stromal cells (Bakir et al. 2020).

In CRC, the TGF-β signalling pathway (Lin et al. 2018a) is genetically inactivated, leading to loss of cell sensitivity to TGF-β tumour-inhibiting action and TGF-β overexpression. Besides stimulating hepatic stromal fibroblasts to produce interleukin11, this cytokine also mediates metastasis (Calon et al. 2012). Furthermore, Wnt/β-catenin signalling is augmented by the hepatocyte growth

factor produced by stromal fibroblasts, enhancing the stem cell potential of CRC cells (Vermeulen et al. 2010; Moez et al. 2019).

1.7.4 Epithelial to mesenchymal transition (EMT)

Tumour cells may use various mechanisms to circulate in the blood, such as EMT (Hamilton and Rath 2018; Fares et al. 2020). Tumour invasion and metastasis depend significantly on the EMT mechanism, which causes loss of epithelial cell polarity and conversion into mesenchymal phenotypes (Figure 1.3), (Gonzalez-Villarreal et al. 2020). Mesenchymal structures are acquired by epithelial cells in primary tumours due to undergoing EMT (Hamilton and Rath 2018). They start to migrate and express matrix metalloproteinase and other proteases, thereby invading locally through the surrounding extracellular matrix (ECM) and stromal cell layers (Bakir et al. 2020). EMT-related epithelial cell conversion is triggered when specific cytokines reactivate cancer cell development (Ribatti et al. 2020). EMT depends on E-cadherin loss (Yang et al. 2008; Ribatti et al. 2020) and comprises the TGF-β signalling pathway, Wnt pathways, and several tyrosine kinase receptor pathways, including those initiated by fibroblast growth factor receptor, EGFR and platelet-derived growth factor receptor (Ribatti et al. 2020).
Ultimately, this process causes a sequence of changes to cell-biology starting with local invasion and then by intravasation the cells invade into nearby blood and lymphatic vessels (László et al. 2021) and then outflow of cancer cells from the nearby lumina facilitates their spread in the body. If they become trapped in a remote site, the cells undergo extravasation into the tissue or organ at that site. If this site provides suitable conditions, the cancer cells will initiate the development of small lumps of cancer cells called (micrometastasis) and eventually undergo proliferation to produce macroscopic lesions (Valastyan and Weinberg 2011), a process known as the invasion-metastasis cascade (Talmadge and Fidler 2010; Lambert et al. 2017; Luo et al. 2017).
EMT increases the stem cell potential of cancer cells and makes them motile and treatment-resistant (Mani et al. 2008; Ribatti et al. 2020), and is suppressed (Ocaña et al. 2012) by EMT-inducing transcriptional regulators. Metastasis typically exhibit epithelial rather than mesenchymal characteristics.
EMT is best understood as a temporary state enabling the spread of cancer cells, which undergo a MET once metastasis has occurred (Korpal et al. 2011).

1.7.5 Stemness

Cancer cells must have the ability of tumour growth to induce metastasis at a remote site. Cells possessing this ability are called metastatic stem cells (MSCs). If it is not already contained in a primary tumour, this stemness can be acquired via phenotypic plasticity after MSC detachment. The presence of MSCs in a primary tumour is indicates a poor prognosis and early distant relapse of primary tumours with an expression of a stem cell-like signature (Merlos-Suárez et al. 2011). Metastasis activation in animal models is caused by primary tumour cells based on stem cell markers (Pang et al. 2010). CRC metastasis is due to cells possessing stem-like features (Kim et al. 2021) such as the ability to self-regenerate in the long term, quiescence and chemotherapy unresponsiveness (Dieter et al. 2011; Kreso et al. 2013). Cells detached from a primary tumour undergo EMT, which is induced by epigenetic modifications like methylation (Hamilton and Rath 2018).

1.7.6 Intravasation, circulation and extravasation

Until extirpated, primary tumours can release numerous cancer cells into the bloodstream (Baccelli et al. 2013; Fares et al. 2020). Experiments reveal that tumour cells could intravasate with the aid of macrophages (Qian 2017), without the prerequisite of local angiogenesis (Wyckoff et al. 2007; Khamis et al. 2012). The circulation patterns determine the organs that cancer cells in the bloodstream can reach, according to where they originate. Acquisition of certain features is necessary for cell survival in the bloodstream (Quintana et al. 2012), and this is enabled by genetic transformations associated with primary tumours (Yachida et al. 2010; Fares et al. 2020). Furthermore, extravasation of cancer cells may occur (Qian 2017) into remote organs such as the brain (Minn et al. 2005; Achrol et al. 2019), lungs (Weis et al. 2004; Gupta et al. 2007; Padua et al. 2008; Wolf et al. 2012) and liver.

1.7.7 Colonisation

Metastasis hinges on colonisation (Bakir et al. 2020) which comprises survival on arrival, micrometastasis development, latency adoption, growth reactivation, recirculation, and even tertiary lesion development in the same or different organs. Although the causes remain unclear, cancer cells decrease at remote sites with a potential culprit for this being the stress of endothelial barrier

permeation, immunity effects, and absence of signals of survival and growth at the remote site (Fares et al. 2020). To avoid being exposed to destructive reactive stroma signals (Bakir et al. 2020) and to enhance the survival and anti-apoptotic pathways, cancer cells must acquire certain features. Survival at the remote site also depends on cancer cells effectively interacting with stromal components, such as macrophages (Chen et al. 2011; Qian 2017), and attaining particular functions, such as the gene encoding the nonreceptor tyrosine kinase Src activity (Fares et al. 2020),enabling the survival of breast cancer cells in the bone marrow (Zhang et al. 2009; Hen and Barkan 2020).

1.8 Stages and progression of colorectal cancer

1.8.1 The tumour-node-metastasis (TNM)

TNM classification (Baqar et al. 2019; Kasi et al. 2020) is a commonly used classification which is based on the anatomic extent (Piñeros et al. 2019) (depth of invasion) of the colon wall into three system (Chiappetta et al. 2020). Tumours (T) means the invasion grade of intestinal wall, node (N) is the grade of lymphatic node participation and metastasis (M) is the grade of metastasis. The ranges of these are TX – primary tumour cannot be assessed stage to T4 – tumour that directly invades other organs and structures. The extent of regional lymph node involvement from Nx – lymph nodes do not present to N2 – metastases in ≥ 4 regional lymph nodes, and presence of distant sites of disease (Mx – metastases cannot be determined to M1 – distant metastases detected) (Greene et al. 2002; Moreno et al. 2018).

1.8.2 The Dukes classification

In 1932 the pathologist called Cuthbert Dukes created a system which divided CRC into three grades: the first grade A (the most differentiated), the second grade B (intermediate) and the third grade C (the least differentiated) (Gabriel et al. 1935; Grinnell 1939; Włodarczyk and Sobolewska-Włodarczyk 2017; Elias 2020). The fourth grade D was added by Turnbull to represent the incidence of metastases to liver and other distant organs (Turnbull 1976). Nevertheless, the American Joint Committee on Cancer (AJCC), suggested it to be no longer used in the 2010. This system has mainly been changed to the more detailed TNM system and in clinical practice is no longer suggested (Edge and Compton 2010).

In CRC progression, apart from the *Apc* gene other important genes are implicated such as *TP53* and *K-Ras* (Kasi et al. 2020). A transcription factor p53 encoded by the *TP53* gene which engaged in cell cycle regulation, programmed cell death and DNA damage. Thus, loss of *TP53* role is significant in cancer development. It has been well-known that inactivation of p53 happens in a variety of human cancers. (Hollstein et al. 1991; Armaghany et al. 2012). The *K-Ras* mutation is approximately found in 8.6% of CRC (El Bali et al. 2021) patients and leads to diminishing of the Ras signalling pathway which is involved in proliferation, growth differentiation, cell survival and a programmed cell death (Migliore et al. 2011).

The Duke's staging classification developed (Akkoca et al. 2014);

Dukes' A: Indicates that the tumor is only in the innermost lining of the colon or somewhat growing into the muscle layer.

Dukes' B: Indicates the tumor has grown all the way through the muscle layer of the colon.

Dukes' C: Indicates the cancer has dispersed to at least one lymph node in the region close to the colon.

Dukes' D: Indicates cancer has metastasised from the origin organ to another of the body organs, such as liver, bones and/or lungs (Dukes 1949; Dukes and Bussey 1958; Koskensalo 2013).

1.9 Physiological roles of Ran

1.9.1 Nucleus and cytoplasm

Ran-GTPase is a small GTPase of the Ras superfamily (Goitre et al. 2014). Ran performs an essential function in cancer growth and development and is overexpressed in several tumours and its overexpression is associated with enhanced aggressiveness of the cancer cells (Boudhraa et al. 2020). Cell survival is dependent many processes that occur in the nucleus and cytoplasm and the trafficking of macromolecules between these two environments. The transport of these macromolecules is through nuclear pore complex (NPC) Nanopores; NPCs are fixed into the nuclear envelope (NE), where the external and internal membranes merge (Kau et al. 2004; Peters 2009; El-Tanani et al. 2016).

Morphologically, the NPC is comparable to a doughnut or hourglass, though rather than circular, its radial symmetry is octagonal around the central axis, and it has quasi-two-fold symmetry on the longitudinal axis across the NE. Each of the eight central cylindrical sections is called a 'spoke', and these form the central channel, which is bound at both ends by the cytosolic and nuclear rings. Also, at each end are eight filaments that branch from the respective rings; on the nuclear side, these interact to form the nuclear basket (El-Tanani et al. 2016). Microscopic studies of NPCs reveal that the extended filaments are 150-200 nm long and have an external diameter of 100-125 nm (Kau et al. 2004; Lin and Hoelz 2019). The radius on each side of the channel is 60-70 nm, narrowing to 25-45 nm in the centre; yet the diameter of the openings of the channel is about 10 nm (Dworetzky et al. 1988). It has been reported that as well as the central channel, some smaller bordering channels are approximately 8 nm in diameter; these are for the transport of small proteins and ions. The NPC is a bilateral, selective filter that rapidly transports diverse molecules, without the need to completely open or close the gateway assembly (Lin and Hoelz 2019).

1.9.1.1 Ran-GTPase

Ran-GTPase is (Sazer and Dasso 2000; Goitre et al. 2014), present mainly in the nucleus and affiliates with various cellular proteins according to the nucleotide. It is attached to (GTP or GDP), which depends on the interaction between Ran and the guanine-nucleotide-exchange factor (Ran-GEF) and GTPase-activating protein (Ran-GAP). The GDP-GTP nucleotide exchange is underpinned by Ran-GEF, while GTP-GDP hydrolysis is stimulated by Ran-GAP (Boudhraa et al. 2020; Zhou et al. 2020). Hydrolysis cannot occur without Ran Gap (Figure 1.5), as GTP hydrolysis by Ran has a poor intrinsic rate (Klebe et al. 1995; Mishra and Lambright 2016).

A member of the Ras superfamily, the small GTPase called Ras-related nuclear protein (Ran) GTPase is not subject to prenylation because it does not have a cysteine residue at its C-terminus, thus differing from other Ras members. Furthermore, its cellular functions (e.g. nucleocytoplasmic transportation, mitosis, centrosome reduplication) are critically dependent on its acidic C-terminal sequence DEDDDL (Yuen et al. 2013). Ran is highly conserved in various species, including yeasts and mammals (Matunis et al. 1996).

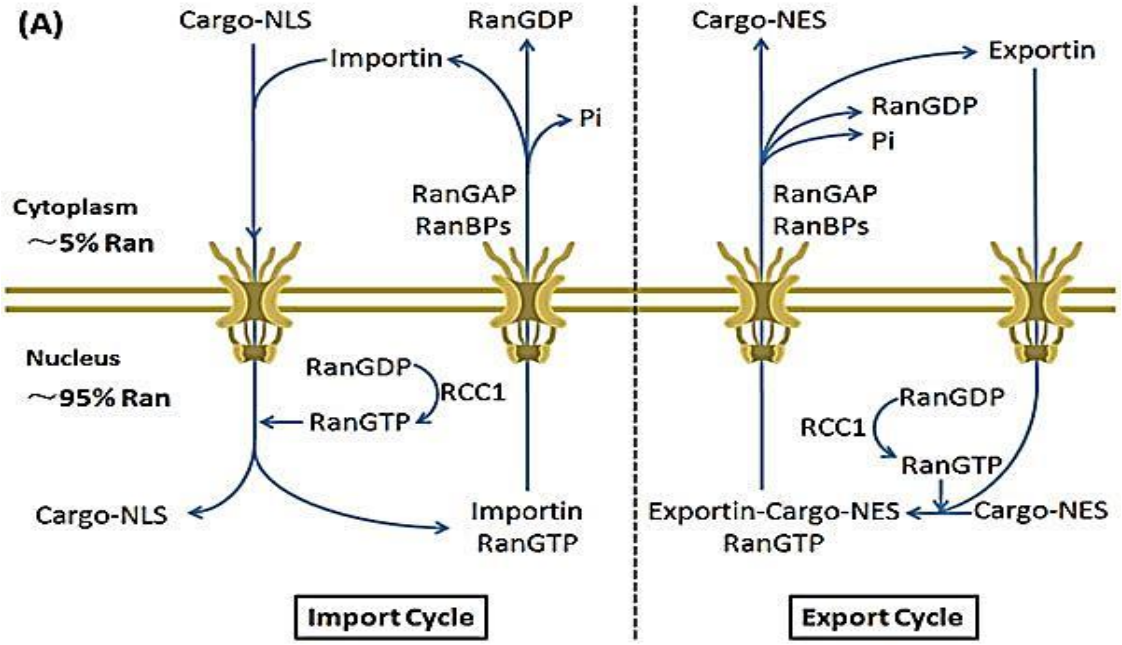

Figure 1.5: Signalling Ran-GTP.

Illustration of the mechanism of nuclear import and export shows in this diagram. 95% nucleic and 5% cytoplasmic RAN shuttles across the NE, with RCC1 maintaining the high nucleus level of Ran-GTP. The cytoplasmic Ran-GDP is due to the action of Ran-GAP and Ran-BPs that enhance GTPase activity. Ran contributes to these processes by forming and dissociating complexes. Ran also assists some proteins which are called regulatory proteins RCC1, Ran-GAP, and Ran-BP1/2. Figure is adapted from (Clarke and Zhang 2008; Strambio-De-Castillia et al. 2010)

The mechanism of nuclear import and export shows in this diagram 95% nucleic and 5% cytoplasmic RAN shuttles across NE, with RCC1 (a Ran-GTP formation catalyst) maintaining the high nucleus level of Ran-GTP (Ren et al. 2020). The cytoplasmic Ran-GDP is due to the action of Ran-GAP and Ran-BPs that enhance GTPase activity. Import left (Figure 1.5), involves importins attaching to cytoplasmic NLS containing cargoes and moving them into the nucleus via NPCs, where the cargoes are released through Ran-GTP attachment to importins (Ren et al. 2020). Followed by cytoplasm recycling of the Ran-GTP-importin complex, this breaks down after Ran-GTP hydrolysis. Export right (Figure 1.5), involves the attachment of the nucleic NES-containing cargoes to exportins and Ran-GTP, before exiting the nucleus. Ran-GTP hydrolysis causes the release of the cargoes from the complex (Ren et al. 2020).

1.9.2 Transport through the nuclear pore (NP)

Small molecules and ions that are no more than 40 kDa and 5-9 nm in diameter passively diffuse through the NPC (El-Tanani et al. 2016). In contrast, molecules such as larger proteins, ribosomes and RNAs, ranging from 40 kDa to 25 MDa and measuring 40-90 nm (Hoelz et al. 2011; Lin and Hoelz 2019), require active transport through the NPC. The rate of transport through the NPC is dependent upon the size of the cargo being transported. Where the radius of the cargo complex is greater than the radius of the channel, the rate of transport declines sharply. There is inconsistency in the literature as to whether facilitated transport and passive diffusion are handled by different pathways, though the body of evidence tends to reject the notion that they are not coupled, and the same NPC channel achieves both. Microscopy studies reveal that in response to various chemical or physical stimulations, for example, changing the concentration of calcium, CO_2 or ATP in the cell environment, the NPC undergoes dynamic conformational shifts (El-Tanani et al. 2016).

The NPC is a complex molecule comprised of about 30 different nucleoporins. Nucleoporins are referred to as Nups followed by a number that generally refers to their molecular mass (Hoelz et al. 2011; Sakiyama et al. 2017; Lin and Hoelz 2019). Due to the octagonal symmetry of the structure, Nups are present in multiple sets ranging from 8 to 48 (varying by species). The full complement of Nups per NPC is estimated to be between 500 and 1000 (Hoelz et al. 2011; Lin and Hoelz 2019). Some Nups are ubiquitous, found on either side of the NPC (Sakiyama et al. 2017), and some are only found in the central structure. There are also mobile Nups that perform different actions in the different stages of the cell cycle (Figure 1.6). The NPC is formed in three concentric layers, with the innermost layer bearing the FG-repeat Nups (El-Tanani et al. 2016).

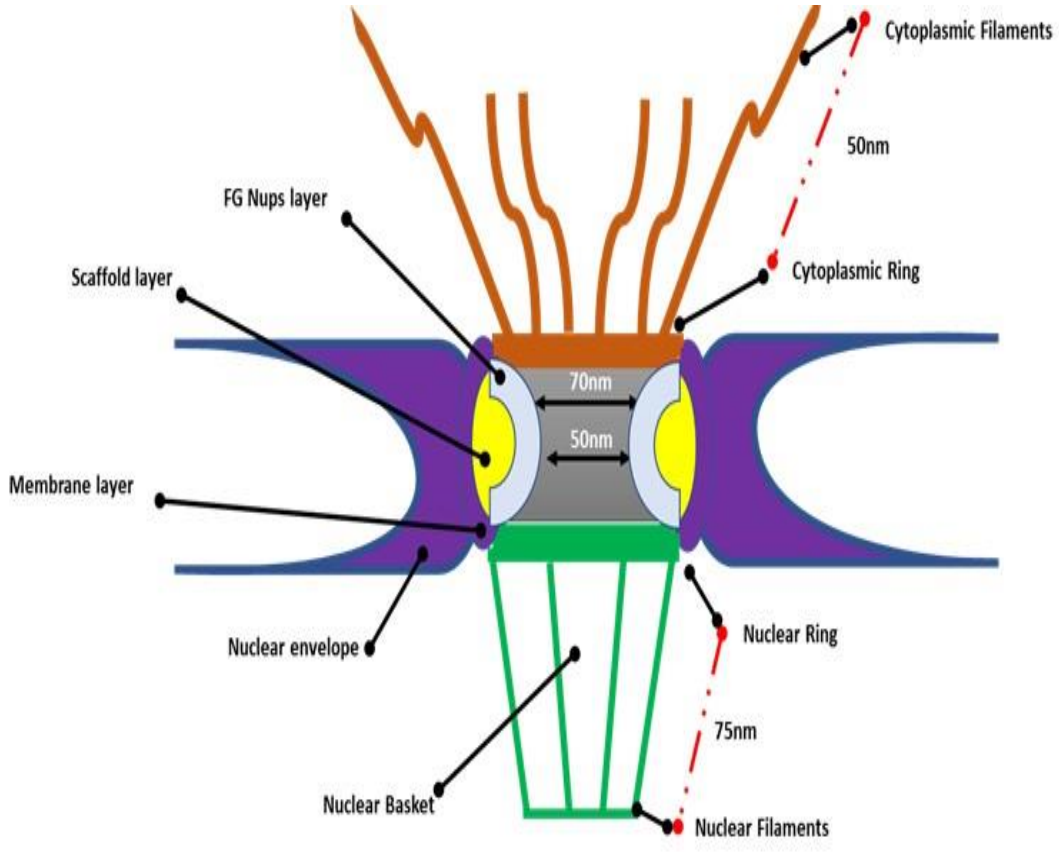

Figure 1.6: Diagram illustrating the nuclear pore complex.

Figure 1.6 is adapted from (Moussavi-Baygi et al. 2011; Lin and Hoelz 2019). The NPC structure is composed of different groups of structural proteins; (nuclear envelope, nuclear basket, nuclear filaments, and cytoplasmic and nuclear rings), (cytoplasmic filaments and rings (brown) and (scaffold layer (yellow), membrane layer (purple) and FG-nups (white) and nuclear basket (green).

1.9.3 Role of Ran on spindle assembly

Homeostasis depends on the Ran cellular functions of nucleocytoplasmic transport, mitotic spindle assembly and centrosome duplication control (Ozugergin and Piekny 2021; Tsuchiya et al. 2021), the disruption of which causes genomic instability and enhances cancer risk. Among the Ran downstream effectors discovered to have cancer involvement are active Aurora A triggered by TPX-2 which needs Ran for the release of TPX-2 from importin (Figure 1. 7), (Gruss and Vernos 2004). Tumour inhibitors such as Lats2 and BRCA1 are phosphorylated by activated Aurora A, and their function is suppressed (Meraldi et al. 2004; Mou et al. 2021).

In nuclear transport, Ran directs the chromosome position by spindle assembly in two ways:

Ran-GEF and RCC1 binds to DNA and histone proteins 2A and 2B (H2A, H2B), for the localisation of spindle assembly on an appropriate position of the chromosome using the Ran-GTP. High concentrations of Ran-GTP in specialised spindle assembly areas attracts importins and produces SAFs assembly factors such as TPX2 and NuMA and then these factors react with receptors at the nucleocytoplasmic region (Guilloux and Gibeaux 2020).

TPX2 is a tubular agent that stimulates spindle formation and is inhibited by importin α/β. When importin α/β react with Ran-GTP, the microtubule formation starts by TPX2 action. Moreover, TPX2 has a role in main protein kinase stimulation called Aurora A that has a role in cell division (Figure 1. 7) (Heald and Khodjakov 2015).

The spindle assembly regulates by Ran gradient around the chromosome-spindle binding point (Fu et al. 2007; Clarke and Zhang 2008; Heald and Khodjakov 2015).

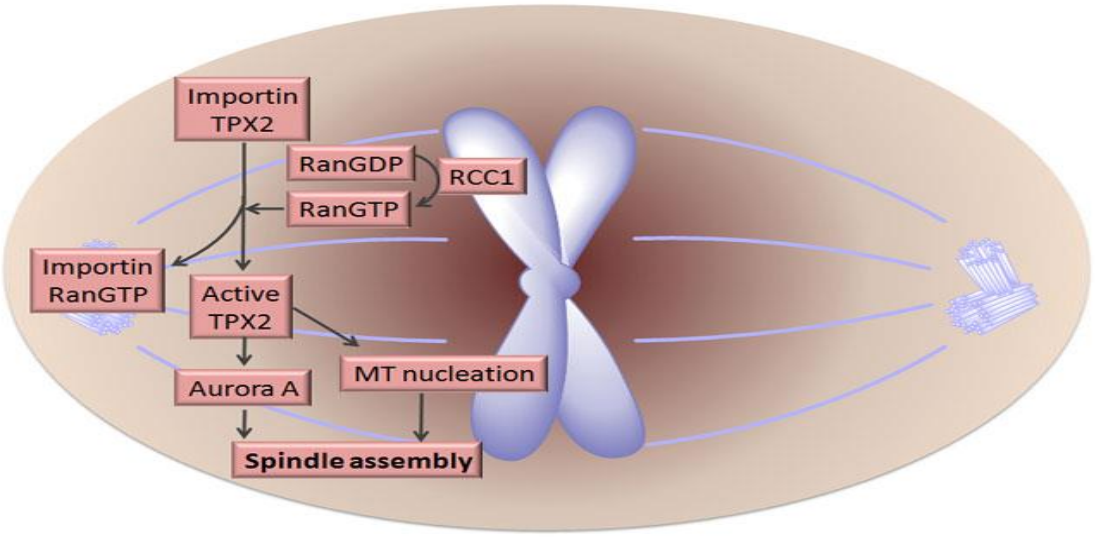

Figure 1. 7: The role of Ran on spindle assembly.

A Ran gradient likely regulates the mitotic spindle assembly. A Ran-GTP gradient is formed around chromosomes (pink shading) due to the association of RCC1 with chromosomes during mitosis. Importins attach to a number of spindle assembly factors, including TPX2, limiting their activity. Ran-GTP occurs in high levels around chromosomes, capturing importins and releasing functional TPX2. The latter supports spindle assembly by stimulating microtubules to nucleate and activate Aurora A (Fu et al. 2007; Clarke and Zhang 2008; Fu et al. 2010).

1.10 The role of Ran in cancer

The first clear indication that Ran was expressed in human tumours was subject of debate until 2006. After that, a tissue array analysis of serous epithelial cell cancers across different disease grades and stages was carried out and confirmed that relationship (Ouellet et al. 2006). Around the same time, screened small interfering RNA (siRNA) libraries against 3700 genes and evaluated cell apoptosis through cytotoxicity assay, reporting that fewer cells of several cancer cell lines such as human colon cancer, non-small cell carcinoma, and human breast carcinoma survived in the absence of Ran and TPX2 (Morgan-Lappe et al. 2007).

Ran also performs a vital role in cancer development and progression (Boudhraa et al. 2020). It is overexpressed in several cancers with prognostic significance and its excessive expression is associated with an increase in aggressive tumour cells *in vitro* and *in vivo* (Kurisetty et al. 2008; Yuen et al. 2016b; Wang et al. 2020b)

Ran is overexpressed in cancer cell lines and tumour tissues at both the mRNA and protein levels matched with normal counterparts (Yuen et al. 2016b). Ran expression is necessary for mitosis of cancer cells but not normal cells. Cancer cells with *K-Ras* activating mutations are more dependent relative on Ran expression than their *K-Ras* wild-type counterparts. Ran expression is required for the survival of cancer cells with hyperactivation of the PI3K/Akt/mTORC1 and Ras/Mek/Erk pathways. Ran is also a poor prognostic indicator in breast, lung, and ovarian cancers and renal cell carcinoma. In addition, researcher have demonstrated that Ran overexpression may engage in a role in the metastatic development of breast and lung cancers, highlighting a novel task of Ran in cancer progression (Yuen et al. 2013).

Furthermore, Ran siRNA experiments on a *K-Ras* isogenic cell line revealed that Ran loss adversely affected *K-Ras* transformed cells through activation of S-phase or transient G2-M phase arrest and then apoptosis. By contrast, Ran loss caused fewer normal cells to die. This discrepancy prompted investigations into Ran functions in human cancer. Mitosis is crucially dependent on Ran, so Ran-mediated mitotic mechanisms are likely important for tumour cells. The fact that Ran is excessively expressed in human cancer tissues compared to non-cancerous tissues prompted studies to explore Ran targets in human cancer via

siRNA techniques. This revealed that abnormal mitotic spindle formation, impaired function of mitochondria, and apoptosis occurred when Ran was inhibited in various tumour cell lines, but not in healthy cells.

The molecular mechanism promoting tumour cell apoptosis when Ran was lost (Altieri 2006; Sheng et al. 2018). Miss-localisation of *TPX* from microtubules occurred due to Ran ablation, and cell apoptosis occurred due to the release of apoptogenic molecules (cytochrome C and Smac) into the cytosol, without the involvement of p53, Bax and Smac, but with mediation by survivin, which contributes to apoptosis in different cancers (Sheng et al. 2018). The pathway underpinning the binding of survivin to *TPX* Crml actively participates in nucleocytoplasmic shuttling and apoptosis prevention (Knauer et al. 2007; Huang and Yeh 2020). Furthermore, reported that Ran inhibition reduced survivin protein levels, causing cell apoptosis, which was reversed by forcing excessive survivin expression. However, reported that Crm1 was unnecessary for survivin binding to Ran, although renewed emphasis was put on the importance of the survivin-Ran complex for spindle formation in cancer cells (Doherty et al. 2011).

1.11 Progressions leading to cancer

Through, the cell division, several proteins are intricate in regulatory the cell cycle. Therefore, some genes were responsible for regulated these proteins roles by mechanisms of transcription and translation. Each adjacent cell is receiving the contact chemical signals from other cells to responds to mechanisms of inhibiting or promoting the cell division (Leake 2014).

A traditional concept of carcinogenesis is numerous of schemes present to stop cell proliferation then unfortunately, leading to divisions but lacking DNA's repairing damages. Thus, finally leading to unrestricted division due to accumulation of mutations (Leake 2014). Furthermore, cancer stem cell and epigenetic effects ideas recently have been announced. Tissues and organs which rapidly renewing are places where cancer initiated, and this type of cancer called a regular type. On the other hand, the type of cancers initiated concerning endocrine glands, for example, brain or thyroid called relatively infrequent (Leake 2014).

1.12 Genes mutation

1.12.1 Oncogenes and tumour suppressors

Some genes yield proteins that distribute and promote the progress of the cell cycle. This is ultimately responsible for causing cancer. They are named "tumour genes" or oncogenes. For example, a single-point mutation in DNA, that is, a change from G to T causes glycine in the normal *Ras*- to yield valine in expressive proteins and is established to be carcinogenic. Proto-oncogenes act as a growth factor or receptor, transcription factor or cytoplasmic signaling factor under normal conditions. Many cancer genes are known in humans which called proto-oncogenes (Kasi et al. 2020), such as, *K-Ras, N-Ras* (Ras = GTPase), L-myc, c-myc and N-myc (MYC = transcription factor), Bcl-2, Bcl-1 (B-cell lymphoma), MDM2 and Erb-B (human epidermal growth factor receptors). The small font italic letter expresses the “gene”, and the capital letter means the protein (Bruford et al. 2020). Several genes and proteins are implicated in various kinds of cancer (Esteller and Herman 2004; Katherine 2017).

In contrast, under normal conditions, several genes yield mediators to inhibit cell proliferation, and their products (proteins) act as nuclear proteins and cytoplasmic signals. Mutations and damages on any of these tumor-suppressors genes would enable the cell to proliferate and continue division. Tumour-suppressors genes consist of *Apc* (adenomatous polyposis coli), p53, BRCa1 (breast cancer type1 susceptibility protein), (Esteller and Herman 2004; Rivlin et al. 2011).

1.12.2 Accumulation of mutations

Tumorigenesis is caused when oncogenes and tumour-suppressors are mutated chemically or by another process. In normal conditions they regulate the cell cycle but in case of mutation all their function fails to provide cell cycle arrest, programmed cell death (apoptosis) and DNA repair (Terabayashi and Hanada 2018). Subsequently, cell proliferation is permitted to continue unchecked leading to cancer formation (Kawada et al. 2012)**.**

Cancer indeed arises with organs and tissues that are rapidly rejuvenating, for example, colorectal canal, especially in the epithelial lining, male gonad, and blood-producing system. Initially, as with the stem cell, its produces ever more differentiated cells, and all genetic information will be copied to the cells along with it through genes. So, the mutated genes will be present in the next

generation of cells. As the tissue cells continue to renew, further DNA mutations will accumulate and with dysfunction of the cell cycle, consequently a cancer will form (Kawada et al. 2012).

1.12.3 Epigenesis and cancer

Epigenesis is altered cellular behaviour without changing the DNA sequence. In healthy cells, CpG islands (CGI) abundant sequences do not show methylation at all in the promoter regions where C-5 cytosine is located, except for 6-8 per cent of CGI, which is methylated in certain tissues (Illingworth et al. 2008; Gerhauser 2012).

The heavily methylated part of CG sites in repetitive sequences are for instance in satellite, centromeric and ribosomal DNA repeats. This restricts the admittance to the transcription machinery and stabilizes chromosomes (Esteller 2007). If any disruption happens to this DNA methylation, the cancer formation will start (Jones and Baylin 2007; Gerhauser 2012).

For instance, the silence of some of tumour suppressive genes and genes with significant biological roles is due to hypermethylation of DNA at CGI regions. In contrast, the genomic instability and chromosomal aberrations have been related to hypomethylation (Goelz et al. 1985; Gerhauser 2012).

Epigenetic control of gene expression is also facilitated by post-translational alterations of the N-terminal tails of proteins (histone). These modifications such as methylation, acetylation, ubiquitinylation, phosphorylation, ADP ribosylation and SUMOylation (small ubiquitin-related modifier) (Abbas 2020; Celen and Sahin 2020) was exposed to be a rescindable post-translational protein modifier grant DNA damage response, cell cycle checkpoint integrity and genomic stability (Knauer et al. 2007; Füllgrabe et al. 2011).

MicroRNAs (miRNAs) are consist of 20–22 nucleotides that known as small non-coding RNAs and prevent the gene expression at the level of posttranscription (Annese et al. 2020). MiRNAs help control vital biological developments such as proliferation, differentiation, development, programmed cell death and are identified to be changed in a range of diseases such as cancer (Calin and Croce 2006; Annese et al. 2020). MiRNAs are produced from the precursor of RNA by a complex system of a protein that arises from a family called Argonaute (Annese et al. 2020) and the ribonucleases Drosha and Dicer and polymerase II-

dependent transcription (Winter et al. 2009; Kim et al. 2017; Shkurnikov et al. 2020). They control formation proteins by the transformation of mRNA, specifically by deficiency in base-pairing to the mRNA 30-untranslated site to suppress protein formation, or by distressing mRNA stability. Individually miRNA is able to regulate numerous genes. miRNA has been involved in cancer beginning and development, and during carcinogenesis, miRNA level is regularly down regulated. Major miRNA mechanisms are deregulation which comprises of epigenetic alterations and genetic which include faults in the processing machinery that is responsible for miRNA formation (Begum et al. 2011).

1.13 Biomarkers

Cancer develops in a multiphase process comprising various mechanisms whereby normal cells become malignant (Nguyen and Duong 2018). Several abilities must be demonstrated by malignant tumours including maintenance of proliferation signalling, growth inhibitor avoidance, resistance to apoptosis, angiogenesis activation, immortalisation, invasion and metastasis (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Such malignant abilities are reflected by biomarkers (e.g. faecal, genomic, epigenetic, serum and microRNA biomarkers) which are molecules generated by cancerous tissue or by normal tissue as a reaction to malignant tumours. They are useful for cancer assessment and diagnosis, as well as for prognosis assessment and monitoring patients undergoing or who have undergone therapy.

1.13.1 Ran-GTPase as a biomarker

Ran-GTPase (Ran) (Boudhraa et al. 2020) plays an vital role in cancer development and progression and is overexpressed in several cancers with prognostic significance and its overexpression is associated with increased aggressiveness of the cancer cells (Yuen et al. 2012; Boudhraa et al. 2020). It enhances metastasis in various kinds of tumours such as colorectal and breast cancer, silencing Ran can reduce the apoptotic resistance, tumour transformation and thus, RAN-GTPase is a potential therapeutic target (Boudhraa et al. 2020).

1.13.2 Serum or blood tumour markers

Carcinoembryonic antigen

The glycoprotein CEA (Yang et al. 2018), is the only CRC serum marker confirmed for clinical applications, as its expression frequently occurs in CRC (Compton 2003; Duffy et al. 2007; Shinozaki et al. 2018). Monoclonal antibodies constitute the basis of current CEA testing. Although non-specific for CRC (Duffy et al. 2007), 20% of CRC patients were reported to have high levels of preoperative CEA (Carpelan-Holmström et al. 1995; Baqar et al. 2019). Follow-up assessment should monitor CEA, as higher levels are suggestive of recurrence or metastasis (Duffy 2001; Carpelan-Holmström et al. 2004; Baqar et al. 2019).

1.13.3 Tissue biomarkers

Matrix metalloproteinases

The zinc-based endopeptidases called matrix metalloproteinases (MMPs) can break down the extracellular matrix (ECM) (Laronha and Caldeira 2020), which are comprised of basement membrane (BM) proteins and extracellular attachments. There are 28 MMPs and at least 23 are expressed in human tissue as well as four MMP suppressors (TIMPs) which are known in humans (Chernov and Strongin 2011; Laronha and Caldeira 2020). Activation of MMPs produced as pro-enzymes are undertaken by other MMPs or serine proteinases (Coussens and Werb 1996; Laronha and Caldeira 2020). The deregulating of MMP activity leads to progression of numerous disorders (Laronha and Caldeira 2020). Both normal (e.g., embryonal development, tissue remodelling, and angiogenesis) and pathological procedures (e.g., inflammation, arthrosis, and cancer) have MMP involvement (Laronha and Caldeira 2020). In particular, tumour invasion and metastasis are facilitated by MMPs through ECM and BM breakdown. MMPs are also crucial for tumour neovascularisation to allow cancer progression (Coussens and Werb 1996; Laronha and Caldeira 2020).

MMP activity can both promote and inhibit apoptosis (Laronha and Caldeira 2020). Proteolytic breakdown of ECM proteins underpins the proapoptotic effects, serving as a ligand for integrin receptors of cell-surface adhesion. In physiological conditions, apoptosis is either intensified or diminished by MMP cleavage of the

ligand of the receptor Fas, which promotes apoptosis (McCawley and Matrisian 2001; Egeblad and Werb 2002; Stamenkovic 2003; Laronha and Caldeira 2020). Cancer cells and surrounding stroma (Laronha and Caldeira 2020) and immune cells show MMP-2 expression (Egeblad and Werb 2002; Calu et al. 2021). MMP-2 expressed in high levels in CRC has been associated with an advanced stage (Barabás et al. 2021; Calu et al. 2021) and poor prognosis (Hilska et al. 2007). Numerous carcinomas are associated with high MMP-7 levels such as CRC (Adachi et al. 1999; Ii et al. 2006; Vočka et al. 2019). Poor CRC prognosis is correlated with MMP-8 in serum (Kantola et al. 2012; Väyrynen et al. 2012).

Trypsinogens and tumour-associated trypsin inhibitor (TATI)

Produced largely in the pancreas, trypsinogens break down dietary proteins and activate another digestive enzymes (Paju and Stenman 2006; Kozakiewicz et al. 2016). Numerous types of cancers, including CRC, express tumour-related trypsinogen-1 and -2 (Oyama et al. 2000; Yamamoto et al. 2003; Koskensalo 2013). CRC prognosis is poor if trypsin is expressed (Yamamoto et al. 2003; Kozakiewicz et al. 2016), while trypsinogen-1 expression is indicative of an advanced stage (Oyama et al. 2000; Williams et al. 2001; Koskensalo 2013).

TATI is a tumour-associated trypsin inhibitor (Koskensalo 2013). In CRC in particular, expression of TATI in tissue is indicative of liver metastasis (Gaber et al. 2009; Koskensalo 2013) and high levels of expression suggest an advanced stage (Higashiyama et al. 1990; Koskensalo 2013).

p53

Around half of CRC cases are linked with mutations in the tumour-suppressor gene *p53* (Loktionov 2020), which are crucial for carcinogenesis (Steele and Lane 2005; Liebl and Hofmann 2021). The nuclear phosphoprotein that is the *p53* translational product behaves like a transcription factor (Liebl and Hofmann 2021). Gene mutation causes a mutated protein *PT*53 to accumulate in nuclei disrupting apoptosis, angiogenesis (Liebl and Hofmann 2021), cell cycle and genomic maintenance (Baas et al. 1994; Vogelstein et al. 2000; Mills 2005). A number of malignancies are associated with the *PT*53 mutation and overexpression (Hollstein et al. 1991; Liebl and Hofmann 2021) and CRC

prognosis is poor when the mutated protein is overexpressed (Kaklamanis et al. 1998) (Manne et al. 1997).

1.13.4 Faecal markers

Faecal haemoglobin

CRC can be detected easily, affordably and with minimal invasiveness via stool-based screening (Duffy et al. 2007; Loktionov 2020). More specifically, CRC is usually screened via Faecal occult blood testing (FOBT), (Huang et al. 2005; Loktionov 2020), which can detect haemoglobin either enzymatically or immunologically. The former measures the peroxidase-like activity of haemoglobin from any bleeding source in the colorectal and upper gastrointestinal tracts, but it often produces false positives, particularly if patients eat certain foods (red meat, fruit, and vegetables) or take non-steroidal anti-inflammatory medication.

1.14 MicroRNA

Significant advances in biological research (Loktionov 2020) and drug discovery have been made possible by genomic technology. To investigate genetic events broadly functional genomic analyses are commonly applied in gene discovery, to identify biomarkers, to categorise diseases and to determine drug targets. Expression arrays and microRNA (miRNA) assays are the newest tools for genomic research (Chen et al. 2009b; Loktionov 2020).

In CRC, miRNA could aid diagnosis and prognosis, as miRNA expression is related to microsatellite instability (MSI) subgroups, involving cancers associated with low MSI and HNPCC (Earle et al. 2010; Müller et al. 2016).

1.15 CRC treatment

Surgery, radiotherapy, and chemotherapy are the major therapy options available to individuals with CRC diagnosis, the majority of whom undergo surgery combined with radiotherapy and/or chemotherapy (Lin et al. 2018b).

1.15.1 Surgery

All individuals with CRC undergo surgery first, as this provides an uncomplicated strategy for managing early-stage CRC (Lin et al. 2018b) and tumour removal via colonoscopy or diagnosis by capsule colonoscopy (Lacy et al. 2002; Amri et al.

2013; Rastogi and Wani 2016). A lengthy, malleable tube of minimal thickness terminating in a light fixture and video camera, the colonoscope enables visualisation of the inner colon through the images conveyed by the camera to a monitor (Stauffer and Pfeifer 2021). Tumours can be excised after their localisation, but removal of a larger portion of the colon is necessary in the event of tumour spread to the muscles in the approximately of the colon wall. Therefore, partial colectomy is needed in cases of advanced disease (Lacy et al. 2002; Rastogi and Wani 2016). Anastomosis is performed once the cancerous colon portion is removed in order to link the colon portions that are left.

Tumours may not be fully eradicated through surgery due to the fact that tumour cell clusters of small dimensions remain and their removal or identification through testing or screening is impossible (Roodman 1999; Liao et al. 2021). Development and proliferation of these cells can be initiated some time following surgery. Cancer progression is the term applied to the renewed cell development if cancer symptoms start to manifest again in three months or earlier (Kufe et al. 2003; Liao et al. 2021), whereas cancer recurrence is the term used to refer to the renewed cancer cell development if symptoms begin manifesting after a year or more (Liao et al. 2021). As such, patients are additionally subjected to radiotherapy and chemotherapy to avoid CRC recurring or progressing in the aftermath of surgery (Kufe et al. 2003; Rodriguez-Bigas et al. 2003; Schwab 2008; Badic et al. 2021; Liao et al. 2021).

1.15.2 Radiotherapy

Radiation therapy (RT) is ionising radiation (IR) emitted by X-rays and gamma rays which trigger not only atom and molecule ionisation but also rupture of DNA double strands (White et al. 2019; Liu et al. 2020). The term of ionising radiation describes removal of atom electrons and cell molecules, leading to ion formation. Furthermore, harmful free radicals are engendered in large proportion by ionising radiation through the removal of one electron from the cellular water. Free radicals (Liu et al. 2020), molecules exhibiting instability and high reactivity as they possess an electron without a pair, which enables donation or acceptance of an electron to/from other molecules, damaging cells more extensively (Olive 1998; Kufe et al. 2003; Lin et al. 2018b). As a cancer therapeutic strategy, radiotherapy is advantageous because, unlike normal cells and tissues, which

are more capable of radiation damage repair, tumours typically possess impaired pathways of recovery from radiation (Lliakis 1991; Olive 1998; Kufe et al. 2003; Liu et al. 2020). In the past ten years radiotherapy for CRC treatment has undergone notable innovation (Tam and Wu 2019; White et al. 2019).

CRC treatment usually involves the following forms of radiotherapy:

(1) External-beam radiation therapy: administration of CRC-targeting radiation generated by an external machine for a period varying from a couple of days to weeks (Rijkmans et al. 2017).

(2) Internal radiation therapy (brachytherapy): this reduces side-effects to other body parts as the source of radioactivity is inserted within patients' rectum (Tam and Wu 2019).

(3) Systemic radiation therapy: insertion into the blood circulation of a radioactive substance that detects and eradicates cancer cells (Tam and Wu 2019).

To make surgery more successful or to attenuate particular cancer-related symptoms, radiotherapy can be performed pre- or post-surgery (Ismaili 2011; van Gijn et al. 2011). Surgical removal of tumours can be facilitated by subjecting the tumour tissue to X-ray radiation (van Gijn et al. 2011; Tam and Wu 2019). On the other hand, cancer recurrence can be reduced through the post-surgery application of X-ray radiation (van der Meij et al. 2016).

1.15.3 Chemotherapy

The therapeutic strategy whereby chemical agents are employed to prevent cancer cells from spreading and proliferating by damaging them is known as chemotherapy (Pentz et al. 2019). Chemotherapy can take a range of forms, but the majority of chemical agents hinder cell division or induce cell apoptosis by targeting particular cell cycle stages (Kufe et al. 2003; Schwab 2008; Lin et al. 2018b). Although to keep cancer from recurring, chemotherapy is generally conducted post-surgery (Schrag et al. 2001; Lin et al. 2018b), it can also be administered pre-surgery to minimise the tumour bulk or prior to radiotherapy to increase the sensitivity of cancer cells to radiation (Nordlinger et al. 2008; Wang et al. 2019). However, the availability of many different types of chemotherapy for CRC treatment has still not shed light on differences in responses according to patient and tumour type (Schwab 2008; Group 2012). The anticipation of drug reaction is important to know as the genomic nature of cancer cells determines

how they respond to chemotherapy. In the following part, the major chemotherapy agents used in CRC treatment are presented (Lin et al. 2018b).

1.15.3.1 Alkylating agents

These include cisplatin and oxaliplatin (OXA), which lack specificity for cell cycle and typically exert their effect during the resting cell stage (Fu et al. 2012). Their action entails cross-linking guanine nucleobases in double-stranded DNA by binding an alkyl group to DNA resulting in strands that cannot be divided. The cytotoxic effect of cisplatin is exerted through hydrolysis that produces a charged platinum complex [Pt(NH3)2Cl(H_2O)] + with high reactivity, which attaches to both genomic and mitochondrial DNA and triggers cell apoptosis by suppressing processes of replication and transcription (Fuertes et al. 2003). Meanwhile, covalent binding with DNA and macromolecules is displayed by reactive species like dichloro (1,2-diaminocyclohexane) platinum (II) (DACHPt) generated from the non-enzymatic conversion of OXA in physiological solutions. Although at first, OXA forms monoadducts guanine, in due course it fosters DNA cross-links between neighbouring adenine and guanine by binding concomitantly to two nucleotide bases. Consequently, DNA replication (Abbas and Dutta 2017) and transcription are suppressed (Kweekel et al. 2005).

1.15.3.2 Inhibitors with cell-cycle specificity:

These include irinotecan (IRI) (Shi et al. 2014), which is a topoisomerase I inhibitor (Topo I), doxorubicin, which is a topoisomerase II (Topo II inhibitor), and paclitaxel.

1.15.3.3 Antimetabolites:

These are molecules comparable to natural cellular substances involved in cellular metabolism and exhibiting cell-cycle specificity in their action (Tiwari 2012; Pérez-Herrero and Fernández-Medarde 2015). One such agent is 5-fluorouracil (5-FU), which suppresses the enzyme thymidylate synthase (TS) of vital importance to produce thymine necessary to synthesise DNA and RNA.

By contrast to the forms of chemotherapy discussed above, which target all cells exhibiting fast division, whether normal or cancerous, targeted therapies focus on particular cancer-related molecular targets and are geared towards hindering cancerous cells from proliferating (Pérez-Herrero and Fernández-Medarde

2015). Cetuximab (Erbitux) and Panitumumab (Vectibix) are two novel targeted therapy agents that single out the epidermal growth factor receptor (EGFR). A protein receptor with three domains, namely, an external domain, a cellular membrane domain and an internal domain, EGFR exhibits an internalisation response with EGF that is triggered by a ligand and facilitates signal pathways participating in cell differentiation and overgrowth (Pettigrew et al. 2016).

Knowledge of the processes through which normal cells are converted to cancerous cells is necessary to attain comprehension of the function of chemotherapy. Basically, chemotherapy targets cancerous cell rapid growth. Epithelial turnover of healthy colon happens every five days, with stem cells grown under a microenvironmental effect promoting self-regeneration of intestinal epithelial cells (Mathonnet et al. 2014). To highlight what a complex disease cancer was and to shed light on the conversion of normal cells into proto-tumoral cells, Hanahan and Weinberg (2000) distinguished six critical attributes of a cancerous cell. Eleven years later, they amended their list of critical attributes with the addition of four others (Hanahan and Weinberg 2011). The ten attributes are growth signal self-sufficiency, avoidance of growth inhibitors, avoidance of apoptosis signals, avoidance of body defence mechanisms, cellular metabolism changes, lack of genomic stability, protracted angiogenesis, activation of tissue invasion and metastasis, and stimulation of inflammation (Hanahan and Weinberg 2000; Kufe et al. 2003; Hanahan and Weinberg 2011).

Some of the attributes previously listed serve as mediators in normal cell conversion into cancerous cells. In the normal cell cycle, epithelial cells undergo death through apoptosis and are substituted with new cells under the regulation of extracellular and intracellular signals (Schwab et al. 2008). This closely controlled process underpins colon homeostasis. A malignant environment can emerge because of homeostatic imbalance, with implications for growth suppression, apoptosis, and carcinogenesis. As explained by several authors, carcinogenesis depends on various epigenetic and genetic alterations aggregating and resulting in aberrant colorectal cell growth (Mathonnet et al. 2014).

In the context of CRC, carcinogenesis is primarily defined by angiogenesis and stem cells with infinite replication capacity, which have consequently been the focus of efforts to formulate new treatment approaches (Mathonnet et al. 2014).

Treatment can be unsuccessful and sensitivity to chemotherapy minimal if mutations and modifications occur in these two characteristic aspects (Hanahan and Weinberg 2011).

The individual patient's situation and cancer type and stage are the factors dictating the choice of chemotherapy (Hanahan and Weinberg 2000). Furthermore, tumour location and the type of chemical compound employed will determine the manner of administration of chemotherapy. Oral administration is possible in the case of chemotherapeutic agents in capsule and pill form and, besides solid forms, oral chemotherapy administration can take liquid form as well. Oral chemotherapy is advantageous because it is more convenient for patients and can be administered at home. Meanwhile, intravenous injections (IV) or cannulas can also facilitate chemotherapy administration (Kufe et al. 2003; Senko 2020). As regards the length of time involved, chemotherapy can range from weeks to months. To make the treatment more effective, multiple chemotherapeutic agents are administered to the majority of patients (Kufe et al. 2003; Park et al. 2018).

1.15.4 Typical agents used in CRC chemotherapy

CRC has been treated with a number of chemical compounds for over six decades (Kufe et al. 2003; Diasio et al. 2021).

1.15.4.1 5-fluorouracil (5-FU)

It was in the 1950s that CRC began to be treated via chemotherapy, with 5-FU being administered in advanced cases (McArdle et al. 1994; Diasio et al. 2021). An uracil analogue, 5-FU has a cytotoxic effect that triggers cell apoptosis when it is integrated into DNA and RNA (Longey et al. 2003; Diasio et al. 2021). Provided a comprehensive discussion of the manner in which 5-FU exerts its effect. In general, 5-FU is administered alongside folinic acid (Folprecht et al. 2016), otherwise called leucovorin. In 1991, the US Food and Drug Administration (FDA) granted authorization for chemotherapy involving the injection of folinic acid in doses of 20 mg/m^2 and then 425 mg/m^2 5-FU over the course of five days of treatment with repetition every four weeks (Jung et al. 2007a; EROGLU et al. 2013; Carlsson et al. 2014). In 2005, the FDA endorsed the novel 5-FU prodrug capecitabine (Xeloda® Tablets, made by Hoffman-LaRoche Inc.) (Diasio et al.

2021) to be administered alongside OXA (Hoff et al. 2001; Van Cutsem et al. 2001; Pfeiffer et al. 2019) for stage-III CRC cases. This treatment has been shown to be more effective compared to 5-FU (Cunningham et al. 2004; Innocenti et al. 2020; Diasio et al. 2021) and is administered in three-week cycles over a period of six months, with two weeks of oral administration of 1250 mg/m^2 capecitabine two times per day and a break of one week (Pentheroudakis and Twelves 2002; Innocenti et al. 2020).

1.15.4.2 Oxaliplatin (OXA)

OXA is a third-generation platinum medication also an agent that is frequently employed in the therapy of CRC (Lin et al. 2015; Madigan et al. 2020). A platinum-based chemical compound, OXA attaches covalently and permanently to the negative DNA charges, thus damaging the cancerous cells (Alcindor and Beauger 2011; Lin et al. 2015). In 2004, the FDA endorsed the administration of OXA alongside 5-FU and folinic acid as an adjuvant treatment for cases of stage-III CRC (Jung et al. 2007b; Pfeiffer et al. 2019).

1.15.4.3 Irinotecan (IRI)

The camptothecin derivative IRI is commonly used to treat CRC as well (Innocenti et al. 2020; Diasio et al. 2021). Carboxylesterase undertakes the hydrolysis of IRI to the active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin). The latter subsequently undergoes conjugation by hepatic uridine diphosphate glucuronosyltransferase to SN-38 glucuronide (SN-38G) (Diasio et al. 2021). Causing cells to die via apoptosis, the Topo I inhibitor SN-38G received endorsement for use in advanced cases of CRC in 1996 (Liu 2000; Diasio et al. 2021).

1.16 Non-cancer medicines used for the treatment of cancer

1.16.1 Pimozide

Pimozide is an antipsychotic medication and has been shown in *vivo* and *in vitro* study to suppress breast cancer cell growth and A549 lung cancer cell proliferation in a dose- and time-dependent manner (Dakir et al. 2018). As well as encouraging apoptosis as established by cell cycle arrest and initiation of double-strand DNA breaks (Dakir et al. 2018). Pimozide, has shown that both level of protein and mRNA was downregulated by Ran-GTPase and Akt. In

addition, the drug repressed the signalling pathway of Akt in a breast cancer cell line MDA-MB-231. Pimozide furthermore repressed the EMT and cell migration (Dakir et al. 2018).

1.16.2 Mebendazole (MBZ)

MBZ is a broad-spectrum anti-anthelmintic medication (AbdusSamad et al. 2016; Andersson et al. 2020). Infestations by different kinds of worms (e.g. pinworms, roundworms, tapeworms, American hookworms, whipworms) are treated by MBZ, (Mukhopadhyay et al. 2002; Doudican et al. 2008; Bai et al. 2011; Pinto et al. 2015; Ahmed et al. 2021), which is termed in the International Union of Pure and Applied Chemistry (IUPAC) as (5-benzoyl-1H-benzimidazole-2-yl) -carbamic acid methyl ester ($C_{16}H_{13}N_3O_3$, M = 295.293 g mol^{-1} (Al-Badr and Tariq 1987). Figure 1.8 illustrates the chemical structure of MBZ. Through selective and permanent suppression of nutrient assimilation in adult intestines that play host to such worms, MBZ gradually inactivates these helminths and causes them to die (Al-Badr and Tariq 1987; He et al. 2018). Furthermore, MBZ targets the protein threads linking the centromere areas of chromosomes, thus interrupting cellular reproduction (Al-Badr and Tariq 1987; Wang 2021). The three polymorphic forms of MBZ (A, B and C) possess distinct therapeutic action and solubility properties, but none of them are soluble in water (Himmelreich et al. 1977; Rodriguez-Caabeiro et al. 1987). The greatest efficiency is attributed to the polymorphic form C (Evans et al. 1999; Brusau et al. 2008), while the polymorphic form B displays the highest solubility, which may explain its toxic nature (Costa et al. 1991; Swanepoel et al. 2003). By contrast, the form with the poorest solubility and greatest instability is polymorphic form A (Camí et al. 2020), and guidelines suggest that the formulation should not contain more than 30% of this form (Evans et al. 1999).

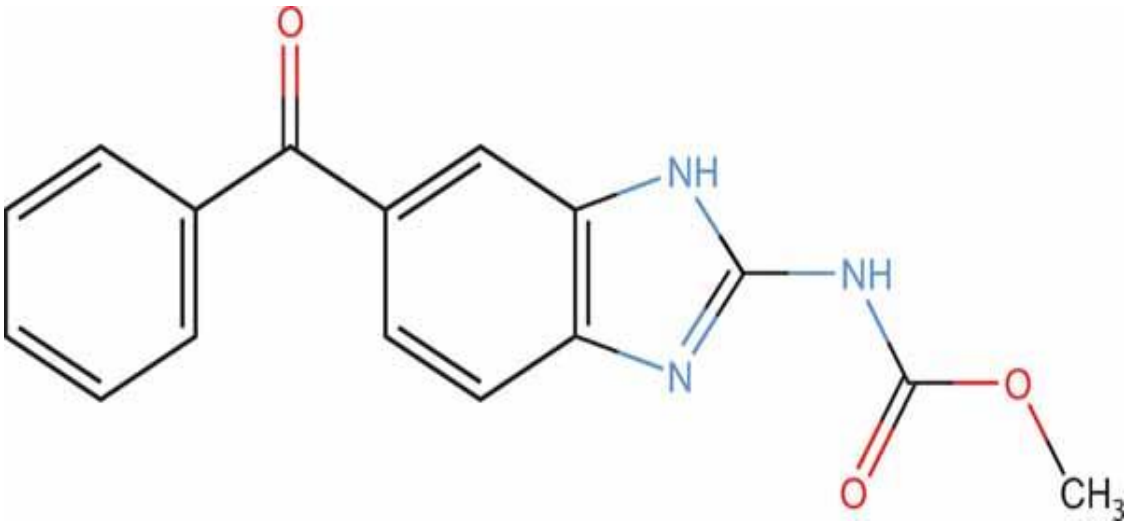

Figure 1. 8: The chemical structure of MBZ.
Adapted from (Gunning et al. 2015).

Tubulin belongs to a superfamily of globular proteins or one of the member proteins of that superfamily in molecular biology basis. Tubulin consists of two isoform α- and β-tubulins that are essential for formation of microtubules through its polymerisation. The microtubules are the main constituent of the cytoskeleton structure of the eukaryotic cells (Gunning et al. 2015). Microtubules have roles in many critical cellular developments, such as mitosis. So, the medications such MBZ that have ability to binding to the tubulin-binding site will cause cancerous cells to die through preventing microtubule dynamics formation, which is essential for DNA segregation, intracellular transport and consequently pushes the cells to divide. Tubulin is depolymerized and thus the functions of microtubules are disrupted.

Microtubules are α- and β-tubulin dimers which bind to GTP and gather onto the microtubules at plus ends (Heald and Nogales 2002; Centelles 2019). Microtubule ends of both α-tubulin subunit is exposed on the (-) end while the β-tubulin subunit is exposed on the (+) termination. Subsequently, the dimer which forms is then merged into the microtubule with the GTP bound molecule incorporated into the subunit of β-tubulin finally hydrolysed through inter-dimer into GDP along the microtubule protofilament (Howard and Hyman 2003; Centelles 2019). On the other side, the α-tubulin subunit that is bound to a GTP molecule is stable through the entire process. Both the tubulin dimer is bound to GTP or GDP of a β-tubulin subunit member influencing the microtubule stability

(Centelles 2019). The dimer related to GTP tends to merge into microtubules, while dimer related to GDP tends to split so this cycle of GTP is essential for the microtubule dynamic instability (Centelles 2019).

MBZ performs this action through selectively preventing the synthesis of microtubules by binding to β-tubulin through a colchicine binding site, thus blocking the tubulin dimers polymerisation in parasite's bowel cells (Lacey 1990; Thakur and Patel 2020). Interruption of microtubules in cytoplasmic leads to hindering of glucose and other nutrient uptakes, then leading to immobilisation gradually and the death of the helminths subsequently (Petry and Vale 2015). MBZ malabsorption in the intestinal tract makes an effective medication for bowel infections therapy with parasitic as well as adverse effects limitation. As MBZ can inhibiting polymerisation of tubulin dimers in mammalian cells (Thakur and Patel 2020), hence disrupting structures of essential microtubules such as spindle mitotic formation (van Linde et al. 2017). Dismantling the mitotic spindle subsequently leads to programmed cell death facilitated by Bcl-2 dephosphorylation which permits Bax (pro-apoptotic protein) to dimerise and initiate apoptosis (Blagosklonny et al. 1997; Levy and Claxton 2017).

The previous study shows that MBZ has a potent antitumor activity (Thakur and Patel 2020) as well as acting as a mild inhibitor to spindle formation, *In vivo*, MBZ inhibited cell growth through induced programmed cell death on healthy spare cells and tumours cells such as squamous cell carcinoma (SC) non-small cell lung cancer (NSCLC) S.C. NSCLC xenografts and in lung metastasis (Sasaki et al. 2002).

Previous *in vitro* experiments have shown that mebendazole prevents a wide variety of factors in tumour formation such as tubulin polymerisation, pro-survival, angiogenesis, and matrix metalloproteinases. MBZ is not only directly cytotoxic but has a synergistic effect with ionising radiation and several chemotherapeutic medications and provokes an antitumoral immune response (Guerini et al. 2019).

1.17 Connectivity mapping (CMAP)

The connectivity mapping concept was first taken from (Lamb et al. 2006; De Abrew et al. 2019; Bibby et al. 2021). It tried to establish a relationship between the type of disease and the gene expression associated with the molecules of the drug or the state of disease and genes.

The purpose of the linkage is to identify which molecules or genes can be used to treat the disease. Through the connectivity map, MBZ (Figure 1. 8) an anticancer drug, was selected from a composite library containing around 1,600 compounds that are clinically used to determine drugs likely to be reintroduced or repurposed for the therapy of colon cancer (Nygren et al. 2013).

Therefore, new medication detection strategies are needed. One of these strategies is the repurposing of a drug in which a new indication of the drug is identified (De Abrew et al. 2019; Bibby et al. 2021). In this approach, well-known drugs and even discontinued drugs with unrecognised cancer activity can progress rapidly in clinical trials of this new indicator since many or all of the necessary documents that support clinical trials are already published (Hurle et al. 2013).

The gene expression perturbations induced by the drug were studied using the contact map (CMAP) for 1,309 compounds (www.broad.mit.edu/cmap) (Lamb et al. 2006; Bibby et al. 2021). From a repurposing perspective, MBZ had been approved for clinical use in humans and was easily accessible for clinical trials. Thus more detailed analysis could be carried out. (Lamb et al. 2006; Pantziarka et al. 2014).

MBZ has significant antineoplastic effects both *in vivo and in vitro*. It has been shown to exhibit potent inhibition in growth of lung cancer cell lines and human adrenocortical carcinoma cells, with slight toxicity to a normal cell (Sasaki et al. 2002; Martarelli et al. 2008; Zhang et al. 2017; He et al. 2018; Williamson et al. 2020).

1.18 Aim of the study

This study will test the hypothesis that the Ran inhibitor MBZ reduces cell invasion and metastasis in CRC.

In this research, I will investigate the impact of MBZ on Ran on three pathways namely proliferative signalling, avoiding programme cell death, and triggering pathways that boost invasion and metastasis as well as immune response.

Objective for the research project:

To Investigate the effect of the Ran Inhibitor (MBZ) on expression levels of genes regulating cell invasion and metastasis in isogenic CRC cell lines (Chapter 3).

To Investigate the effect of MBZ on proliferation of isogenic CRC cell lines (Chapter 4)**.**

To Investigate the effect of MBZ on association of K-Ras and Pten mutation with Ran expression as well as cell invasion and metastasis in isogenic CRC cell lines (Chapter 5).

Chapter 2
Materials and Methods

2. Materials and methods

This chapter lists all the materials and methods used to generate experimental data presented in chapters 3, 4 and 5.

2.1 Materials

2.1.1 Cell lines

The European Collection of Animal Cell Cultures and American Type Culture Collection (Cree 2011), provided the human isogenic colorectal, breast and American Type Culture Collection provided lung cancer cell lines (Cree 2011) (Table 2.1).

Table 2.1: Origin and identification code of colorectal, breast and lung cancer cell lines.

Cell lines	Origin	Identification code
A549	Human epithelial lung carcinoma	ATCC® CCL-185
DKO-3 Wt.	Human colorectal	RRID: CVCL-9799
DLD-1 *K-Ras* Mt.	Human colorectal adenocarcinoma	ATCC® CCL-221
HCT-116 *K-Ras* Mt.	Human colorectal adenocarcinoma	ATCC® CCL-247
HCT-116 *K-Ras* Wt.	Human colorectal	NC1205813
HCT-116 *Pten -/-* Mt.	Human colorectal carcinoma	NCBI-5728
MDA-MB231	Human breast adenocarcinoma	ECACC-92020424

Key: ATCC: American Type Culture Collection, NCI: National Cancer Institute, ECACC: European Collection of Animal Cell Culture, RRIDs: Research Resource Identifiers and NCBI: National Center for Biotechnology Information.

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2.1.2 Materials

Chemicals used during this study are summarised in (Table 2.2).

Table 2.2: Chemicals and their suppliers.

Product	Catalogue number	Supplier
2 -Mercaptoethanol	M3148	Sigma-Aldrich, UK
5 ml Precision PLUS qPCR Master Mix with ROX at a reduced level premixed with SYBR Green	PPLUS-LR-SY-5ML	Primer-Design, UK
Acrylamide 30%	A6050	Sigma-Aldrich, UK
Albumin bovine serum	B4287	Sigma-Aldrich, UK
Ammonium per sulphate (APS)	A3678	Sigma-Aldrich, UK
Bromophenol blue	B8026	Sigma-Aldrich, UK
Chemiluminescent substrate	RPN2106	GE Healthcare
Complete mini protease cocktail inhibitor	11836153001	Roche, UK
Cryovials	G122263	Scientific Lab
Crystal violet	C3886	Sigma-Aldrich, UK
Dimethyl sulphoxide (DMSO)	D8418	Sigma-Aldrich, UK
Ethanol	E/0600/05	Fisher, UK
Ethylene Glycol-Bis (2aminoethylether) N, N, N', N' Tetra acetic Acid (EGTA)	E4378	Sigma-Aldrich, UK
Glacial acetic acid	320099	Sigma-Aldrich, UK
Glycine	BPE381-1	Fisher scientific
Magnesium chloride	M8266	Sigma-Aldrich, UK
Marvel dried skimmed milk powder 1% fat		Marvel, UK
MBZ (Poole, UK)	M0215000	Sigma-Aldrich, UK
Methanol	BPE1105	Fisher scientific
Nitrocellulose membrane	IPVH00010	GE Healthcare
PBS 500 ml	806552	Sigma-Aldrich, UK

Phosphatase cocktail I inhibitor	P2850	Sigma-Aldrich, UK
Phosphatase cocktail II inhibitor	P5726	Sigma-Aldrich, UK
Precision nanoScript 2 reverse transcription kit.	RT-nanoScript2	Design, UK
Pre-stained page ruler	SM0671	Fermentas
QCM ECMatrix, 24-well (8 µm), fluorometric	ECM554	Merk-molpore, UK
RNeasy Mini Kit Plus	74134	Qiagen, UK
Sodium chloride	7647-14-5	Fisher scientific
Sodium dodecyl sulphate (SDS)	S/S200/53	Fisher scientific
Tetra methyl ethylene diamine (TEMED)	T9281	Sigma-Aldrich, UK
Tween 20	P2287	Sigma-Aldrich, UK

2.1.3 Media and cell culture materials

Cell culture medium and biological materials used to maintain cells are listed in (Table 2.3).

Table 2.3: Cell culture consumables.

Product	Catalogue number	Supplier
Dulbecco's Modified Eagle's Medium	D5796	Sigma-Aldrich, UK
Foetal Bovine Serum	F4135	Sigma-Aldrich, UK
L-Glutamine	G6392	Sigma-Aldrich, UK
Penicillin and streptomycin	P4333	Sigma-Aldrich, UK
RPMI-1640 medium HEPES modification Gibco	R5886	Sigma-Aldrich, UK
Sodium pyruvate	S8636	Sigma-Aldrich, UK
Trypsin/EDTA (Life Technologies, Invitrogen™)	25200-056	Sigma-Aldrich, UK

2.1.4 Primary antibodies.

Antibodies used during this study are summarised in (Table 2.4).

Table 2.4: Primary antibodies with species, dilutions, catalogue number and supplier details.

Antibodies	Species/ isotype	Catalogue number	Dilution	Supplier
AKT	Rabbit polyclonal	# 9272	1:1000	CST-UK
BAD	Rabbit polyclonal	# 9239	1:1000	CST-UK
BAK	Rabbit polyclonal	# 12105	1:1000	CST-UK
BID	Rabbit polyclonal	# 2002	1:1000	CST-UK
BIM	Rabbit polyclonal	2933#	1:1000	CST-UK
C5a	Mouse polyclonal	Ab11876	1:100	Abcam
Caspase 3	Rabbit polyclonal	9665#	1:1000	CST-UK
Caspase 7	Rabbit polyclonal	12827#	1:1000	CST-UK
Caspase 9	Rabbit polyclonal	9508#	1:1000	CST-UK
Cleaved caspase 3	Rabbit polyclonal	9664#	1:1000	CST-UK
Cleaved caspase 7	Mouse polyclonal	8438#	1:1000	CST-UK
Cleaved caspase 9	Rabbit polyclonal	7237#	1:1000	CST-UK
Cleaved PARP	Rabbit polyclonal	5625#	1:1000	CST-UK
IL-1α	Rabbit polyclonal	50794#	1:1000	CST-UK
P53	Mouse polyclonal	Ab131442	1:1000	Abcam
P90rsk1	Rabbit polyclonal	AHP2710	1:2000	Bio-Rad
P-AKT	Rabbit polyclonal	9271#	1:1000	CST-UK
PARP	Rabbit polyclonal	9542#	1:1000	CST-UK
P-Bcl-2	Rabbit polyclonal	2827#	1:1000	CST-UK
Phospho-BAD	Rabbit polyclonal	5284#	1:1000	CST-UK
P-MAPK	Rabbit polyclonal	4370#	1:2000	CST-UK
PUMA	Rabbit polyclonal	12450#	1:1000	CST-UK
Ran	Rabbit polyclonal	ab155103	1:2000	Abcam
β-Actin	Mouse polyclonal	A531	1:10000	Sigma

2.1.5 Secondary antibodies

Secondary antibodies used against primary antibodies are listed in (Table 2.5).

Table 2.5: Secondary antibodies with their dilution, species and supplier.

Product Antibody	Species/isotype	Catalogue Number	Dilution	Supplier
Anti-Rabbit IgG HRP conjugate	Donkey	NA934	1:2000	Dako, UK
Anti-Mouse IgG HRP conjugate	Sheep	NXA931	1:10000	Dako, UK

2.1.6 Glass and plastics

The glass and plastic consumable material used are listed in (Table 2.6).

Table 2.6: Equipment and consumables and their supplier

Equipment and materials	Supplier
10 ml glass pipettes	Sarstedt, Germany
24-well microtitre plate	Sarstedt, Germany
25 ml glass pipettes	Sarstedt, Germany
6-well microtitre plate	Sarstedt, Germany
96-well microtitre plate	Sarstedt, Germany
CCD camera	Leica, Wetzler, Germany
Cell culture flasks	Corning, Fisher Scientific, UK
Centrifuge mistral 3000	MSE, GMI, Albertville, USA
Cover glass	VWR, UK
Coverslip	Poole, UK
Cryovials	Sarstedt, Germany
Electrophoresis power supply	Bio-Rad, UK
Electrophoresis tank	Bio-Rad, UK
End frosted slides	VWR, UK
Eppendorf® tubes	Sigma, UK
Flow digital incubator	Flow Labs, UK
Fluorescent microscope	Leica, Wetzler, Germany
Freezer -20°C	Sanyo, ultra-low, Japan
Freezer -80°C	Sanyo, ultra-low, Japan
Fume hood	Maich-Aire, Bolton, UK
Ice maker (Scotsman AF 100)	Namur, Belgium
Light microscope	Nikon, Japan
Liquid nitrogen dewar	Bio-star, UK
Magnetic stirrer SMI	Stuart Scientific, Essex, UK
Microcentrifuge	Sanyo-MSE, GMI, Albertville, USA
Microscope	Leica, Wetzler, Germany
Neubauer haemocytometer	Poole, UK
Neubauer improved haemocytometer	Sigma, UK
pH meter	Dunmow, UK
Pipette tips 10, 200, and 1000ul	Sarstedt, Germany
Pipettes	Gilson, USA
Slides	BD H Merck, Poole, UK
Sterile 15 ml tubes	BD Swindon, UK
Sterile 30 ml universals	BD Swindon, UK
Sterile 50 ml tubes	BD Swindon, UK
T25 tissue culture flasks	Sarstedt, Germany
T75 tissue culture flasks	Sarstedt, Germany
Water bath	Grant instruments, UK

2.1.7 Genes classified based on function

Table 2.7: List of genes and primers sequences used in quantitative RT-PCR. All were selected from NCBI primer bank.

Gene	Primer sequences	Tm °C	Size (bp)
AKT1	*F (5-3') AGC GAC GTG GCT ATT GTG AAG*	71	6527.2
	R (5-3') GCC ATC ATT CTT GAG GAG GAA GT	72	7104.6
AKT2	*F (5-3') AGG CAC GGG CTA AAG TGA C*	70	5887.8
	R (5'-3') CTGTGTGAGCGACTTCATCCT	70	5143.5
AKT3	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8
B2M	*F (5-3') CCA AGG AAG GCG TCT AAG GC*	72	61777
	R (5-3') CTT TCG AGC GCA ACC ACT TTG	71	6358.2
Bcl-2	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8
C5a	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8
IL1B	*F (5'-3') ATG ATG GCT TAT TAC AGT GGC AA*	68	7103.6
	R (5'-3') GTC GGA GAT TCG TAG CTG GA	70	6214
IL-1α	*F (5'-3') AGA TGC CTG AGA TAC CCA AAA CC*	71	7011.6
	R (5'-3') CCA AGC ACA CCC AGT AGT CT	69	6032
Mcl-2	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8
Ran	*F (5-3') TCT GGC TTG CTA GCA AGC TCA*	69	6438.2
	R (5-3') GCT GGG TCC ATG ACA ACT TCT	70	6398.2
VEGFR1	*F (5'-3') TTTGCCTGAAATGGTGAGTAAGG*	70	7629.54
	R (5'-3') TGGTTTGCTTGAGCTGTGTTC	71	4533.1
VEGFR2	*F (5'-3') GGCCCAATAATCAGAGTGGCA*	72	5555.7
	R (5'-3') CCAGTGTCATTTCCGATCACTTT	69	4678.1

Note: (NCBI) National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA (NCBI 2018).

2.2. Methods

2.2.1 Cell culture

A class II microbiological cabinet swabbed with 70% ethanol was used for aseptic work (Cree 2011).

There are two methods to manipulate gene expression. Firstly, the knockdown RNAi method by the short interfering siRNA or the short hairpin RNA shRNA or secondly the newer knock-out methods that contain the transcription activator-like effector nucleases (TALENs) (Joung and Sander 2013), zinc finger nucleases

(ZFN) (Lander et al. 2016), and the clustered regularly interspaced short palindromic repeats CRISPR/Cas9 method. The RNA portion of the CRISPR is called a director RNA that directs enzyme Cas9 to attack the DNA sequence. Cas9 cuts the genome at this site to edit the genomic materials. CRISPR can create deletions and/or insert new DNA sequences in the genome materials of cells (Varshney et al. 2015). The scientists create isogenic cell lines *in vivo* through a technology called homologous recombination to manipulate the gene by knock-in or knock-out (Li et al. 2020).

To achieve homologous recombination in the research laboratory, one must produce and separate DNA fragments possessing genome sequences that share of the genome characteristic that is to be modified. These separate fragments can be inserted into single cells or taken up by cells consuming special chemicals. Meanwhile, after insertion into a cell, these fragments of DNA can recombine with DNA of cells to substitute the targeted part of the genome materials (Li et al. 2020). This technology compares between the normal and the mutated cell lines.

2.2.1.1 Growth medium

According to the specifications of each cell line (Cree 2011), Roswell Park Memorial Institute (RPMI-1640) medium and Dulbecco's Modified Eagle Medium (DMEM (1X) +Glutamine MAX), were used for cell culture. 5 ml L-glutamine (2 mM final concentration) and 10% (v/v) fetal calf serum (FCS) were added to 500 ml medium, which was kept in storage in aliquots at 4-8°C.

2.2.1.2 Cell revival from liquid nitrogen

To ensure long-term storage (Cree 2011), human CRC cells were frozen in liquid nitrogen. About 1×10^6 cells in the dimethylsulphoxide-supplemented medium were present in every cryovial (Nunc) in liquid nitrogen (Cree 2011). After removal from liquid nitrogen, a cryovial was subjected to 60 second incubation at room temperature before being placed in a 37°C water bath until complete defrosting. Before it was opened, the cryovial was wiped with 70% (v/v) ethanol, and the defrosted cells were gradually supplemented with an equivalent amount of RPMI-1640 medium that had been previously warmed. The cell suspension was moved into a 30 ml sterile plastic tube with 10 ml RPMI-1640 medium. The cells were subjected to 5 minutes pelleting through 1000 rcf centrifugation. After removal of

the freezing medium, suspension of the cell pellet in 10 ml of new RPMI-1640 was performed again before being moved to a T75 cell culture flask and placed in an atmosphere of 37°C and 5% CO_2.

2.2.1.3 Cell maintenance and sub-culturing

Different human CRC mutant types and wild types were cultured in a sterile micro-flow class II cabinet (Cree 2011).

According to cell type, cell maintenance took the form of monolayer cultures in the T75 flask (75 cm^2 cell culture, 10 ml complete RPMI-1640 medium enhanced with 10% v/v FCS, 200 mM L-glutamine and 100 mM sodium pyruvate, or DMEM (1X) +Glutamine MAX.

The cell maintenance conditions were in an atmosphere of 5% CO_2 and 37°C. Passaging of the cells was performed when they reached 75-80% confluence when the medium was eliminated and the flask contents were rinsed twice with 10 ml PBS. Two ml of 0.25% trypsin-EDTA solution was afterwards used for trypsinisation followed by 5-minutes of incubation at 37°C. Trypsin/EDTA action was deactivated through the addition of 10 ml medium upon cell detachment from the flask (Cree 2011). The trypsinised cells were subjected to 5-minute 1000 rcf centrifugation at room temperature. After elimination of the supernatant, a cell pellet suspension in 10 ml of fresh culture medium. Subsequently, the cells were counted, and fresh T75 flasks with a suitable volume of complete medium were seeded with the specified quantity of cell suspension, followed by incubation (Van Meerloo et al. 2011).

2.2.1.4 Cell counting

Ten microliters (10 µl) of cell suspension was added to each chamber of a Neubauer haemocytometer, and the cells were counted in ten one mm^2 areas of the haemocytometer using an inverted microscope (Olympus, CK2, X20 objective lens magnification) (Cree 2011), and a mean count was determined (Van Meerloo et al. 2011). cell count was calculated to be (mean of 10 cell count) × 10^4cells/ml medium (Van Meerloo et al. 2011).

2.2.1.5 Cell line frozen stocks

20% (v/v) serum and 7-10% (v/v) cryoprotectant dimethylsulphoxide (DMSO) in RPMI-1640 medium made up the freezing medium employed to produce frozen

cell stocks. Cells were left to grow in the T75 flask until around 70% confluence, after which the medium was eliminated and 5 ml ice-cold PBS was used for cell washing. Trypsinisation allowed scraping of cells from the flask (Cree 2011), followed by addition of growth media and transfer into a 30 ml sterile plastic tube. The cells were subjected to 5-minute pelleting through 1000 rcf centrifugation, followed by re-suspension in 0.5 ml freezing medium A (20% (v/v) serum in RPMI-1640 medium). This was followed by addition of about 0.5 ml freezing medium B (20% (v/v) serum, 18% (v/v) DMSO in RPMI-1640 medium). This approach helped to avoid cells suffering toxic shock due to being exposed too rapidly to cryoprotectant. According to the quantity of pellet cells, 1 ml aliquot was added to each cryovial. The cryovials were frozen overnight at -80°C before being moved to liquid nitrogen to be stored over the long-term (Doyle and Griffiths 1998; Van Meerloo et al. 2011).

2.2.1.6 MBZ preparation,concentration and treatment conditions

Preparation of 10 mM stock solution involved weighing and dissolving 0.45 mg Ran inhibitor (MBZ) (Sigma-Aldrich, St. Louis, MO) in 1000 µl DMSO and then stored at -80°C. Additional serial dilution was undertaken to prepare the target concentration for loading on the test plates.

The concentrations of MBZ were selected according to the published data and outcomes of the MTT assay. The dose response curve of MBZ cells treated with MBZ for 48 h is shown in (Figure 4.1 to Figure 4.3) with the IC_{50} at 0.5 µM. The concentration of MBZ selected is 0.5 µM for all the treatments throughout this thesis to make sure that none of the detected effects were due to cytotoxicity.

The IC_{50} in previous studies show that MBZ inhibits cell proliferation of two commonly used HNSCC cancer cells lines CAL27 and SCC15 at IC_{50} 0.2 µM and at 0.5 µM respectively (Zhang et al. 2017). MBZ at 0.3 and 10 µM, significantly decrease levels of VCAM-1, IL6 compared to its control (Rubin et al. 2018). Another *in vitro* study showed that MBZ inhibited cell proliferation in human CRC cell lines between 0.20-0.81 µM (Guerini et al. 2019).

2.2.1.7 MTT assay

The principle underlying the MTT assay is the capacity of viable cells to generate an insoluble purple formazan product from a soluble yellow tetrazolium salt (MTT:

3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide) via mitochondrial succinate dehydrogenase. The viable cell count determines the quantity of formazan generated. After dissolving the product in DMSO, spectrophotometry is performed to measure solution absorbance (Mosmann 1983; Van Meerloo et al. 2011).

2.2.1.8 MBZ cytotoxicity assessment

An MTT assay (Sumantran 2011) was conducted to determine how cytotoxic MBZ was for isogenic pairs of the human colorectal cell lines HCT-116 *K-Ras* Wt. Vs. HCT-116 *K-Ras*, DLD-1 *K-Ras* Mt. vs DKO-3 Wt. and HCT-116 *Pten* -/- vs. HCT-116 Wt. 200 µl RPMI-1640 medium was introduced in the first lane of a 96-well plate, acting as blank. 1×10^4 /ml tumour cell lines were plated in a final volume of 180 µl medium in the following lanes and subjected to 24 h incubation at 37°C and humidified atmosphere with 5% CO_2. 20 µl MBZ in various concentrations 0.05, 0.1, 0.25, 0.5 and 0.75 µM was added to every seeded well besides the blank control, with ongoing drug exposure for 24, 48, 72 and 96 h at 37°C and 5% CO_2. The drug was changed with fresh medium at 24 h intervals for the wells that assessed for 48, 72 and 96 h.

Post-incubation medium elimination was followed by addition of 20 µl MTT solutions (5.0 mg/ml) and 180 µl fresh medium in all wells. The plate underwent 4 h incubation under the same conditions, followed by 5-minute 1000 rcf centrifugation (Sumantran 2011). Subsequently, the supernatant was eliminated and 150 µl DMSO was introduced per well, mixing energetically with the pipette to break down the formazan crystals. A Multiskan Plus spectrophotometer (Lab-systems Group, UK) was employed to measure solution absorbance at 540 nm. Subtraction of the mean background absorbance (i.e., an average of the blank well) from the test well mean absorbance gave the adjusted mean absorbance for every concentration, and an associated dose-response curve was created. The least squares method was applied in Microsoft Excel to determine the IC_{50} values for compound against every cell line. All sample testing and all experiments were done in triplicate. Untreated wells of each cell lines were used as the control for calculation % of cell viability.

2.2.1.9 MTT assay reagent preparation

Preparation of 12 mM MTT stock solution involved the addition of 20 ml sterile water to 100 mg MTT, followed by vortex mixing until dissolution. Filtration or centrifugation was performed to eliminate the un-dissolved material. The prepared MTT solution was stored in the dark for up to a month at 4°C.

Data analysis

The percentages of growth deficit were calculated as:

Percent of growth inhibition = 100 × (mean optical density of control isogenic cells 0.1% DMSO) – (mean optical density of treated isogenic cells with tested MBZ concentration)/ (mean optical density of control isogenic cells 0.1% DMSO). This equation was employed to measure the cytotoxic action of MBZ through assessing the half maximal cytotoxic effect concentration (CTC50; µmol/L) (Bhattacharjee et al. 2019).

IC_{50} calculation

IC_{50} value for MBZ inhibitor for each cell lines were calculated from the following equation:

$IC_{50} = (50-A)/ (B-A) \times (D-C) +C$

Where:

A= the first point on the curve, stated as % inhibition, that is less than 50%.

B= the first point on the curve, stated as % inhibition, that is greater than or equal to 50%.

C= the concentration of inhibitor that gives A% inhibition.

D= the concentration of inhibitor that gives B% inhibition.

2.2.1.10 Scratch assay seeding density

The scratch assay relies on cell density/well to obtain a pre-scratching confluent monolayer. Therefore, the ideal seeding concentration for every cell line was established by seeding the cell lines in 24-well plates at varying concentrations for 24, 48 and 72 h. At first, the cell lines were seeded in concentrations of 1×10^4, 1×10^6 and 1.5×10^6 a cells /ml and underwent 24 h incubation at 37°C and 5% CO_2. Concentration appropriateness for seeding was confirmed if a monolayer became visible after 24 h and reproducible formation of a confluent monolayer occurred. If this did not happen, the cell lines were subjected to further incubation. Confluent monolayer formation was achieved by different cell lines at varying seeding concentrations and incubation intervals (Figure 2.1).

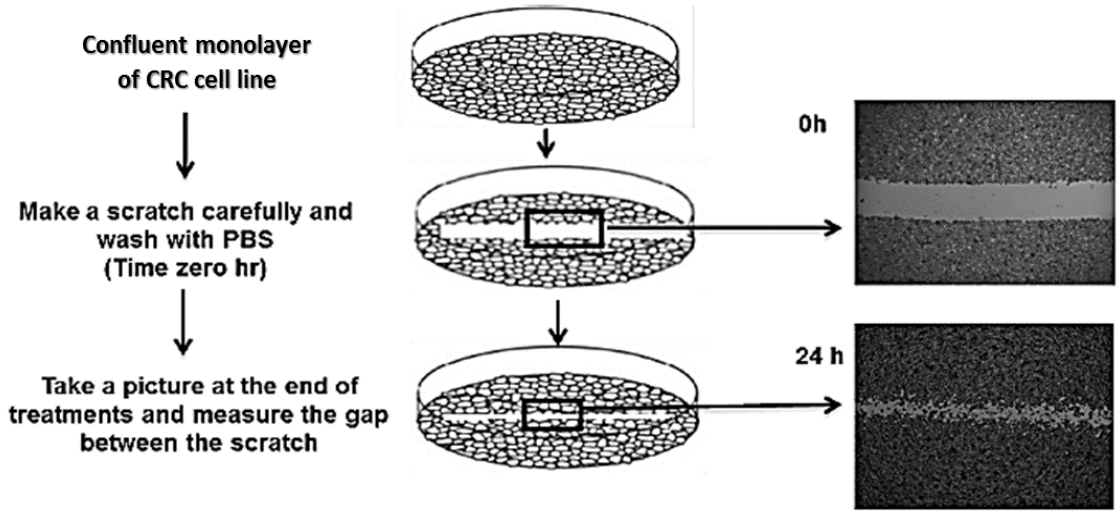


Figure 2.1: Schematic diagram of migration assay
Three hundred cells of isogenic CRC were seeded into each well of 6-well plate while waiting for it to become fully confluent. Later after the full confluency happens, the wound was made with a 200 µl tip and then washed with phosphate buffer saline solution to remove the debris. Finally, the seeded wells were treated with different compound concentration and time interval. At the start time (0 h) and the end time (24, 48 and 72 h), the pictures were taken, and the gap between the two edges of the scratch was measured with the help of an image J processing program designed for scientific multidimensional images. Modified from (Eccles et al. 2005; Decaestecker 2007; Moreno-Bueno et al. 2009).

Data analysis

All experiments were repeated three times independently unless otherwise specified. Quantitative analysis of chem. Then was conducted via Image J software. Measurement of distances between scratch sides at particular intervals involved comparison of images from time (0h) to the final point of wound closure was calculated by formula (CFA0-CFA1)/CFA0×100, and the outcome was

contrasted against control to determine the migration proportion (wound closure% with compound/wound closure% of control) ×100 where CFA0 and CFA1 denote wound size or cell-free area, graphs were drawn with GraphPad software.

The results are presented differences between two groups were evaluated with unpaired *Students t* test (Two tailed) as the mean and ±SD of three independent experiments and *p* value also calculated by unpaired *student t*-test (Two tailed) as the mean ±SD of three independent experiments P (***$p<0.001$* were statistically highly significant, P **$p<0.01$* and, * indicates $p<0.05$ was statistically significant.

CFA0 =Area of wound at zero h.

CFA1= Area of wound at different times interval h.

2.2.1.11 Invasion assay (Transwell invasion assay)

In a transwell invasion assay a micro porous insert membrane lined with extra cellular matrix (ECM) is widely used. The ECM obstructs the membrane holes to prevent non-invasive cell lines from traveling through the membrane. In contrast, the highly invasive cell lines can dissolve ECM by a proteolytic enzyme such as MMP-collagenases and move through the ECM layer and adhere to the bottom of the micropore membrane (Figure 2.2). There are several detection methods depending on the migrated cell lines which have passed through the membrane pores. One of them is, some of them involved cotton swabbing for non-invasive cells on the top of insert then staining invasive cells with DAPI and counting them with a light microscope. A more widely accepted method which is a Boyden chamber assay where the cell lines are detached, lysed, and stained using fluorometric detection. The method gives broad accessibility to several cell culture sizes and inserts. This technique is an endpoint assay and has disadvantages if simple cell staining processes are used. Non-invaded cells, which stay on the upper side of the transwell insert, must be removed prior to staining of the invasive cells at the bottom of the membrane. This is generally done by eliminating the cells with a cotton swab, which often turns out difficult, non-quantitative and of variable success. I strongly suggested using fluorescent dyes then lyse the cells and quantify them in a plate reader for reliable results. The transwell invasion assay is the most regularly involved and has been used

to assess human colon and rectal cancer (Zhang et al. 2012), invasion melanoma (Kreiseder et al. 2013). QCM24-well transwell™ cell invasion assay fluorometric kits provided by Merck-Millipore are generally involved, because of their well-standardised assay requirements and protocols.

2.2.1.12 Invasion boyden chamber assay

A Cell invasion assay was carried out using the QCM24-well transwell™ cell invasion fluorometric kit assay provided by Merck-Millipore with ECMatrix™-coated inserts, according to the manufacturer's instructions. This kit offers an efficient system for a quantitative assessment of the invasion of tumor cells line throughout a model basement membrane.

Suspended cells at a concentration of 1.0 $\times 10^6$ cells/ml of HCT-116 *K-Ras* Mt. and Wt. were prepared in serum-free medium. The plates and reagents were kept at 25°C before starting the essay for best results. The experiment was performed in a cell culture hood and insert holder with forceps was disinfected with 70% ethanol. Then 300 µl of prewarmed 0% serum medium was added to the inside of the inserts. This was left for 30 minutes at Rt to rehydrate the ECM layer. Subsequently, 250 µl of medium was discarded from the inserts carefully to prevent the microporous membrane from distressing. 250 µl of the 1.0 × 10^6 cells/ml cell suspension was loaded inside each insert. Into the lower chamber, 500 µl of 10% serum medium was inserted. The plate was incubated at 37°C in a CO_2 incubator for 24 - 72 h. At the end of experiment cell suspension was pipetted out from inside the insert and 225 µl of prewarmed cell detachment reagent was added to the clean well and then the invasion chamber was placed in. The insert was incubated at 37°C for 30 minutes The cells were completely moved to detached solution by tilting the insert several times back and forth through the incubation period. The insert was removed from the well. A mixture of 300 µl Lysis buffer and 4 µl CyQuant GR dye regent was prepared and 75 µl of this solution was added to each well having invaded cells with 225 µl cell detachment solution. This green-fluorescent dye shows intense fluorescence augmentation when attached to cellular nucleic acids. Plates were incubated for 15 minutes at Rt. 200 µl of the mixture was transferred to a 96-well fluorescence plate for quantification using a fluorescence plate reader with a 480/520 nm filter.

The fluorescence measurements were reported as RFU (relative fluorescence unit) values.

2.2.1.13 Transwell assay manual technique

HCT-116 *K-Ras* Mt. and Wt. cells were grown in RPMI-1640 of 0% serum for 24 h prior to testing. Cell suspensions of 150 µl (67×10^4 cells/ml) (diameter 6.6 mm, pore size 0.8 µm) were placed into the insert from ThinCert greiner bio-one™ in 0% serum medium. 600 µl of medium with chemoattractant 10% serum was added to the lower plate well. The drug at concentrations of 0.5,1 or 2 µM of MBZ was added to the suspension cell into the upper part of the insert and 0.1% of DMSO was added to control well. The plate was incubated for 48 h at 37°C, 5% CO_2. Subsequently, the suspension cells were discarded, and the inner of the insert was rubbed with a cotton swab to removed non-invasive cells on the top of the insert and fixed with 70% v/v ethanol. The membrane was cut on a tissue slide then invasive cells were stained with DAPI and counted with a light microscope**.** The total numbers of invasive cells on the insert membrane were calculated in four random fields and the percent of control was calculated (total invasive cells number treated with MBZ multiplied by 100 then divided by the total invasive cell number of control).

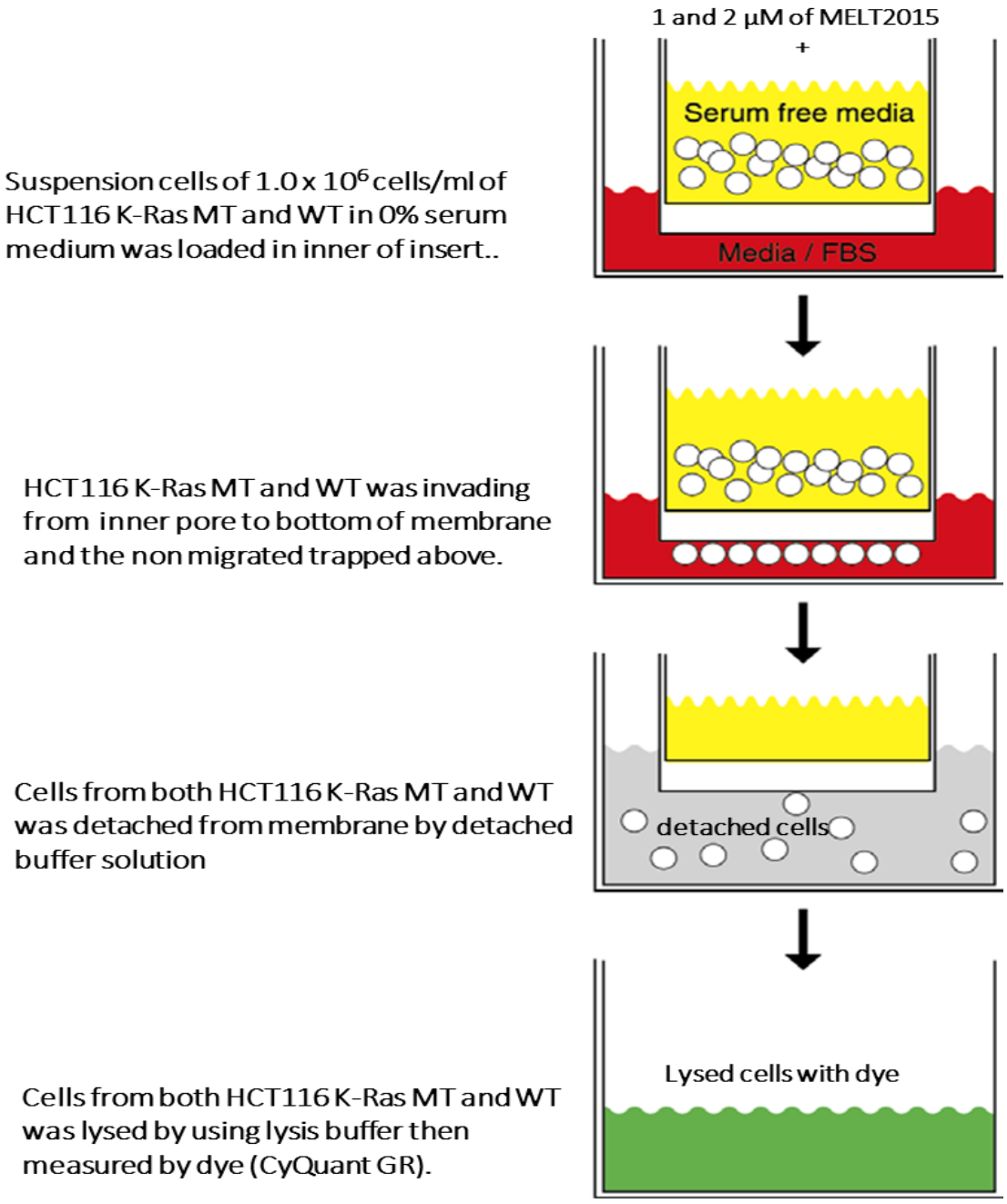


Figure 2.2: Schematic diagram of the cell invasion assay.

Data analysis

All experiments were repeated three times independently unless otherwise specified. Statistical analyses were achieved using Excel software. The results are presented as differences between two groups, assessed by a *Students t* test (Two tailed) as the mean ±SD of three independent experiments P (****p<0.001* were statistically highly significant, P ***p<0.01* and, * indicates *p<0.05* was statistically significant.

Pictures of the migrated cells were taken a Leica fluorescence microscope at 200× magnification power and using five representative fields to each membrane. The migrated cells were calculated by means of Image J software as follows: the images were opened through Image J software, then converted from the blue channel to binary and counted. The mean number of cells was calculated from three independent experiments.

2.2.1.14 Colony formation assay

To achieve adherence to the plastic surface 24 h prior to treatment approximately 300 cells were added to a 6-well plate in complete medium, and equal distribution was ensured by shaking the plate. After 24 h, the growth medium was substituted with drug-containing medium to expose the cells to the drug. After exposure and a change of medium, the cells were incubated for 10-12 days under conditions of 37°C and 5% CO2 (Sumantran 2011). Subsequently, the colonies became macroscopically observable, at which point the culture was terminated through medium elimination and using PBS to wash the cells twice. 95% methanol and glacial acetic acid (1:7) was used for 10 minutes to fix the colonies (Guzman et al. 2014), which were then dried and stained with 0.5% crystal violet for 45 minutes. Water was then used to wash the plate thrice, after which it was left to dry. The stained plates were imaged, and the colonies were counted after they incubated for 10-12 days at 37°C. The dose-response curves were used to determine the 50% inhibiting dose. Every treatment was conducted thrice. Colony fixation (Guzman et al. 2014) solution was prepared in a falcon tube where 1 ml glacial acetic acid and 7 ml methanol were mixed. Crystal violet (Guzman et al. 2014) solution was made by dissolving 0.5 g of crystal violet powder in 100 ml distilled water.

Data analysis

All experiments were repeated three times independently unless otherwise specified. Pictures of the colonies cells were taken using a Sony camera to each well. The colonies cells were counted by means of Image J software by opening the images through Image J software (Guzman et al. 2014), then converted to the blue channel to binary and counting. Statistical analyses were achieved using Excel software. Colony formation was normalised to DMSO controls for cell lines in the presence MBZ.The differences between two groups were assessed with an unpaired *Students t* test (Two tailed) as the mean ±SD of three independent experiments P (***$p<0.001$ were statistically highly significant, P **$p<0.01$ and, * indicates $p<0.05$ was statistically significant.

2.2.2 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was carried out to analyse gene expression. This was quantified by SYBR Green qPCR Master Mix method probes using SYBR select Master Mix on a Real Time PCR system (Primer Design). Relative expression was quantification by using the comparative Ct method (2-ΔCt). Transcript level of B2M was employed as an endogenous control. The genes selected for expression analysis were purchased from (Invitrogen by Thermo Fisher Scientific), and their sequences forward (5′-3′) and reverse (5′-3′) and annealing temperatures applied for qRT-PCR are listed in (Table 2.7).

2.2.2.1 Cell harvesting

HCT-116 *K-Ras* Mt. and Wt. cells exposed to 0.5 µM of MBZ for 48 h were washed with cold PBS and separated from the flask surface using trypsin solution, then cells were collected in a 20 ml universal tube and centrifuged at 1,000 rpm for 5 min. The RPMI-1640 medium was discarded, and the pellet was washed with PBS 5 ml. The cell lysates were moved to a 1.5 ml Eppendorf tube and centrifuged at 3000 rpm for 5 minutes. Subsequently, PBS was discarded, and the RNA extraction procedure carried out for the qRT-PCR technique.

2.2.2.2 RNA isolation and cDNA synthesis

Total RNA was extracted from HCT-116 *K-Ras* Mt. and Wt. by using RNase mini kit reagent (Qiagen Valencia, CA, USA). The quantity of RNA was assessed using a Nano Drop (Shimadzu Biotech), then one micrograms of total RNA were

reverse transcribed and amplified with gene-specific primers using Nano Script 2RT reverse transcriptase PCR kit (Primer design). These are listed in (Table 2.8) and (Table 2.9).

Table 2.8: List of annealing step reagents

Component	One reaction
RNA template up to 1 µg	× **µl**
RT primer yellow	**1 µl**
RNase/DNase free H_2O	× **µl**
Final Volume	**10 µl**

The equivalent amount to 1µg of RNA template sample from each HCT-116 *K-Ras* mutant and HCT-116 *K-Ras* wild type cell line after quantification was added to reagents in (Table 2.8), then to a thin walled 0.2 ml labelled PCR tubes RNase/DNase free H_2O was added to complete the final volume of reaction to 10 µl. Then a lid was applied to each tube and at that point the reaction was carried out at 65°C for 5 minutes by using a thermal cycler (MJ Research PTC-200 Peltier). Subsequently, the 0.2 ml PCR tubes were cooled in an ice bath.

Table 2.9: List of extension step reagents

Component	One reaction
nanoScript2 4× Buffer Black	**5 µl**
dNTP mix 10 mM Orange	**1 µl**
RNase/DNase free water White	**3 µl**
nanoScript2 enzyme White	**1 µl**
Final Volume	**10 µl**

10 µl of each reaction was prepared according to the manufacturer protocol as in (Table 2.9).

10 µl of this mixture was added to each to a thin walled 0.2 ml labelled PCR tubes on ice. Their lids were shut tightly, and each PCR tube was vortexed and incubated at 42°C for twenty minutes by using a thermal cycler (MJ Research PTC-200 Peltier). They were then incubated at 75 C^{o} for ten minutes and at the end of the reaction cDNA was kept at -20°C until use. Complementary DNA (20 µl) was diluted in 80 µl of distilled water.

2.2.2.3 qRT-PCR

The qRT-PCR 96-well plate (MicroAmpTM) was set up and the samples loaded in triplicate for target genes using B2M as a housekeeping gene in a UV-irradiated hood of ABI 7500 Real-Time PCR system (Applied Biosystems®). Relative expression was assessed by a comparative Ct method (2-ΔCt). The analysis was carried out by using reactions of total volume 20 µl containing of 1 µl of each primer mixed together (forward and reverse) for the target genes e.g. Ran gene and reference housekeeping gene B2M. 10µl, of SYBRGreen from Primer Design 2x Precision Plus Master Mix, RNase/DNase free water 4 µl (Primer Design) and template cDNA 4 µl. Finally, 16 µl of this mixture in (Table 2.10) was loaded in each well plus 4 µl of template cDNA, subsequently the plate was closed with plastic plated sealer then centrifuged for 2 minutes at 1000rpm. Each assay was carried out 3 times in triplicate according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments- MIQE Guidelines (Bustin et al. 2009). The calculation of expression gene level was performed by the delta delta threshold cycle (2 - ΔΔCT) method and the gene of interest expression level is stated relative to the gene of reference for each sample (Schmittgen and Livak 2008).

Table 2.10: List of reagents for quantitative RT-PCR

A. Reaction setup

Component	One reaction
Maxima SYBRGreen/ROX qPCR Master Mix 2X	**10 µl**
Forward Primer	**1 µl**
Reverse Primer	**1 µl**
Water nuclease free	**4 µl**
Template cDNA	**4 µl**
Total Volume	**20 µl**

Table 2.11: Thermal cycling conditions

B. Thermal cycling conditions

A Three step cycling protocol was used.

Step	Temperature ℃	Time	Number of cycles
Optional: UDG pre-treatment	**50**	**2 min**	**1**
Initial denaturation	**95**	**10 min**	**1**
Denaturation	**95**	**15 s**	**40**
Annealing	**60**	**30 s**	
Extension	**72**	**30 s**	

Data analysis

All PCR data are reported as mean ± SEM. All tests were repeated at least 3 times independently. Statistical analysis between two groups was achieved using *Student's t*-test and was used to calculate the statistical significance of differences. The values for *$p<0.05$ was defined as statistically significant P ***$p<0.001$ were highly significant, P **$p<0.01$ was statistically significant. Prism 6 software was used for drawing the graph. Control (Un-treated sample) was considered equal to one and treatment with different drugs concentrations were calculated consequently.

Calculation was carried out through software bio systems applied using following equation:

Algorithm ΔΔCt

1. ΔCt = Ct (gen of interest) – Ct (housekeeping gene like GAPDH).
2. ΔΔCt = ΔCt (treated sample) – ΔCt (average of non-treated control).

Doubling of molecules in every cycle:

$$F = 2^{-\Delta\Delta Ct}$$

2.2.3 Western blot

Western blotting, also called protein blotting or immunoblotting is used to detect the existence of a particular protein in total proteins extracted from whole cells or tissues. Fluorescent western blotting allows several proteins to be detected and measured in one sample with fluorescent antibodies with high sensitivity and specificity.

2.2.3.1 Immunoblotting

2.2.3.1.1 Extract proteins from whole cell lysates

A whole-cell lysate from growing cells was collected by trypsinisation. Cells were washed with ice-cold PBS and lysed on ice by RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 1% (v/v) NP- 40, 0.5% (w/v) deoxycholate, 150 mM NaCl, and 0.1% (w/v) SDS, supplemented with sodium orthovanadate, phenylmethylsulphonyl fluoride with phosphatase cocktail I, II inhibitors and protease inhibitors to lyse the cells. The lysate was centrifuged at 13,000 rpm for 15 minutes at 40°C to remove cellular debris and then the supernatant was transferred to a fresh centrifuge tube. A Bradford protein assay was carried out to measured protein concentrations of the cell lysates at OD 595. Total proteins were immediately exposed to SDS-PAGE and western blot analysis or stored at -20°C overnight or, for longer-term storage, transferred to -80°C freezers.

2.2.3.1.2 Determination of protein concentration (Bradford assay)

The Bradford assay was established by Marion M. Bradford (Bradford, 1976). It is a colorimetric protein assay that measures the protein concentration of samples. The samples subsequently undergo a colorimetric measurement at 595 nm wavelength with a spectrophotometer. To determine the actual concentration of a protein, a standard curve was plotted using absorbance and a varying amount of some known protein. Bovine serum albumin (BSA) 2mg/ml was used as standard. A range of masses of BSA (0, 2, 1, 0.5, 0.25, 0.125 and 0.0625 mg) were dissolved in total volume 50 µl of water. A mass of 2 mg BSA in 50 µl of water was added to Eppendorf tube which labelled with 2 mg and similarly for 1 mg, 0.5 mg BSA and so on. Subsequently, a suitable amount (1.5 ml) of Bradford reagent was added to all Eppendorf tubes (standard and samples) then mixed gently using pipette and vortex and then left them for 15 minutes. Later a 1 ml of each tube was transferred to a cuvette to measure the absorbance by multiskan spectrometry at 590 nm. A standard curve was produced by the Excel program based on a linear regression. Consequently, the proteins concentration in all unknown samples was determined by plotting from the BSA data to generated standard curve and calculated using the equation shown below in (
Figure **2.3**).

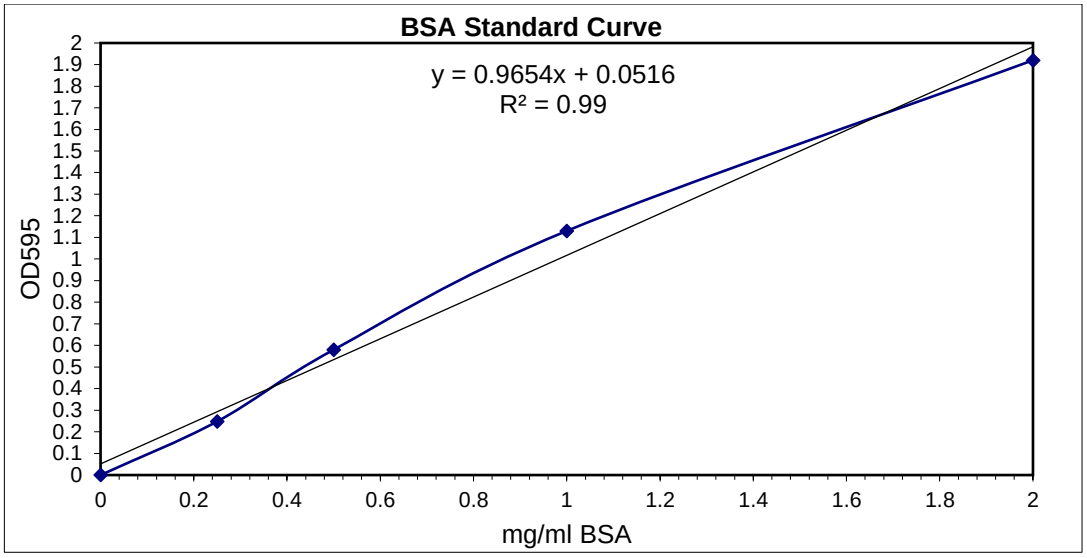


Figure 2.3: Standard curve for protein concentration determination by the Bradford assay.

2.2.3.1.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The process by which large molecules such as proteins are separated by an electric field is called electrophoresis. A polyacrylamide gel is used as an adjuvant and SDS breaks down the proteins. This technique is called SDS-PAGE gel electrophoresis. The molecular weight is the basis of separation of the proteins. SDS removes secondary structures in the protein and provides a negative charge for the proteins, so that the distance which the protein travels is related to the size of the protein and, thus, its molecular weight.

2.2.3.1.4 Sample preparation for SDS-PAGE

Protein extracts of 40 µg were placed into Eppendorf tubes and 1x SDS loading buffer (2% SDS, 62.5 mM Tris pH 6.95, 5% β-Mercaptoethanol and 10% Glycerol) was added to these.

2.2.3.1.5 SDS-PAGE

The gel casting equipment from (Mini-PROTEAN 3 - Bio-Rad, UK) was set-up following the manufacturer's guidelines. SDS-PAGE gel contains two parts, a resolving, and a stacking gel.12% resolving gel was prepared by mixing the ingredients defined in (Table 2.12), and then a cassette gel 1 mm (Bio-Rad) was poured into the gel contents. The gel was left at RT for polymerization, after which isopropanol solution was put on top of the cassette to prevent the gel from forming air bubbles.

Stacking gel was made by mixing ingredients listed in (Table 2.12), and isopropanol solution was discarded and stacking gel poured onto the top of the resolving gel. A 1 mm, 10-well comb was cleaned with 70% ethanol and dried by a clean paper towel, then put in the gel cassette and left the gel to polymerize. The stacking gel was involved in contracting the resolution of the optimum protein. The stacking gel has less % of acrylamide solution and pH than with the resolving gel, which permits the proteins to arrive at the resolving gel.

Table 2.12: Composition of three 12% polyacrylamide gels

Solutions	Resolving gel 12%	Stacking gel
Water	**14.7 ml**	**12.3 ml**
30% Acrylamide mix	**18 ml**	**9 ml**
Tris pH 8.8 (1.5 M)	**11.4 ml**	**--**
Tris pH 6.8 (1 M)	**--**	**2.25 ml**
SDS (10%)	**450 µl**	**180 µl**
APS (10%)	**450 µl**	**180 µl**
TEMED	**18 µl**	**18 µl**

After the stacking gel was set, the gels were installed into an electrophoresis container from Bio-Rad and then fill up with running buffer (25 mM Tris, 190 mM Glycine, 35 mM SDS) then the combs were detached. The protein samples were boiled for 5 minutes at 90°C, then vortexed and introduced in the lanes. 4 µl of Page ruler pre-stained protein ladder was loaded in one lane per gel, and 80 volts was applied to the tank until entering the resolving gel where the voltage increased to 110 V.

2.2.3.1.6 Western transfer

After SDS-PAGE electrophoresis finished, the gel was exposed to electrophoretic transfer to transfer the proteins to the membrane. Western transfer apparatus (Bio-Rad) was set up conferring to guidelines of manufacturers and so western transfer buffer (20% Methanol, 25 mM Tris, 192 mM Glycine) was used to fill the tank. The transfer tank was put it in an icebox to keep the transfer reaction temperature cool until the end of the reaction, and the transfer carried out for 2 h at 300 amp.

2.2.3.1.7 Immunodetection of proteins

Nitrocellulose membranes (Table 2.2) were blocked in 5% semi-fat milk (Table 2.2) in PBST-20 (0.1% Tween), after transfer for 1 h at RT to diminish non-target protein from binding to non-specific antibodies on a rocking platform. Then the membrane was incubated with the specific primary antibody and specific dilution (Table 2.4).

Table 2.4) overnight at 4°C on a rocking platform. The next day, membranes were washed three times with PBST-20 for 15 minutes and incubated for 1 h on a rocking platform with a 1:2000 dilution of the secondary relevant antibody (Table 2.5) and washed again three times with PBST-20 for 15 minutes on a rocking platform. Proteins were visualised using substrate from GE healthcare Amersham™ western ECL Substrate (Table 2.2), following manufacturer's guidelines and developed using a Bio-Rad ChemiDoc™ MP system.

2.2.3.1.8 Densitometry

The protein bands were photographed and then proteins were quantified by using NIH Image J software (NIH Image, Bethesda, MD, USA) (Yousif 2014)**.** The readings of three independent experiments were measured for each band and at that point, each band was normalised to its corresponding band of β-actin. The average of the three readings of each experiment were calculated and used to draw chart bars by GraphPad software. The average and standard deviation readings were assessed by using the values of 3 independent experiments.

2.2.3.1.9 Membrane stripping

Nitrocellulose membranes were swamped in fresh prepared stripping buffer (Mild Stripping Solution, Abcam) and incubated for 15 minutes at RT. Then, the membrane was washed two times with PBST-20 for 10 minutes and two times with PBS and then incubated with specific primary antibody at 4°C on a rocking platform overnight.

2.2.3.1.10 Data analysis

Data are assumed as mean ±SEM. All experiments were repeated at least three times. Statistical analysis between two groups was achieved using *Student's t*-test (Two tailed) and performed using Excel software. The values for $*p<0.05$ was defined as statistically significant P $***p<0.001$ were high significant, P $**p<0.01$ was statistically significant. Prism 6 software was used for drawing the graph. Control (Un-treated sample) was equal to one and the effect of different drugs concentration were calculated consequently.

Pictures of the membrane was taken using Bio-Rad imaging system Doc, were calculated by means of Image J software by first opening the images through

Image J software, then detecting the bands and measuring them. Mean number of bands was calculated from 3 independent experiments.

Chapter 3

Results

Investigate the effect of MBZ on expression levels of genes regulating cell invasion and metastasis in isogenic colorectal cancer cell lines

3. Effect of MBZ on expression of mRNA and proteins that regulate metastasis.

3.1 Introduction

The active *K-Ras* proto-oncogene which occurs due to point mutation exists in about 30%-40% of human CRCs and more than 90% of the mutations are focused in codons 12 and 13 (Shirasawa et al. 1993; Xu et al. 2021). This disease can be modelled in a reduced system using cell lines with similar genetic make-up. The isogenic human cell lines generally refer to two that have precise similar genetic makeup, except for one gene such as *K-Ras* and *Pten* (DeWeirdt et al. 2020). The authors state that the non-transgenic (wild type cell line) and the transgenic cell line result in identical genes. There are techniques available that can modify the DNA of cells, which can be used as a disease model. For instance, cancer cells frequently have alterations in their DNA, and consequently this can be copied into an isogenic cell line (DeWeirdt et al. 2020).

Modification of the DNA means it has potential to generate two isogenic cell lines one with the alterations related to cancer progress and the other without those alterations. These cell lines offer an effective comparison, as it can be hard to find linked cells that express genes in a similar way and are widely used for studying cancer .This will help to find potential novel therapies (DeWeirdt et al. 2020).

This type of cell lines was achieved from (ATCC): American Type Culture Collection and (NCI): National Cancer Institute which can be used to investigate diseases with a genetic basis as a model. Cancer is considered as one of these diseases that can be studied using the isogenic cell line as models. There are a variety of isogenic engineered cell line products and different oncogenic control mutations such as mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) interactive signaling pathways. Many point mutations approximately 150-point mutations engineered through some genes are available such as MAPK pathway line, diverse mutations in *EGFR, K-Ras, Pten* and *B-Raf* (Torrance et al. 2003; Ince et al. 2005; Cairney et al. 2017). So, a good reason to select this type of isogenic cell line with a point mutation such as *K-Ras, Pten* is to study the effect of MBZ on MAPK and Akt pathways and subsequent cell proliferation, metastasis, and migration. The mutation in *K-Ras* will increase Ran-GTPase activity and the cell becomes more dependent to Ran-

GTPase to cope with cell division. Thus, the activated *K-Ras* gene triggers the leading role in colorectal tumorigenesis by transformed cell differentiation and cell growth behaviors (Boudhraa et al. 2020) (Shirasawa et al. 1993). *Pten* deletion mutation would help us study the inhibition effect on the cell proliferation as well as to overcome its biological activity to stop the cell division. Using these two cell-lines the activity of MBZ on mutant cell lines could be studied and compared to their wild type counterparts. CRC tumorigenesis through changed cell differentiation and cell growth was shown after the activation of the *K-Ras* gene (Shirasawa et al. 1993; Ro et al. 2019; Soleimani et al. 2019), so this is another reason to use this type of mutation of isogenic cell line.

The use of parental cell lines as control in this study allows assessing any oncogenic effects following either loss of *Pten* or activating *K-Ras*.

The *K-Ras* mutation makes this gene constitutively active which means it is transcribed frequently that in contrast to the facultative gene, which is transcribed only when they needed (Ganten and Ruckpaul 2006; Mitra et al. 2021).

3.1.1 Mebendazole

MBZ is known as a tubulin inhibitor (Velan and Hoda 2021) and acts through selectively preventing the synthesis of microtubules by binding to β-tubulin through the colchicine binding site, thus blocking of tubulin dimers polymerisation in parasites present in bowel (Lacey 1990; Thakur and Patel 2020). Tubulin consists of two isoform α- and β-tubulins that are essential for formation of microtubules through its polymerisation. The microtubules are the main constituent of the cytoskeleton structure of the eukaryotic cells (Gunning et al. 2015). Microtubules have roles in many critical cellular developments, such as mitosis. So, medications such as MBZ that have the ability to bind to tubulin will eventually lead to cancerous cells to die through preventing microtubule dynamics formation, which is essential for DNA segregation, intracellular transport and consequently pushes the cells to divide (Petersen and Baird 2021). Through depolymerizing of tubulin MBZ may lead to disrupting the functions of microtubules (Gunning et al. 2015).

3.1.2 MBZ in cancer cells

Several studies were carried out to determine the effect of MBZ, on the expression of several genes and pathways using different cell lines. Firstly, MBZ inhibited *VEGFR2* autophosphorylation with 1–10 μM and with an IC_{50} of 4.3 μM of on the human umbilical vein endothelial cell (HUVECs). Other examples of MBZ effects are impaired tumor progression through angiogenesis (Pantziarka et al. 2014), prevented cell proliferation in human CRC cell lines (Guerini et al. 2019).

In previous studies it was shown that the expression of essential proteins in tumor samples that play roles in initiation of adenoma and development of tumor such as Bcl-2 and pro-inflammatory cytokines (such as IL-6 and IL-1β and TNF*)* was inhibited after treatment with MBZ (Guerini et al. 2019; Sung et al. 2019).

An increase in apoptosis markers cleaved caspase -9 and PARP was confirmed on M-14 and A-375 human melanoma cell lines when treated with MBZ (Doudican et al. 2013; Pinto et al. 2015; Pinto et al. 2019). The antitumor effect of MBZ was confirmed *in vivo* by using M-14 xenograft model, the MBZ showed repressed tumor growth (Guerini et al. 2019), MBZ in the nanomolar range prevents kinases activity that are involved in cancer pathways (Simbulan-Rosenthal et al. 2017). MBZ exhibited weak cytotoxicity when used as a single medication. MAPK/ Erk pathway was inhibited in combination, with trametinib which downregulated its downstream targets, and enhanced the expression of indicators of apoptosis (Guerini et al. 2019).

Previous research using MBZ found repressed cell invasion in H295R, and SW-13 cell lines at an IC_{50} of 0.085 μM and induced apoptosis. This was mediated through cytochrome c, caspase -9, and caspase -3 release (Guerini et al. 2019). Nevertheless, MBZ has showed other direct mechanisms on the tumour cell, which includes protein kinase inhibition, pro-apoptotic activity and anti-angiogenesis (Rubin et al. 2018).

To develop strategies of metastasis prevention, it's necessary to an understanding of how cancer cells grow, proliferate, metastasise, and interact with their surrounding environment.

The aim of this chapter was to study potential mechanisms of MBZ as a Ran inhibitor on immune suppression and anti-angiogenic target on isogenic pairs of colorectal cell line rather than its well-known anti-tubulin activity.

3.2 Materials and methods

3.2.1 Selected genes and their sequences

Table 3.1: List of specific primer sequences used in quantitative RT-PCR in this chapter.

Gene	Primer sequences	Tm °C	Size (bp)
A. Reference gene- Control			
B2M	*F (5-3') CCA AGG AAG GCG TCT AAG GC*	72	61777
	R (5-3') CTT TCG AGC GCA ACC ACT TTG	71	6358
B. Inflammatory response genes			
IL1β	*F (5'-3') ATG ATG GCT TAT TAC AGT GGC AA*	68	7103.6
	R (5'-3') GTC GGA GAT TCG TAG CTG GA	70	6214
IL-1α	*F (5'-3') AGA TGC CTG AGA TAC CCA AAA CC*	72	7011.6
	R (5'-3') CCA AGC ACA CCC AGT AGT CT	68	6032
C5a	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8
Ran	*F (5-3') TCT GGC TTG CTA GCA AGC TCA*	70	6438.2
	R (5-3') GCT GGG TCC ATG ACA ACT TCT	72	6398.2
C. Angiogenesis genes			
VEGFR 1	*F (5'-3') TTTGCCTGAAATGGTGAGTAAGG*	70	7629.54
	R (5'-3') TGGTTTGCTTGAGCTGTGTTC	71	4533.1
VEGFR 2	*F (5'-3') GGCCCAATAATCAGAGTGGCA*	72	5555.7
	R (5'-3') CCAGTGTCATTTCCGATCACTTT	69	4678.1

Note: (NCBI) National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA (NCBI 2018).

3.3 Results

3.3.1 Study the effect of Ran inhibitor MBZ, on expression of genes and proteins in isogenic colorectal cancer cells.

Isogenic CRC cell lines were used to determine the effect of MBZ on expression levels of several gene mRNA and proteins well-known to be involved in cell survival, migration, metastasis, immune response, and apoptosis. HCT-116 *K-Ras* Mt. and Wt. cell lines were selected due to their aggressive growth which mimics the clinical conditions. To find the effect of MBZ and assess possible changes in gene expression, reverse transcription PCR and western blotting were used.

Moreover, several genes that are implicated in cell survival, migration, metastasis, immune response, and apoptosis were investigated using a qRT-PCR technique.

Cancer cells respond to deprivation in nutrient through increasing pro-angiogenic factor expression such as vascular endothelial growth factor (VEGFR) and interleukin-8 (IL-8) which play an essential role in new vascular formation, invasiveness and finally metastasis (Ferrara 2001) thus attempting to increase nutrient supply or migration to a nutrient-rich environment rich with nutrients for survival (Marjon et al. 2004).

3.3.1.1 Effect of MBZ on expression of Ran mRNA in isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells using qRT-PCR.

Ran-GTPase (Ran) is a Ras-related protein that's associated with regulation of cell cycle, nuclear-cytoplasmic transportation, and cell transformation (Clarke and Zhang 2008; Rensen et al. 2009; Lim and Leprivier 2019)**.** Ran was shown to play an essential role in the survival of cancer cells and cancer progression (Xia et al. 2008; Yuen et al. 2012). Upstream factors that initiate Ran expression and activity, such as Ras and PI3K pathways have been known. These are responsible for enhanced cell proliferation, cell survival, and cancer migration (Yoon et al. 2008; Lim and Leprivier 2019). Its expression has also been demonstrated to increase with progressive disease stage of CRC and other cancers and its overexpression is correlated with augmented aggressiveness of the cancer cells (Yuen et al. 2012).

To determine gene expression of Ran upon treatment with MBZ qRT-PCR was used. The isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells grown and treated with 0.5 µM MBZ over a time course of 48 h. Cells were collected and analysed to evaluate Ran mRNA level. Ran expression was significantly down regulated in both HCT-116 *K-Ras* Mt. *p<0.001*and HCT-116 *K-Ras* Wt. *p<0.001* cells treated with 0.5 µM MBZ, by comparison with un-treated control. There was significant inhibition of Ran mRNA up to 60% in Mt. by 0.5 µM MBZ and 40% Wt., after 48 h in both cell lines respectively, as shown in (Figure 3.1).

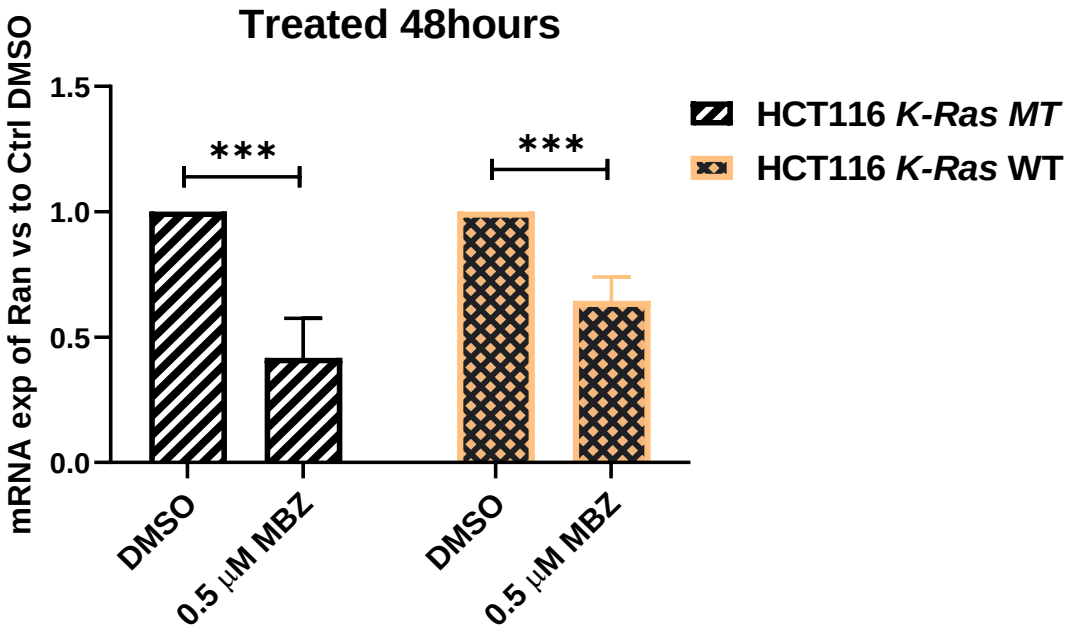
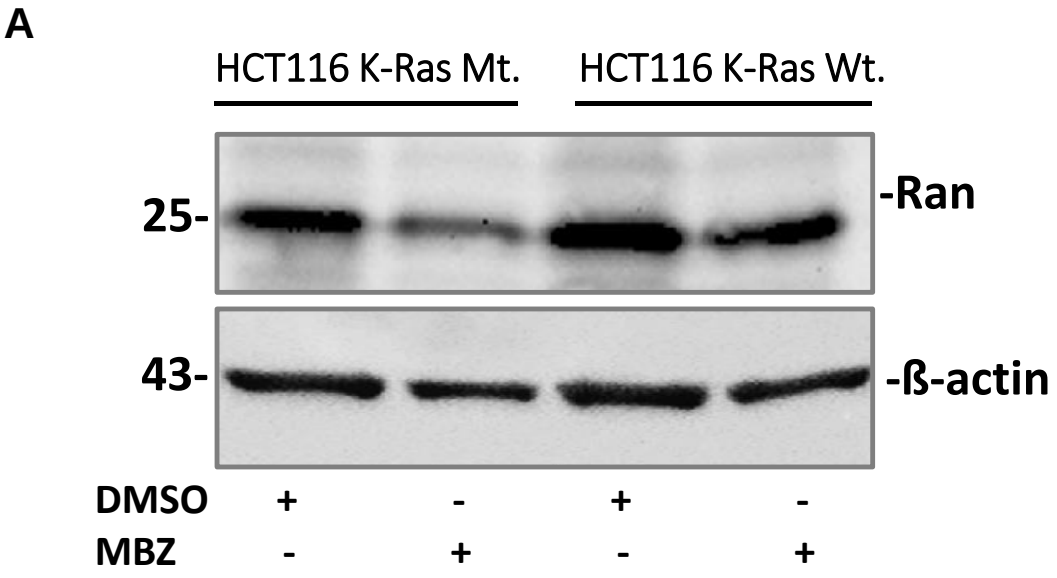


Figure 3.1: MBZ impact on relative mRNA expression level of Ran on HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells by qRT-PCR.
Isogenic colorectal cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. were left un-treated or treated with 0.5 µM MBZ for 48 h. Histograms (Figure 3.1) indicating Ran mRNA levels in the un-treated cells were equal to endogenous housekeeping gene B2M and those in the treated cells were calculated accordingly. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test. ***indicates *p<0.001*.

3.3.1.2 Effect of MBZ on expression of Ran protein in human isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells using western blot.

A western blot investigated the impact of MBZ on Ran protein expression on isogenic human CRC cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. Apoptosis is promoted when Ran was knocked down using potent shRNA (Yuen et al. 2012). The cells line was treated for 48 h with 0.5 µM MBZ while 0.1% DMSO was used as a control. Cells were extracted and their protein concentration was measured by a Bradford assay. Then a similar amount of protein was exposed to SDS-PAGE and subsequent western blotting. The protein levels and densitometry of Ran protein expression shown was normalised to the control (0.1% DMSO in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells). The results show that 0.5 µM MBZ after 48 h significantly inhibits Ran protein expression in HCT-116 *K-Ras* Mt. $p<0.001$and to a lesser extent in the HCT-116 *K-Ras* Wt. (Figure 3.2). Lastly, both isogenic cell lines when treated with MBZ showed a significant decrease in the levels of Ran protein after 48 h compared with that of the un-treated cells. However, the observed decrease is more significant in the HCT-116 *K-Ras* Mt. rather than HCT-116 *K-Ras* Wt. (Figure 3.2).



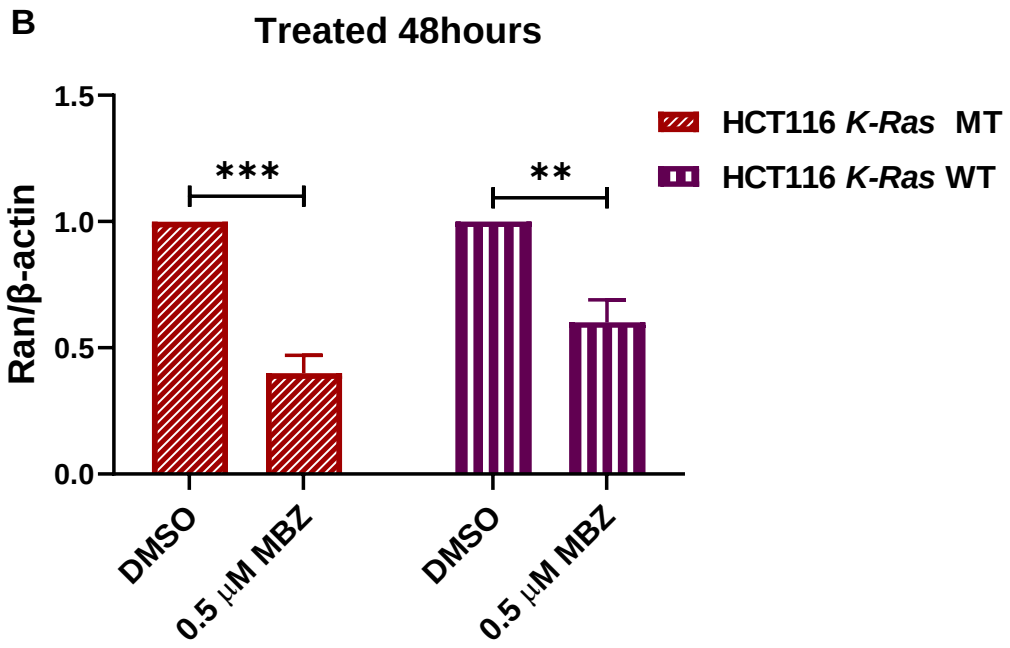


Figure 3.2: Ran protein expression on HCT-116 K-Ras Mt, and HCT-116 *K-Ras* Wt. cells line after exposure to MBZ.

(A) Isogenic CRC cell lines, HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. were left untreated (0.1% DMSO) or treated with 0.5 µM MBZ as indicated. Normalised cell extracts were subjected to western blotting. β-actin was used as loading control. Data presented is representative of three independent experiments as shown in (Figure 3.2).

(B) The histogram (Figure 3.2). shows densitometry of protein expression. Error bars represent mean ± SEM from three independent experiments. Statistical analysis was performed using unpaired *Student's T*-test. ** indicates *p<0.01* and *** *p<0.001*.

3.3.1.3 Effect of MBZ on expression of C5a mRNA in HCT-116 *K-Ras* Mt. and Wt. cells using qRT-PCR.

In CRC a strong inflammatory response is well-known to be connected with improved prognosis in various studies (Roxburgh and McMillan 2012), showing the importance of an effective immune response in regulatory tumour progression (Schreiber et al. 2011).

A risk factor and initiating process for CRC is chronic inflammation of the colon epithelium. Deregulated Prostaglandin-Endoperoxide Synthase 2 (PTGS2, aka COX2) and enhanced amounts of prostaglandin E2 (PGE2) serve as mediators of inflammation and tumorigenesis (Terzić et al. 2010). These encourage β-catenin/TCF4-mediated transcription and upregulation of the anti-apoptosis protein Bcl-2 (Greenhough et al. 2009).

Furthermore, inflammatory cell infiltration and enhanced pro-inflammatory cytokines further drive tumour development and progression (Terzić et al. 2010). Intestinal polyp formation in the ApcMin/+ mouse coincides with enhanced pro-inflammatory cytokines such as TNF, IL1β, IL6 which is most likely driven by constitutively activated NFKB1 (McClellan et al. 2012).

The immune system's role throughout cancer progress is complicated involving widespread mutual interactions between adaptive and innate immune cells, genetically transformed cells, their mediators and structural constituents of the tumor microenvironment (De Visser et al. 2006; Kraus and Arber 2009).

The potent mediator of inflammation is called complement-derived anaphylatoxin C5a, and it has immunomodulatory activities involving the inducement IL-1, IL-6 and IL-8 and TNF-α (Cavaillon et al. 1990; Schindler et al. 1990; Montz et al. 1991; Ember et al. 1994; Guo and Ward 2005; Wetsel et al. 2016; Chen et al. 2020a). C5a is also a known inducer of chemoattraction and leukocytes degranulation. It likewise encourages contraction of smooth muscle, mast cells to release histamine and thus increase vascular permeability and vasodilation.

Complement system activation is a main pathway that leads to several inflammatory reactions in various illnesses including cancer. Subsequently, this activation leads to cleavage of the C5 fragment producing the anaphylatoxin C5a. C5a wields a prevailing pro-inflammatory activity via interactions with the classical G-protein coupled receptor C5aR and non-G protein-coupled receptor C5L2

(GPR77) which are present on several immune and non-immune cells (Woodruff et al. 2011).

The reason to select C5a as an inflammatory target is due to its immunomodulatory activities involving the inducements of IL-1, IL-6, and IL-8. This act is responsible for promoting tumorigenesis by controlling many cancer hallmarks and multiple signaling pathways such as survival, proliferation, programmed cell death, angiogenesis, invasiveness, and metastasis, and, most significantly, metabolism.

So, if C5a was down regulated by MBZ, this means it will facilitate inhibition of downstream pro-inflammatory targets such as IL-1, IL-6, and IL-8.

To determine gene expression of C5a in isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells were grown and treated with 0.5 µM MBZ for 48 h. Cells were collected and C5a mRNA level was evaluated using qRT-PCR. C5a expression was significantly down regulated in both HCT-116 *K-Ras* Mt. *p<0.001* and HCT-116 *K-Ras* Wt. *p<0.01*cells treated with 0.5 µM MBZ in comparison to un-treated controls. There was significant inhibition of C5a mRNA by 60%, *p<0.001* Mt. and 60%, *p<0.01* Wt. after 48 h, in both cell lines respectively after 0.5 µM MBZ, as shown in (Figure 3.3).

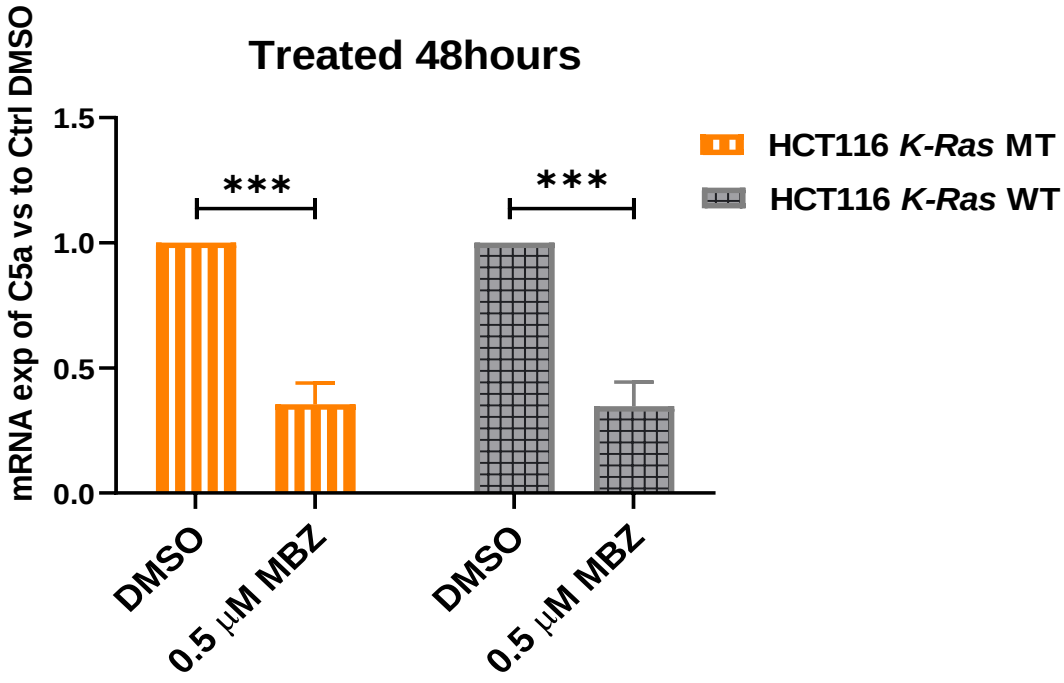
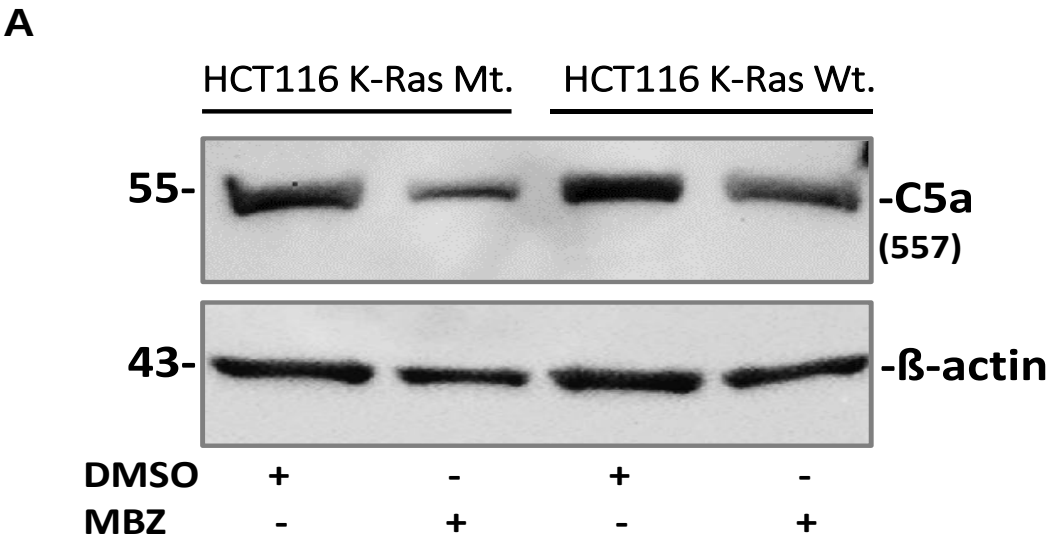


Figure 3.3: MBZ impact on relative mRNA expression level of C5a in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells by qRT-PCR.

HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells were left untreated or treated with 0.5 µM MBZ for 48 h. The histograms indicate C5a relative expression in both cell lines. The C5a mRNA levels were standardised to the un-treated cells with endogenous housekeeping gene B2M and those in the treated cells were calculated accordingly. Error bars represent means SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test. ** indicates $p<0.01$ and *** $p<0.001$.

3.3.1.4 Effect of MBZ on expression of C5a protein in HCT-116 *K-Ras* Mt. and Wt. cells using a western blot.

A western blot was carried out to examine the impact of MBZ on C5a protein expression on isogenic human CRC cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. Cell lines were treated for 48 h with 0.5 µM MBZ with 0.1% DMSO as a control. Cells were extracted and their protein concentration was assessed by a Bradford assay. Then a similar amount of protein was subjected to western blot analysis. The protein levels and densitometry of C5a proteins expression shown in (Figure 3.4) was normalised to β-actin. The results show that 0.5 µM MBZ after 48 h significantly inhibits C5a protein expression in HCT-116 *K-Ras* Mt. *p<0.001* (Figure 3.4). However, when compared to the HCT-116 *K-Ras* Wt., 0.5 µM MBZ *p<0.01* appears to inhibit Ran to lesser extent (Figure 3.4). Lastly, both isogenic cell lines when treated with MBZ demonstrated a significant decrease in the levels of C5a protein after 48 h of treatment when compared with that of the un-treated cells. However, the reduction is more significant in the HCT-116 *K-Ras* Mt. rather than HCT-116 *K-Ras* Wt.



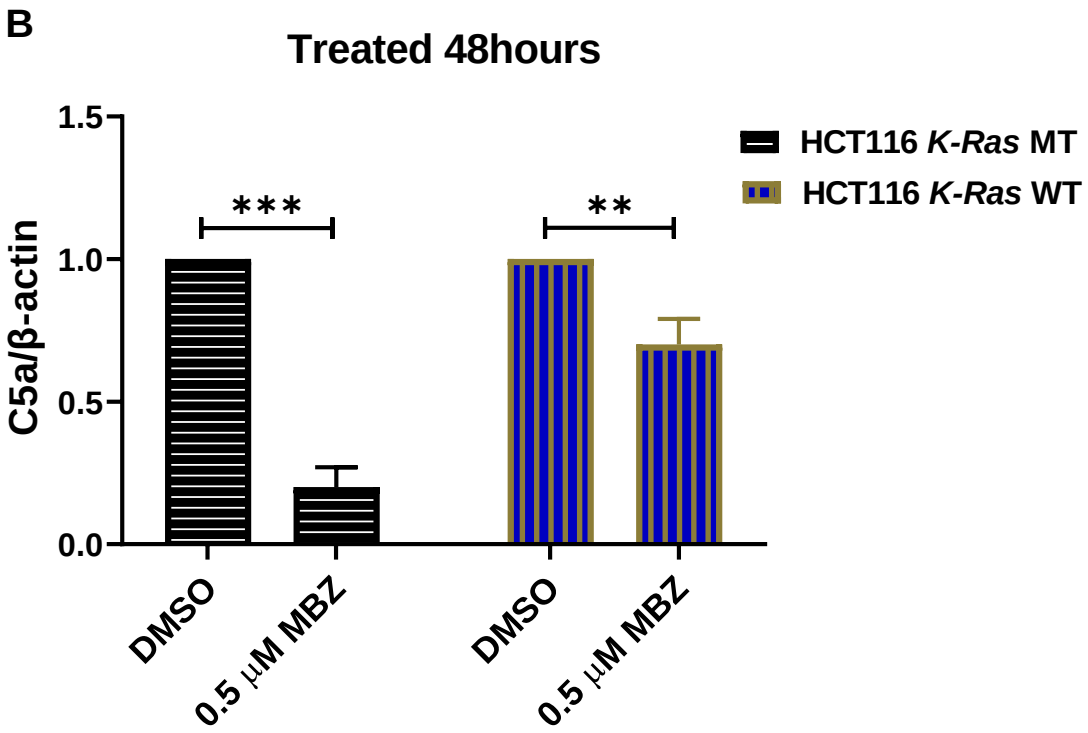


Figure 3.4: C5a protein expression in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells line after exposure to MBZ.

(A) HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. were left untreated 0.1% of DMSO or treated with 0.5 µM MBZ as indicated. Whole cellular extracts were isolated, and equivalent amounts of protein were analysed by western blotting. β-actin was used as loading control. Image presented is representative of three independent experiments.

(B) The histogram indicates densitometry of protein expression. Error bars represent mean ±SEM from three independent experiments. Statistical analysis was performed using unpaired *Student's t*-test. ** indicates *p<0.01* and *** *p<0.001*.

3.3.1.5 Effect of MBZ on expression of IL-1α mRNA in HCT-116 *K-Ras* Mt. and Wt. cells by qRT-PCR.

A vital constituent of the pro-tumorigenic microenvironment is the existence of inflammatory cells and the ensuing chronic inflammatory response. Even though chronic inflammation is a well-established driver in various cancers (Mantovani et al. 2008), inflammation additionally causes harm role in cancers without obvious primary inflammatory etiologies. This phenomenon, described through a tumor's ability to employ inflammatory immune cells to the local microenvironment to aid both tumor progression and development, is stated as tumor-elicited inflammation (TEI) (Grivennikov et al. 2012; Dmitrieva-Posocco et al. 2019).

IL-1 plays a crucial role in encouraging IL-17 production via T helper (Th17) cells in humans and mice in situations of autoimmunity and inflammation (Zhou et al. 2009; Coccia et al. 2012). Methods to prevent the IL-1 pathway are being used in therapies for inflammatory conditions such as gout, familial Mediterranean fever and rheumatoid arthritis (Dinarello et al. 2012). In spite of its well-known role in continuing immune-mediated inflammatory responses, the main role of IL-1 signaling as a controller of TEI and CRC in autochthonous models is ambiguous. IL-1 signaling is engaged in the creation of pro- or anti-tumor immune responses, as well as in the progress of bowel inflammation related to tumorigenesis (Voronov et al. 2003; Bersudsky et al. 2014; Malik et al. 2016; Dmitrieva-Posocco et al. 2019).

The fundamental mechanisms of IL-1 mediated stimulation of metastasis are intricate and comprise a rise in angiogenesis and inducement of endothelial cell adhesion molecules. For example, VCAM-1 encouraged by IL-1 is predictable through VLA4 that is articulated on melanoma cells, although the E-selectin ligand is existing on colon cancer cells (Voronov et al. 2003; Mantovani et al. 2018). Furthermore, IL-1 encourages endothelial cells and stromal cells to express proangiogenic cytokines e.g. IL-8 (Mantovani et al. 2018).

The target genes that are used in tumor cell survival include Bcl-2, Mcl-1, proliferation AKT1/2/3,c-Myc, Cyclin D1, Cyclin B, angiogenesis HIF1alpha, VEGF, metastasis MMP2, MMP9, cell adhesion ICAM-1, VCAM-1, inflammation IL-6, IL-1β, IL-1α and C5a (Waldner et al. 2012).

In order to determine gene expression of IL-1α in isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells were treated with 0.5 µM MBZ for 48 h. Cells were collected IL-1α mRNA level was evaluated using qRT-PCR. IL-1α expression was significantly down regulated in both HCT-116 *K-Ras* Mt. *p<0.05* and in HCT-116 *K-Ras* Wt. *p<0.05* for cells treated with 0.5 µM MBZ, in comparison with un-treated. There was significant inhibition of IL-1α mRNA by 30% in HCT-116 *K-Ras* Mt. and 25% significant change in HCT-116*K-Ras* Wt. after 48 h as shown in (Figure 3.5).

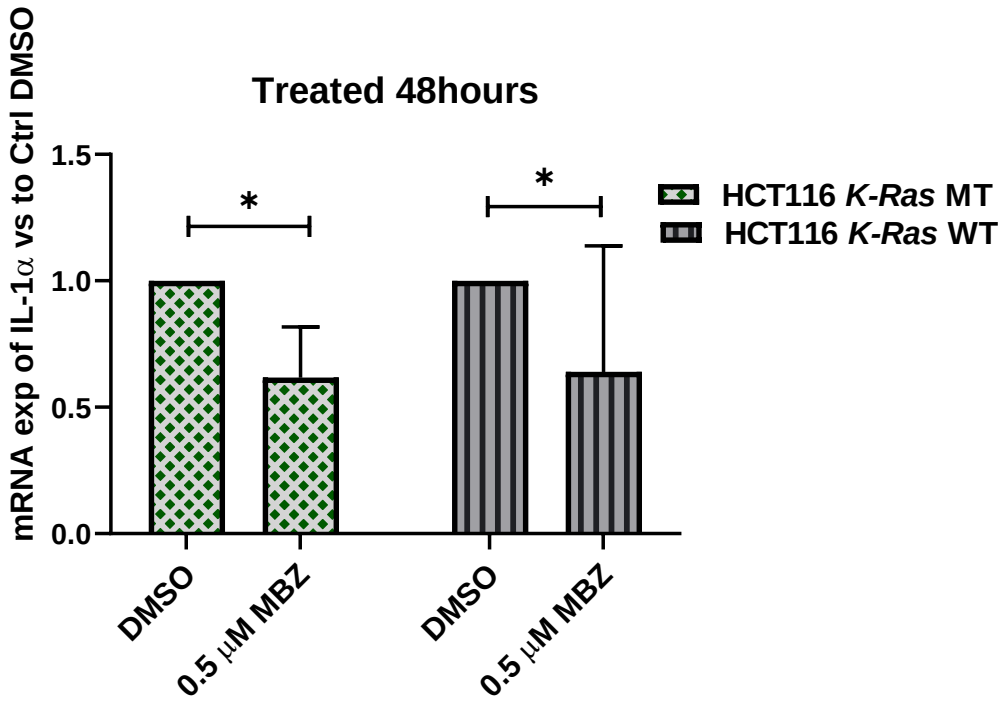


Figure 3.5: MBZ impact on relative mRNA expression level of IL-1α on HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells by qRT-PCR.
HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. were either un-treated or treated with 0.5 µM MBZ for 48 h. The histograms (Figure 3.5) indicating IL-1α relative expression in HCT-116 *K-Ras* cells. The IL-1α mRNA levels in the un-treated cells were standardised to endogenous housekeeping gene B2M and those in the treated cells were calculated accordingly. Error bars represent means SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test. * Indicates *p<0.05* and ns indicates *p>0.05*.

3.3.1.6 Effect of MBZ on expression of IL-1β mRNA in HCT-116 *K-Ras* Mt. and Wt. cells by qRT-PCR.

Interleukin-1β is an inflammatory cytokine existing in stimulated immune cells. Solid tumours, such as colon cancer, are infiltrated via many immune effector cells comprising lymphocytes and macrophages by secretion of platelet-derived endothelial cell growth factor (Takahashi et al. 1996; Takahashi et al. 2020). In both cases of cancer, primary and metastatic, the infiltration of immune effector cells happens. The studies have shown that infiltrating cells might participate in the angiogenesis of human colon cancer (Takahashi et al. 1996; Aguilar-Cazares et al. 2019). Interleukin-1β initiation of VEGF in human colon cancer cells is considered as another mechanism as it allows to the infiltrating cells to participate in tumour angiogenesis (Akagi et al. 1999; Voronov et al. 2014).

The previous report established that IL-1β encouraged VEGF mRNA expression in two types of cell lines, namely human colon cancer HT29 and SW620 (Akagi et al. 1999; Voronov et al. 2014). To determine gene expression of IL-1β in isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells were treated with 0.5 µM MBZ over a time course of 48 h. Cells were collected and IL-1β mRNA level was evaluated using qRT-PCR. IL-1β expression was significantly down regulated in HCT-116 *K-Ras* Mt. and in HCT-116 *K-Ras* Wt. it was up-regulated significantly. There was significant inhibition of IL-1β mRNA by 60% in HCT-116 *K-Ras* Mt. and two-fold and half up-regulation after 48 h, in Wt. cell line respectively, as shown in (Figure 3.6).

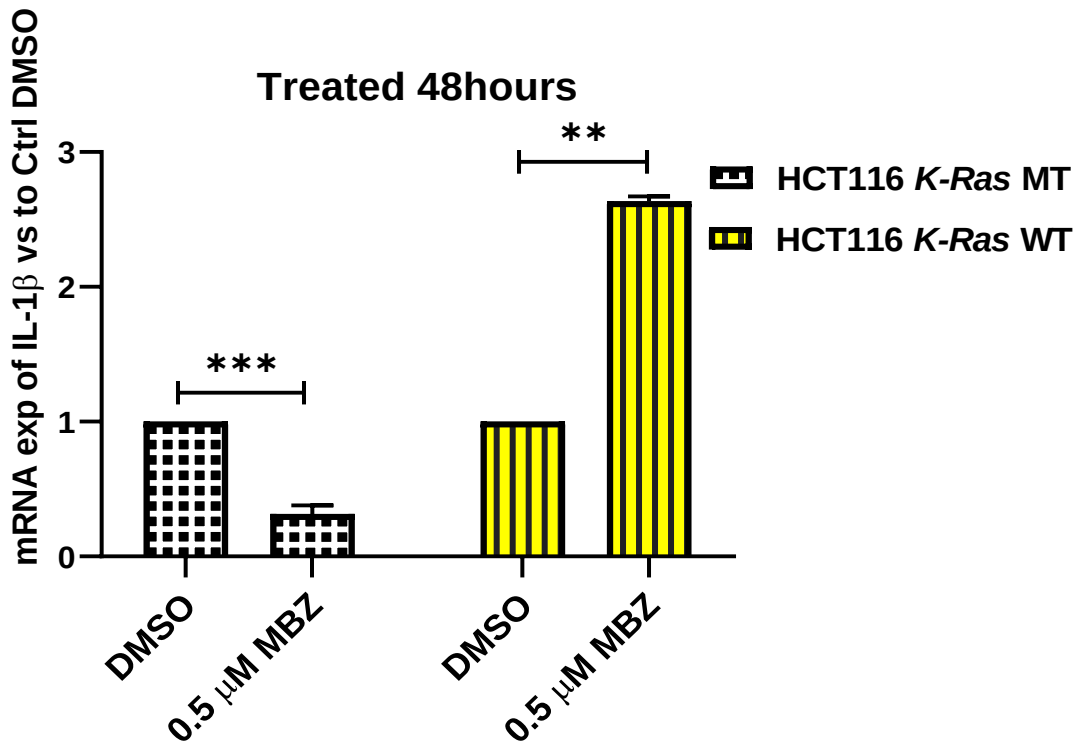


Figure 3.6: MBZ impact on relative mRNA expression level of IL-1β in HCT-116*K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells by qRT-PCR.

HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. were either un-treated or treated with 0.5 µM MBZ for 48 h. The histogram (Figure 3.6) indicates IL-1β relative expression in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. standardised with endogenous housekeeping gene B2M and those in the treated cells were calculated accordingly. Error bars represent means SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test. * Indicates p<0.05, ** *p<0.01* and *** *p<0.001*.

3.3.1.7 Effect of MBZ on expression of VEGFR1/ VEGFR2 mRNA in HCT-116 *K-Ras* Mt. and Wt. cells by qRT-PCR.

Angiogenesis is defined as the physiological procedure through which new blood vessels are created from pre-existing blood vessels. It facilitates progress, menstruation, pregnancy, skeletal muscle hypertrophy and wound healing, but also participates to pathological circumstances such as neovascular disorders like retinopathy, psoriasis, rheumatoid arthritis, AIDS/Kaposi sarcoma, and tumorigenesis of cancer. Angiogenesis is a complicated and highly ordered procedure that depends on widespread signaling networks to connect between and within endothelial cells, their vascular smooth muscle cells and pericytes (associated mural cells) and immune cells.

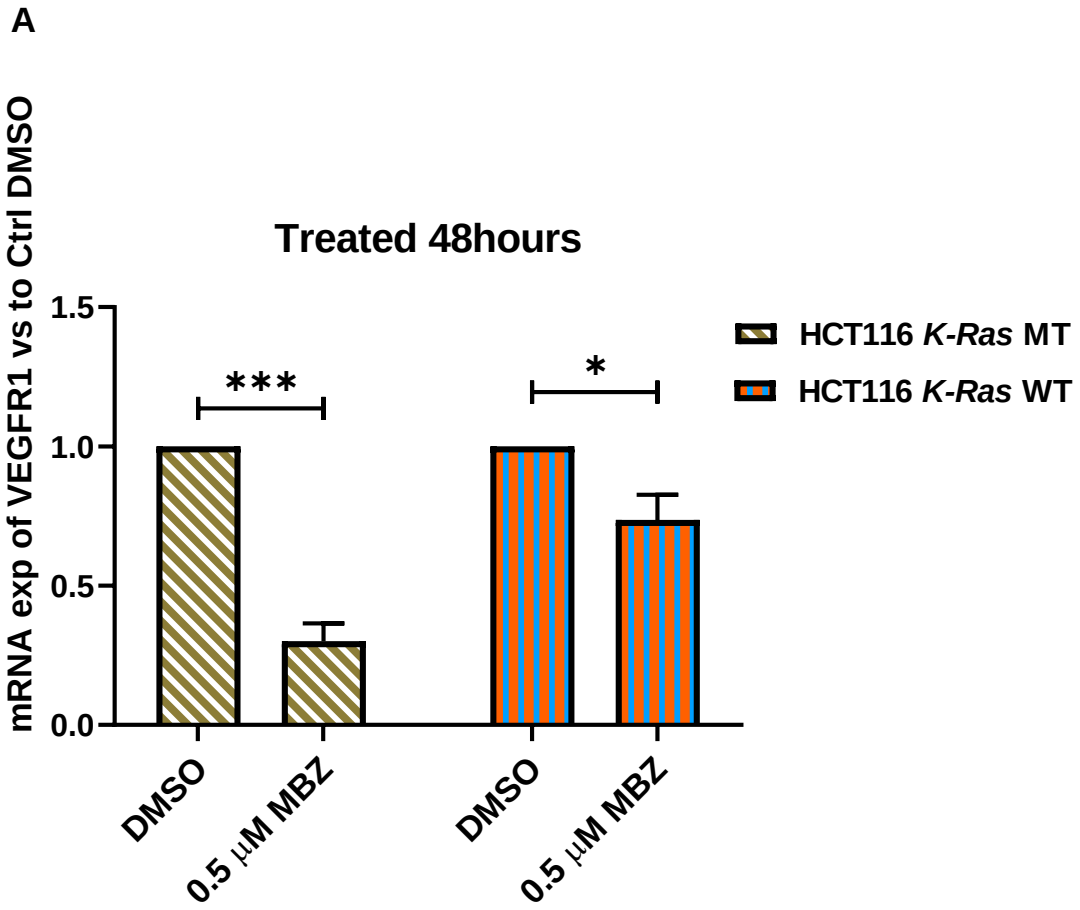
Vascular endothelial growth-factor receptors (VEGFRs) are essential for controlling vasculature especially neo-vasculature. VEGFR1 is essential for the regulation of hematopoietic stem cells and has a major role in migration of macrophages and monocytes, while VEGFR2 and VEGFR3 have crucial roles in vascular endothelial functions and lymph endothelial cells (Olsson et al. 2006; Lacal and Graziani 2018).

A family of proteins that are called vascular endothelial growth factor (VEGF) are essential for angiogenesis. VEGF have multiple isoforms comprising VEGF-A, B, C and D isoform. Each of them plays an essential role in various angiogenic steps starting from an embryonic step through to lymphatic angiogenesis (Olsson et al. 2006; Lacal and Graziani 2018).

The vascular endothelial growth-factor/ vascular endothelial growth factor receptors pathways are also activated throughout colon tumour progression (Markowitz and Bertagnolli 2009; Terzić et al. 2010; Lacal and Graziani 2018). Vascular endothelial growth-factor activation participates in the inflammatory process and encourages angiogenesis (Angelo and Kurzrock 2007; Aguilar-Cazares et al. 2019). Intricate linked pathways such as chronic inflammation and continued angiogenesis lead to the development and progression of CRC.

There are several similarities in pathological and physiological angiogenesis process in terms of signaling causing subsequent changes to cell role and performance which may be new therapeutic choices to fight illness (Chung et al. 2010; Chung and Ferrara 2011).

In order to determine gene expression of VEGFR1/ VEGFR2 in isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells were treated with 0.5 µM MBZ for 48 h. Cells were collected and VEGFR1/ VEGFR2 mRNA level was evaluated using qRT-PCR. VEGFR1/ VEGFR2 expression was significantly down regulated in both HCT-116 *K-Ras* Mt. and Wt. cells. There was significant inhibition of VEGFR1/ VEGFR2 mRNA by 70% after 48 h, in HCT-116 *K-Ras* Mt. cell lines as shown in (Figure 3.7).



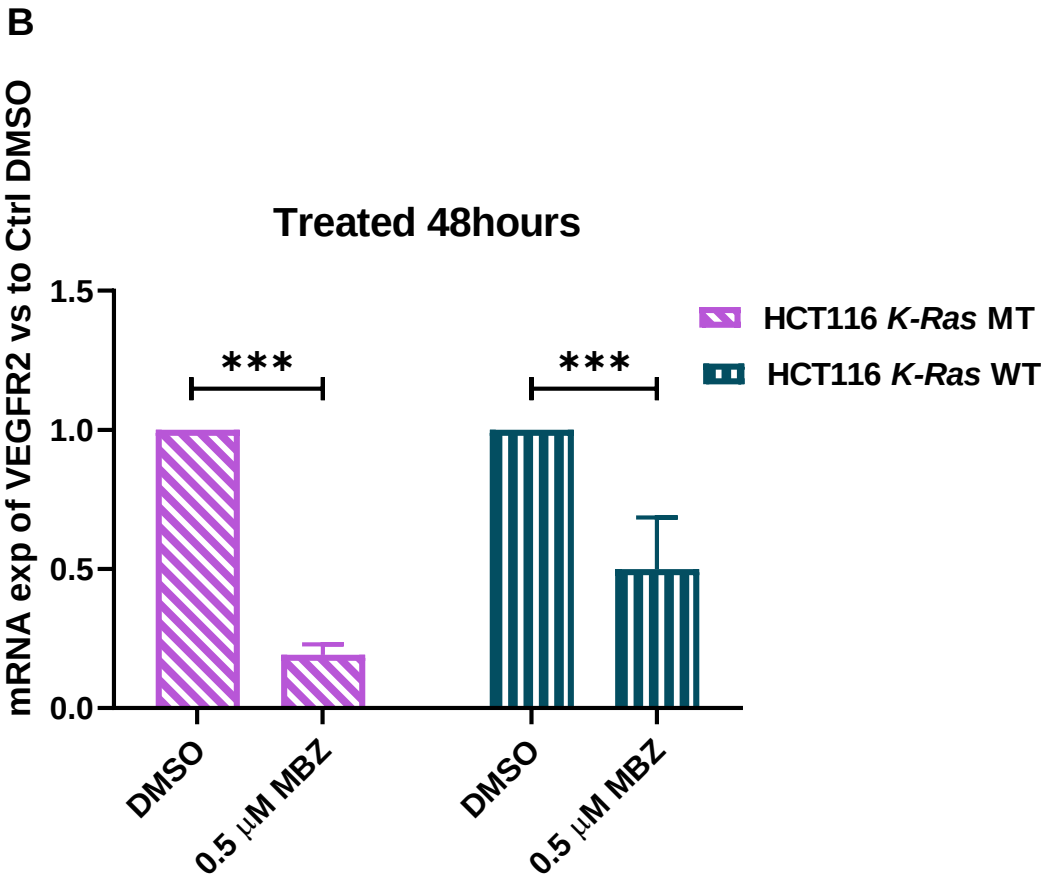


Figure 3.7: MBZ impact on relative mRNA expression level of VEGFR1/ VEGFR2 in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells by qRT-PCR.

HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells were either un-treated or treated with 0.5 µM MBZ for 48 h and mRNA isolated. The histograms indicate (A) VEGFR1/ (B) VEGFR2 relative expression in HCT-116 *K-Ras* Mt. and Wt. standardised with endogenous housekeeping gene B2M. Error bars represent means SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test. * Indicates $p<0.05$ and *** $p<0.001$.

3.4 Discussion

The effect of MBZ on expression levels of several mRNA and proteins which play crucial roles in cell survival, migration metastasis and immune response was analysed in isogenic pairs of colorectal cancer cell lines by qRT-PCR and western blotting. The results showed that Ran mRNA and protein expressions was down regulated by MBZ in both HCT-116 *K-Ras* mutant and wild type after 48 h. Both mRNA and protein expression of C5a was significantly down regulated in both cell lines however, the observed reduction was more significant in the HCT-116 *K-Ras* Mt. compared with Wt. which may be due to highly sensitivity of mutant cell line to MBZ.

The mRNA levels of IL-1α, IL-1β, VEGFR1 and VEGFR2 in HCT-116 *K-Ras* Mt. and Wt. cells showed differential expression. mRNA IL-1α expression was down regulated in both cell lines whereas mRNA of IL-1β was significantly down regulated in HCT-116 *K-Ras* Mt. but up-regulated in Wt. mRNA. VEGFR1/ VEGFR2 expression was significantly down regulated in both HCT-116 *K-Ras* Mt. and Wt. which are consistent with previous study (Bodhinayake et al. 2015).

MBZ is thought to act as a Ran-GTPase inhibitor for cancer cells that overexpress Ran, its protein product and mRNA.

This result shows that Ran inhibitor MBZ are strongly down regulated Ran mRNA and protein. Up to date, there are no published studies regarding effect of MBZ on Ran in both levels in the cell lines described here. There are some studies that looked at MBZ effect on different types of cancer such as breast and lung cancer cells. A patent application describes how Ran was down regulated by MBZ in cancer cell lines such as HCC827, HCC827-GR5 (El-Tanani 2017). Those results are consistent with the results described here in both *K-Ras* mutant and wild type at both protein and mRNA levels of Ran.

The previous study was conducted to assess the significant effect of some mutations such as the activated *K-Ras* genes in carcinoma of human colon cell line such as HCT-116 and DLD-1, where the cell lines were disrupted at the activated *K-Ras* gene through homologous recombination technology compared to wild type (parental cell lines). They found that the cell proliferation capacity was decreased and the capacity of the cells to anchorage-independent growth has vanished in an in *vitro* and nude mice study. Consequently, CRC tumorigenesis through changed cell differentiation and cell growth was shown due to the

activated *K-Ras* gene as it plays a critical role (Shirasawa et al. 1993; Margetis et al. 2017). This is a second reason to use this type of mutation of isogenic CRC cell line to make the cell very active and rapidly proliferating. The use of parental cell lines in this study allows assessment of any oncogenic effects following the loss of *Pten* or activation of *K-Ras* (to eliminate any false results caused by presence of mutation).

The previous research used a specific shRNA to inhibit Ran with the purpose of examining its involvement in breast and lung cancer tumorigenesis. However, our work here has also treated both HCT-116 *K-Ras* mutant and wild type by MBZ for 48 h and has showed that Ran mRNA and protein expressions are down regulated and MBZ mimics the effect of specific knock down using Ran-shRNA. According to these studies, it has been shown that silencing Ran led to induce aapoptosis, in both breast and lung cell cancer (Yuen et al. 2012).

Previously, in human colorectal cell lines (HT29 or SW480 adenocarcinoma xenografts) MBZ produced a reduction in the volume and weight of tumours (Guerini et al. 2019). Moreover, a previous study revealed that down regulation of Ran in TOV112D TetR and TOV1946 TetR parental cell lines affects proliferation of cells through encouraging caspase-3 associated cell death (Barrès et al. 2010). Previous results from our laboratory have shown that MBZ has direct effects on Ran transcription and translation level (El-Tanani 2017).

MBZ down-regulates C5a which suggests effects on innate immune response to cancer cells as well as having direct anti-tumour properties resulting from inhibition of Ran. MBZ may consequently play a role in immunotherapy due to its effects on C5a via up-regulation of T and NK natural killer cells (El-Tanani 2017). These results are consistent with the results described in this chapter shown in both *K-Ras* mutant and wild type of proteins and mRNA levels of C5a.

The reduction in VEGFR1, VEGFR2, IL1α, IL1β and immune response in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. when treated with MBZ agrees with previous published studies carried out on tumour samples of ApcMin/+ polyps (Sung et al. 2019). It was previously reported that when the cells are exposed to MBZ they show a reduction in tumour angiogenesis, repressed VEGFR2 kinase activity and reduced expression of proteins that are essential for pro-inflammatory cytokines (VEGF, IL1α and IL-1β), (Guerini et al. 2019; Sung et al. 2019).

Infiltration of inflammatory cell and augmented pro-inflammatory cytokines C5a, IL1α and IL-1β promote tumor development and progression (Terzić et al. 2010). The VEGF/VEGFR2 pathways are also activated during colon tumor progression (Markowitz and Bertagnolli 2009; Terzić et al. 2010). VEGF activation contributes to the inflammatory process and encourages angiogenesis (Angelo and Kurzrock 2007; Lacal and Graziani 2018). Complex interconnected pathways such as chronic inflammation and sustained angiogenesis work in performance to drive formation and progression of CRC (Williamson et al. 2016; Ahmed et al. 2020). In CD14 depleted (PBMC) cultures MBZ reduced the release of IL1β (Rubin et al. 2018), supporting the current data.

High levels of Ran-GTP encourage cell division, suggesting an oncogenic role of Ran in CRC. Inhibition of Ran expression provides a possible therapeutic strategy promoting apoptosis (Barrès et al. 2010). A previous study used a specific shRNA to inhibit Ran with the purpose of examining its involvement in breast and lung cancer tumorigenesis (Yuen et al. 2012). Recently it has been shown that pimozide reduced the tumour volume by 60%-70% and *in vivo* prevented breast cancer tumour metastasis through its effect on Ran-GTPase (Dakir et al. 2018).

The hypothesis that MBZ inhibits Ran-GTPase on isogenic human CRC cell lines chapter.

The *K-Ras* wild-type cells have normal apoptosis, replicative senescence, and cell arrest. So, in the case of Mt., the first line of defence in case of *K-Ras* activation was growth arrest. It can be established that the *K-Ras* Wt. gene is involved as a tumour suppressant and is nowhere to be found in several types of cancer progression (Zhang et al. 2001; Kent 2018).Thus, when this gene mutates cancer progresses (McCoy et al. 1983; Kranenburg 2005; Bielski et al. 2018; Kent 2018; Marbaniang and Kma 2018). Permanent activation of the *K-Ras gene* due to a mutation leads to GTP hydrolysis termination, This is consistent with results shown with *K-Ras* Mt. cells lines when treated with DMSO and compared with ones which treated with MBZ.

The Ras mutations make monoclonal antibodies to epidermal growth factor receptors (EGFRs) ineffective (Karapetis et al. 2008; Neitzel et al. 2020).

RAN overexpression has been associated with augmented aggressiveness of different type cancer cells *in vitro* and *in vivo* (Kurisetty et al. 2008; Chen et al. 2016; Sheng et al. 2018; Wang et al. 2020a). Moreover, *in vitro* studies have

established that silencing the RAN gene with SiRNA or shRNA induces apoptosis in cancer cells compared to normal cells and in activated *K-Ras*-mutant cells. Cancer cells are found to be more sensitive to deviations in RAN status than normal cells (Kurisetty et al. 2008).

3.5 Conclusion

RAN, a member of the Ras oncogene family, is a gene that encodes the GTP-binding nuclear protein Ran. RAN gene overexpression is detected in several types of cancers and this overexpression produces a poor prognosis. In this chapter, I have shown that MBZ has significant inhibition on Ran pathway in CRC cell lines such as HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. which are proliferation, migration, angiogenesis genes. Ran inhibition down regulates immune response in a dose-dependent manner in mutant rather than wild type cell lines. This is consistent with using Ran specific shRNA as previously published (Yuen et al. 2012).

It is also known that reduced Ran-GTPase activity due to mutation in *K-Ras* gene led to reduction in several genes controlloing cell division, invasion and metastasis which is similar to the effect of MBZ. The next steps are to investigate MBZ effect on apoptosis by a western blot and cellular toxicity in the next chapter**.**

Chapter 4

Results

Investigate the effect of MBZ on proliferation of isogenic CRC cell lines.

4. Effect of MBZ on cell proliferation and apoptosis in isogenic colorectal cancer cell lines.

4.1 Introduction

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is a procedure that can be used to investigate cell viability, proliferation rate or compound toxicity (Van de Loosdrecht et al. 1994; Kumar et al. 2018). The principle underlying the MTT assay is the capacity of viable cells to generate an insoluble purple formazan product from a soluble yellow tetrazolium salt (MTT: 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide) via mitochondrial succinate dehydrogenase. The viable cell count is proportional to the quantity of formazan generated which can be measured (Mosmann 1983; Mbeh and do Nascimento 2015).

An MTT assay was carried out to investigate the impact of MBZ on isogenic human CRC cell lines HCT-116 *K-Ras* Mt., HCT-116 *K-Ras* Wt., DLD-1 *K-Ras* Mt. and DKO-3 Wt. This would assess any cytotoxic and anti-proliferative effect of MBZ as a potential anti-cancer drug. Previous reports show that MBZ has a cytotoxic effect in a dose dependent manner in hepatocellular carcinoma (HCC) cells (Younis et al. 2019).

Therefore, CRC cell lines were treated for 24, 48, and 72 h with a range of concentrations (0.05, 0.1, 0.25, 0.5, 0.75 and 1 µM MBZ) while 0.1% DMSO act as a control. The sensitivity of CRC cell lines is different from HCT-116 *K-Ras* Mt. being the most sensitive IC_{50} 0.35 µM and HCT-116 *K-Ras* Wt. being the most resistant IC_{50}1.51 µM. IC50 was calculated as mentioned in section 2.2.1.9 data analysis (Bhattacharjee et al. 2019).

In previous reports, MBZ has anti-cancer properties with varying IC_{50} values in different types of cancers both *in vitro* and *in vivo* (Guerini et al. 2019). For example, in lung cancer cells 0.16 µM (Mukhopadhyay et al. 2002; Guerini et al. 2019), in melanoma cell lines 0.32 µM (Doudican et al. 2008) as well as has 10 µM to 3.7 µM (Doudican et al. 2012), in an adrenocortical carcinoma model 0.23 µM (Martarelli et al. 2008; Doudican et al. 2012; Pantziarka et al. 2014), in cholangiocarcinoma cells 1 µM (Sawanyawisuth et al. 2014), in glioblastoma multiforme cells 0.1 µM (Bai et al., 2011) and gastric cancer cells AGP01 0.59 µM (Pinto et al. 2015).

Several studies demonstrated that MBZ binds to tubulin and interrupts the structure of microtubules in AGP01 cells. MBZ also repressed the migration and invasion of gastric cancer cells. These outcomes show MBZ can be used alone or in combination with chemotherapeutic drugs in advanced gastric cancer cases (Pinto et al. 2015; Pinto et al. 2019).

The objective of other study was to examine the role of MBZ on malignant ascites cells.

MBZ (0.5 µM and 1.0 µM) augmented caspase -3 and caspase -7 activities (Pinto et al. 2015; Pinto et al. 2019). The objective of this study was to examine the role of MBZ on malignant cancer cell lines through pro-apoptotic family proteins Bad, Bax, Bak, puma, Bim and Bid. A caspase -3/ caspase -7 western blot assay was done to assess the mechanisms of apoptosis of MBZ in HCT-116 *K-Ras* Mt. and Wt. cell lines.

Programmed cell death has a crucial role in normal embryonic growth, tissue homeostasis and the development and function of the immune system. Conversely, processes that prevent normal programmed cell death improve cell survival and can lead to tumors. Therefore, the deregulation of the Bcl-2 family plays a vital role in the formation of tumors. Moreover**,** the Bcl-2 family also plays a key role in triggering infectious, neurodegenerative, and autoimmune diseases. An autoimmune disease, such as type 1 diabetes, may result from improperly programmed cell death, and schizophrenia may arise as a consequence from an abnormal ratio of pro and anti-apoptotic factors (Strasser et al. 2011). Proteins in the Bcl-2 family perform further functions in other cellular processes, such as the morphology and metabolism of mitochondria but these have not yet been studied. Regulation of apoptosis occurs through a balance of pro-apoptotic and anti-apoptotic factors.

4.1.1 Apoptosis

Apoptosis (Ahmed et al. 2019) is a major cell death process that can counteract tumour development and growth. Due to this it is frequently de-regulated in several types of cancers (Ramesh and Medema 2020).

4.1.1.1 Bcl-2 family (B cell lymphoma-2 (Bcl-2)

The Bcl-2 proteins are small proteins in size between 18 kDa and 28 kDa and control programmed cell death by regulating mitochondrial permeability. The family is sub-divided into 3 categories. The first subcategory are anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xl and Mcl 1, which comprise of 4 Bcl-2 homology domain (BH) and are in the outer wall of mitochondria and prevent the release of cytochrome c. The second subcategory, the pro-apoptotic members, which are structurally analogous to the anti-apoptotic proteins (BH.1, 2 and 3), include Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer), but do not have BH4 regions. The last subcategory, pro-apoptotic BH3 only proteins include Bim, Bad (Bcl-2-associated death promoter), Bid, puma. All members of the Bcl-2 family seem to be able to bind to ER and mitochondrial membranes in addition to being presented in cytosol subsequent to an apoptotic signal (Ramesh and Medema 2020). Preliminary studies have shown that for pro-survival, Bcl-2 proteins bind to the mitochondrial outer membrane, blocking the release of cytochrome c, thereby preventing the activation of cascade 9. On the other hand, pro-apoptotic members, for example, Bax and Bad form an active pro-apoptotic complex with Bcl-2 and Bcl-xL when translocated to mitochondria. This step is inhibited by survival factors responsible for Bad phosphorylation which leads to its cellular isolation. Following signaling through Fas; the Bid in cytosol is cleaved into an active fragment (tBid) by caspase-8 then translocated to mitochondria. Both pathways are death receptor signaling, and the mitochondrial cell death is linked through the effect of caspase-8 (Edlich 2018; Green 2019).

Although both pathways work together, they have independent functions (Green 2019) as well as a role in intrinsic pathway which can be blocked by pro-survival factor proteins of the Bcl-2 family. These pro-survival factor proteins of the Bcl-2 could stop release of cytochrome c and caspase activation (Chen et al. 2018b). The contribution of Bcl-2 was proposed to stimulate apoptosis by increasing the release of cytochrome c from mitochondria after binding to apoptosis protease activating factor-1 (Apaf-1) then forming an active complex of Apaf-1 and caspase-9. However, the interactions actions of the Bcl-2 family are much more complicated than expected initially (Ramesh and Medema 2020). Apoptosome is a protein complex formed by cytochrome C, Apaf-1, (d)ATP, and initiator caspase

(Li et al. 1997; Saleh et al. 1999; Chen et al. 2018b). Then this complex raises the affinity of Apaf-1 to ATP, which is responsible for supplying the energy for the formation of apoptosome (García-Aranda et al. 2018). Throughout the formation of the apoptosome, the pro-caspase -9 is activated which leads to the activation of both caspase -3 and caspase -7 effectors.

In response to death stimuli Bax and Bim move to the mitochondria, thereby eliminating the survival factor which leads to DNA damage and p53 causes transcription of Bax, Noxa and puma. Hereafter cytochrome c released from mitochondria then binds to Apaf-1, forming the active complex of Apaf-1 and caspase -9.

Although the mechanism (s) that regulate mitochondrial permeability and cytochrome C release during programmed cell death are not completely understood, Bcl-xL, Bcl-2, and Bax may play an important role in voltage-dependent anion channel regulation (VDAC) to cytochrome c release.

The effect of MBZ on isogenic colorectal cell lines such as HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. was investigated to identify the drug's mechanisms of action on apoptosis and associated signaling pathways responsible for metastases formation, and angiogenesis. The change of level of pro-survival factor proteins and pro-apoptotic proteins after cells were treated with MBZ was assessed in human adrenocortical carcinoma (AC), (Martarelli et al. 2008), and lung cancer (Sasaki et al. 2002) and different colorectal cell lines (Hiraishi et al. 2019), but not on isogenic CRC cell lines such as HCT-116 *K-Ras* Mt., and HCT-116 *K-Ras* Wt. This study will investigate the mechanism of action of MBZ on isogenic colorectal cell lines through pro-survival family factor proteins and pro-apoptotic family proteins.

In addition, Bad can form an active pro-apoptotic complex with Bcl-2 and Bcl-xL when translocated to mitochondria and trigger the apoptosis by release of cytochrome c. This step is inhibited by survival factors that are responsible for Bad phosphorylation, e.g., protein kinase B (Akt), which leads to its cellular isolation**.**

Similarly Bid, Bim and Bad activation does not involve cleavage of caspase. There are several isoforms of Bim in existence due to alternate mRNA splicing: BimEL, BimL, and BimS (O'Connor et al. 1998; Clybouw et al. 2012; Fedele et al. 2020). In normal cells, BimEL and BimL form part of the dynein motor complex of

the microtubule-these are associated through binding to dynein light chain LC8 and are thus incapable to encourage cell apoptosis (Williams et al. 2018). Death-inducing stimuli, e.g. a lack of serum, encourage the release of BimEL or BimL from the dynein motor complex (Torres-Martinez et al. 2021). As soon as they are secreted BimEL or BimL enter the mitochondria, binding to Bcl-2 occurs and these lose their anti-apoptotic activity (Moustafa-Kamal 2018). BimS does not seem to cooperate with the microtubule-complex but is capable to binding to Bcl-2 (Puthalakath et al. 1999).Therefore, BimS performs its vital role as a constitutive death stimulus (Williams et al. 2018). In contrast to Bid, Bim does not seem capable of binding to pro-apoptotic proteins such as Bax and Bak (O'Connor et al. 1998; Pentimalli 2018).

The pro-survival Bcl-2 proteins block apoptosis, whereas the Bax-like proteins Bax/Bak encourage cell death. Therefore, it is challenging to determine which proteins are the primary effectors of cell survival/apoptosis (Chen et al. 2020b).

The initiation of a third well-characterized BH3-only protein, Bad, is regulated by phosphorylation. Survival signals derived from growth factors lead to Bad phosphorylation and its retention in the cytosol in an inactive form bound to 14-3-3 scaffold proteins. Death-inducing stresses such as growth factor deficiency result in the dephosphorylation of Bad and its detachment from 14-3-3. Bad then binds to and inhibits Bcl-2 and thereby promotes cell death. Re-exposure of the cells to growth factors deactivates Bad via phosphorylation facilitated by PI (3)-kinase (Kapodistria et al. 2018).

4.1.1.2 p53

Puma (p53 upregulated modulator of apoptosis) is an entirely mitochondrial protein which binds to Bcl-2 and Bcl-XL through a domain called BH3. Puma exogenous expression causes very rapid and profound programmed cell death. Depending on its expression, p53 dependence and biochemical properties, puma could be a direct mediator of p53 related apoptosis (Li 2021).

The extrinsic death receptor pathway initiates the stimulation of a caspase cascade, and the mitochondria, intrinsic pathway changes the balance in the Bcl-2 pro-survival family towards the puma pro-apoptotic members, encouraging formation of a complex called the apoptosome, and subsequent apoptosis through caspase pathway. The influence of these two apoptotic pathways may

be improved after they congregate through Bid, which is a p53 target. p53 can also encourage cell death by a transcription-independent mechanism under specific circumstances (Haupt et al. 2003; Ho et al. 2019).

TP53 is the gene encoding the cellular tumour antigen p53 is without doubt the most prominent tumour suppressor gene (Hamroun et al. 2006; Ho et al. 2019). Indeed, the p53 reactivation in tumour cells is a promising approach for cancer therapy (Xu et al. 2019), and many drugs targeting p53 are now under preclinical and clinical trials assessments (Vousden and Prives 2009; Bykov et al. 2018). The p53 role as an anti-cancer agent is associated with its capability to induce programmed cell death by the pathway called intrinsic mitochondria apoptosis. The vital site for starting this path is mitochondrial outer membrane permeabilisation (MOMP) that is regulated through members of the pro-survival Bcl-2 protein family and involves the stimulation of its pro-apoptotic members such as Bax or Bak (Suhaili et al. 2017). p53 induces some pro-apoptotic members of Bcl-2 family including puma, Bax, Noxa and Bid as transcription factors and suppresses the transcription of some anti-apoptotic genes, such as those encoding Bcl-2 and Bcl-xL (Laptenko and Prives 2006; Vousden and Prives 2009; Pemberton et al. 2021).

The regulation of cellular p53 levels is triggered by feedback of an autoregulatory loop with mouse double minute 2 homolog (Mdm2) (negative regulator) which is an E3 ubiquitin ligase that binds to p53 thus targeting p53 for proteasomal degradation (Bang et al. 2020). Dissociation of the Mdm2–p53 complex due to phosphorylation of both p53 and Mdm2 and DNA-damage by the checkpoint kinases ATM, Chk1, ATR and Chk2 subsequently increase p53 levels that mainly occur in the nucleus (Marine et al. 2006; Miranda et al. 2006; García-Cano et al. 2020).

Furthermore, Mdm2 conjugates to mono-ubiquitin residues of p53. This modification encourages the passage of p53 into the mitochondria and cytosol, thus playing a role in the transcription-independent apoptosis (Li et al. 2003; Marchenko et al. 2007; García-Cano et al. 2020). p53 has three biological roles in response to stress stimuli with the main one being a transcriptional inducer that binds to a significant number of genes promoters raising their levels of expression (Kim and Deppert 2004; Laptenko and Prives 2006; Levine et al. 2016; Mijit et al. 2020).

4.1.1.3 The cyclin-dependent kinase inhibitor (p21)

p21 encourages the arrest of the cell cycle in response to several stimuli (Hoeferlin et al. 2011). Unexpectedly, p21 might also encourage apoptosis by two mechanisms such as p53-dependent and p53 independent through exposure to cellular stresses (Shamloo and Usluer 2019; Dowaidar 2021). Precisely how p21 encourages programmed cell death is not completely understood but may be through both p53-dependent and p53-independent up-regulation of Bax as a pro-apoptotic protein leading to the tumour necrosis factor family of death receptors activation or through DNA repair effects (Yu et al. 2001; Shamloo and Usluer 2019). Published reports have shown that p21 has a pro-apoptotic role (Shamloo and Usluer 2019), and involved in apoptosis (Abbas and Dutta 2009; Nguyen et al. 2019).

4.1.1.4 Caspases

Caspases are referred to as cysteine-aspartate proteases which are involved in apoptosis (Teng and Hardwick 2015). They specifically cleave their substrates subsequent an aspartate residue (Earnshaw et al. 1999; Callus and Vaux 2007; Julien and Wells 2017). Caspases are usually made as inactive pro-caspases i.e., zymogens (Julien and Wells 2017). An apoptotic stimulus is needed to be activated by either proteolytic cleavage by way of other caspases or by autocatalysis to develop an active enzyme (Cohen 1997; Shi 2002; Julien and Wells 2017). The consensus sequence QAC(R/Q/G) G, is highly conserved at the active sites although the substrate recognition motif varies among caspases (Thornberry 1998; Reis 2012).

4.2 Materials and methods

4.2.1 Materials

Materials that are used in this chapter are described earlier in chapter 2 section 2.1.

Table 4. 1: List of gene-specific primer sequences used in quantitative RT-PCR. All were selected from NCBI primer bank.

Gene	Primer Sequences	Tm ^{o}C	Size(bp)
A. Reference gene – control			
B2M	*F (5-3') CCA AGG AAG GCG TCT AAG GC*	72	61777
	R (5-3') CTT TCG AGC GCA ACC ACT TTG	71	6358.2
B. Apoptotic marker			
Bcl-2	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8
Mcl-1	*F (5'-3') CAG TAA GGC AGG CTG TAA AAG A*	69	6842.4
	R (5'-3') TGG AGC TGG TAG ACC CTC G	72	5845.8

4.2.2 Methods

MTT: Methods that are used in this chapter are described earlier in chapter 2 section 2.1.

4.3 Results

4.3.1 Effect of MBZ on cell viability (MTT assay).

The cytotoxicity is stated as IC_{50}, which is the concentration of MBZ at which colorectal isogenic cell lines survival is inhibited by 50%. The optical density at 540 nm was measured for each CRC cell line separately.

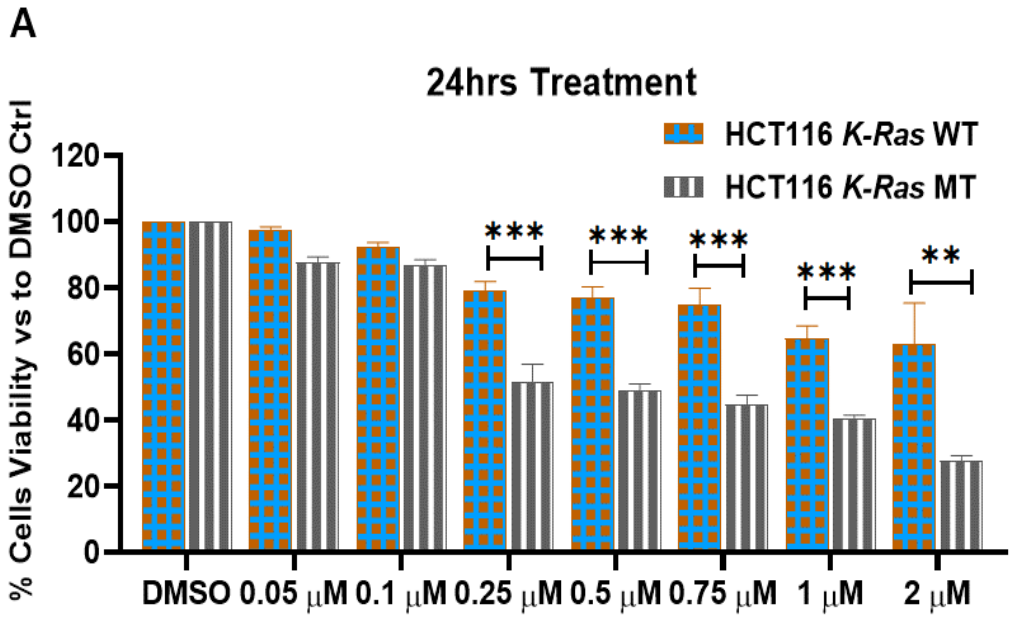
4.3.1.1 Effect of MBZ on HCT-116 *K-Ras* Wt. and HCT-116 *K-Ras* Mt. cells using MTT.

The MTT assay is a technique that measures the effect of MBZ at different concentrations and exposure times on isogenic pairs of human colorectal cell lines. Data show that after 24 h exposure of MBZ to HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells, a marked reduction in cell viability was produced compared to control (DMSO). Nevertheless, the exposure to MBZ did not affect cell viability of HCT-116 *K-Ras* Wt. more than 50% when compared to control DMSO treated cells. 2 µM concentration of MBZ was cytotoxic to HCT-116 *K-Ras* Mt. as 27% of these cells survived though with HCT-116 Wt. 72% survived after 24 h incubation. The IC_{50}, values were 0.486 ± 0.25 µM and 3.743 ± 1.2 µM for these cells respectively (Figure 4.1).

These outcomes were statistically significant for Wt. $p < 0.05$, for 24 h exposure at 0.5 µM, at 0.75 µM and 1 µM as well as significant $p<0.05$, at 0.5 and 0.75 µM, and $p<0.001$, at 1 µM for HCT-116 *K-Ras* Mt. Furthermore, due to the non-toxic effect within this concentration range for these cell lines it will be used in scratch and colony formation assays later in next chapter.

After 48 h HCT-116 *K-Ras* Mt. cell viability decreased significantly when compared to the cells that were exposed to the MBZ for 24 h ($p= 0.001$). In addition, the Mt. and Wt. cell line exposed to MBZ for 24 h, when After 48 and 72 h of drug exposure we observed that the cells sensitivity to the compound increased and the cell survival reduced sigificantly when the concentration increased on the mutant cell line than wild type. On exposure to drug concentrations 0.05, 0.1, 0.25, 0.5, 0.75 and 1 µM, after 48 h the % of start was 87% at the lowest concentration and 27% at the highest concentration for Mt. while survival at these drug concentrations was 97 to 62% for Wt. The IC_{50} respectively for the cell lines was 0.335±1.5 µM 0.679 ± 0.39 (mean SD) and the *p*-value was significant at $p<0.001$ for 0.25, 0.5, 0.75 and 1 µM (Figure 4.1).

After 72 h of cell exposure the absorbance values from treated cells decreased significantly when compared to the cells that were exposed to the MBZ for 48 h p=0.003. IC_{50}, values were 0.436 µM ± 0.09 and 0.886 µM± 0.36 respectively for the two exposure times (mean SD). Furthermore, after 72 h exposure, the % of Mt. cell survival was 53, 50 and 39% respectively at drug concentrations of 0.25, 0.5, 0.75 µM but Wt. had higher survival at these concentrations of 82, 75 and 61% IC_{50}, values were 0.436 µM ± 0.09 and 0.886 µM± 0.36 for 72h and 48h exposure times respectively (mean SD). The *p*-value was significant for at 0.25, 0.5 and 0.75 µM which were p<0.01 (Figure 4.1).



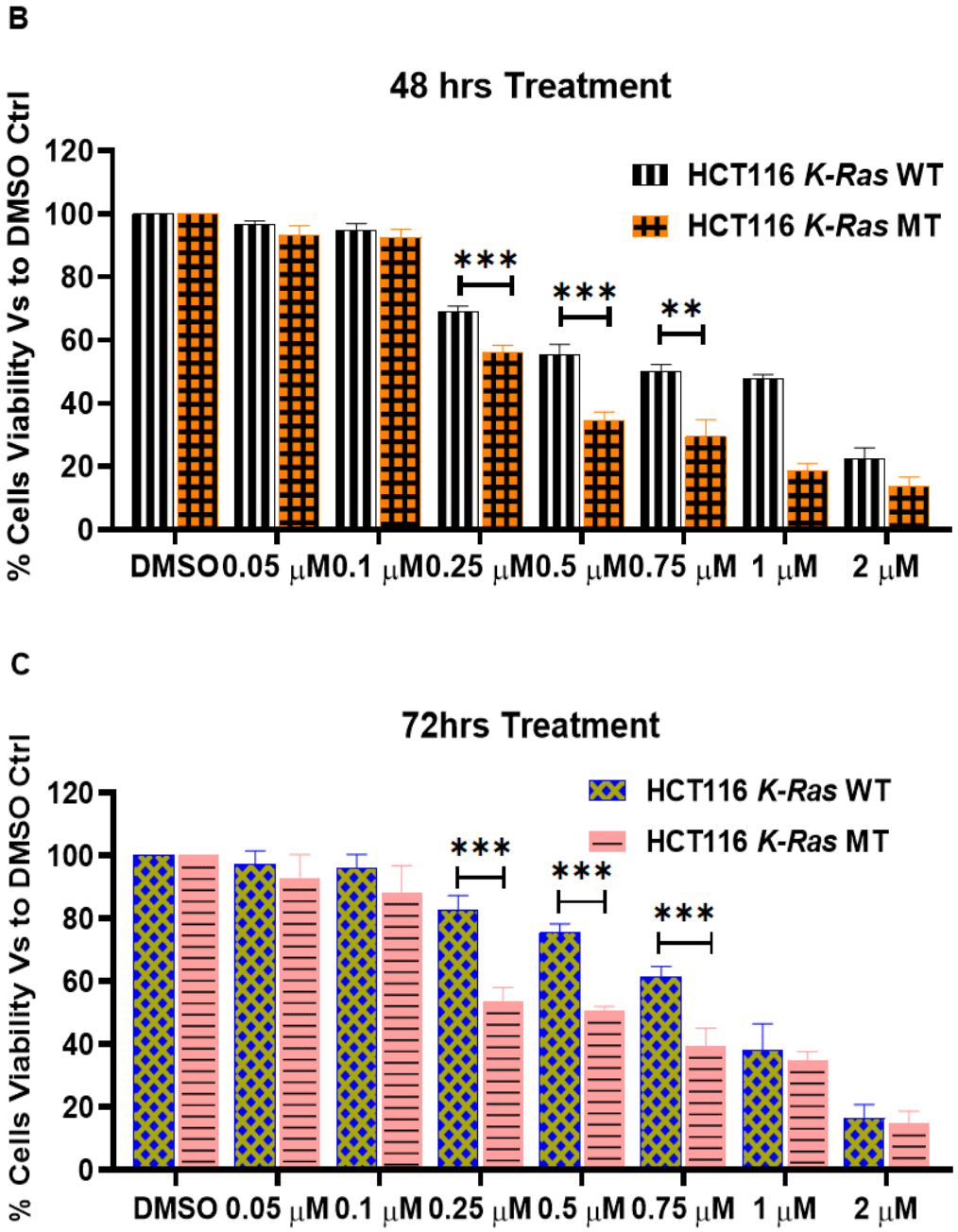


Figure 4.1: Cytotoxic effects of MBZ on HCT-116 *K-Ras* Mt. and Wt. cells, at (A)24, (B)48 and (C)72 h.

1×10^4 HCT-116 *K-Ras* Mt. and Wt. cells in a 96-well plate were treated with different concentration (0.05 - 2 µM) of MBZ or with vehicle (DMSO) for (A) 24, (B) 48 and for (C) 72 h. On day five MTT solutions were added, and absorbance was measured at 540 nm using a colourimetric plate reader. The percentage viability is calculated by the formula as described in methods (Section 2.2.1.9). The data represent results from at least three independent experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* and P ***p<0.01*).

4.3.1.2 Effect of MBZ on DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells using MTT.

The MTT assay showed that a 24 h exposure of DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells to MBZ produced a marked reduction in cell viability when compared to control (DMSO). After 48 h, the absorbance values from treated cells decreased significantly when compared to the cells that were subjected to the MBZ for 24 h (p= 0.05), with the statistical parameter determined by using unpaired *student t* test.

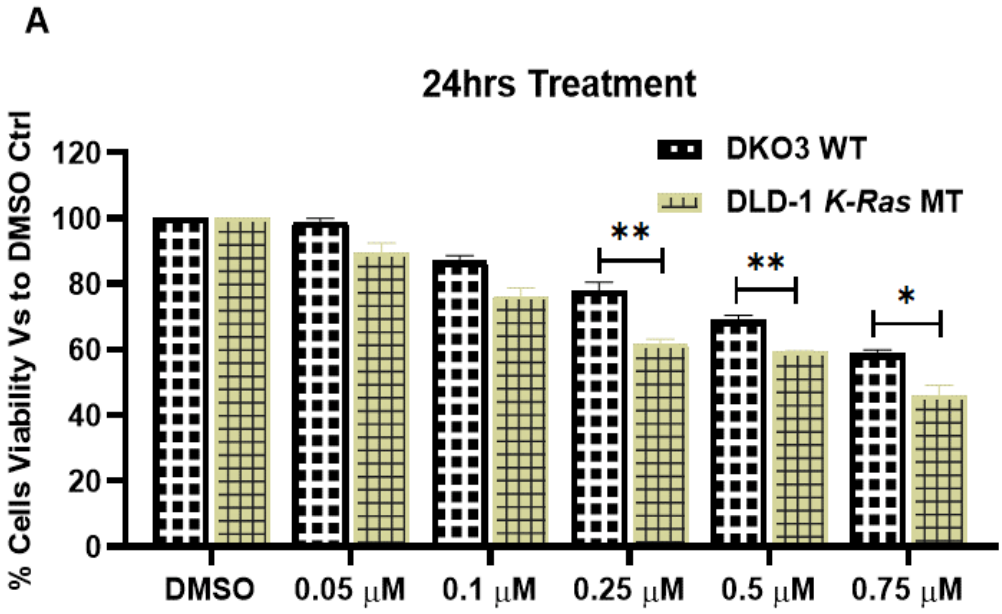
In addition, same cells lines of Mt. and Wt. exposed to Ran inhibitor for 24 h, when compare this result, after 48 and 72 h of drug exposure, have observed that the cells sensitivity to the compound increased and the cell survival reduced sigificantley when the concentration increased on the isogenic pairs cell line.

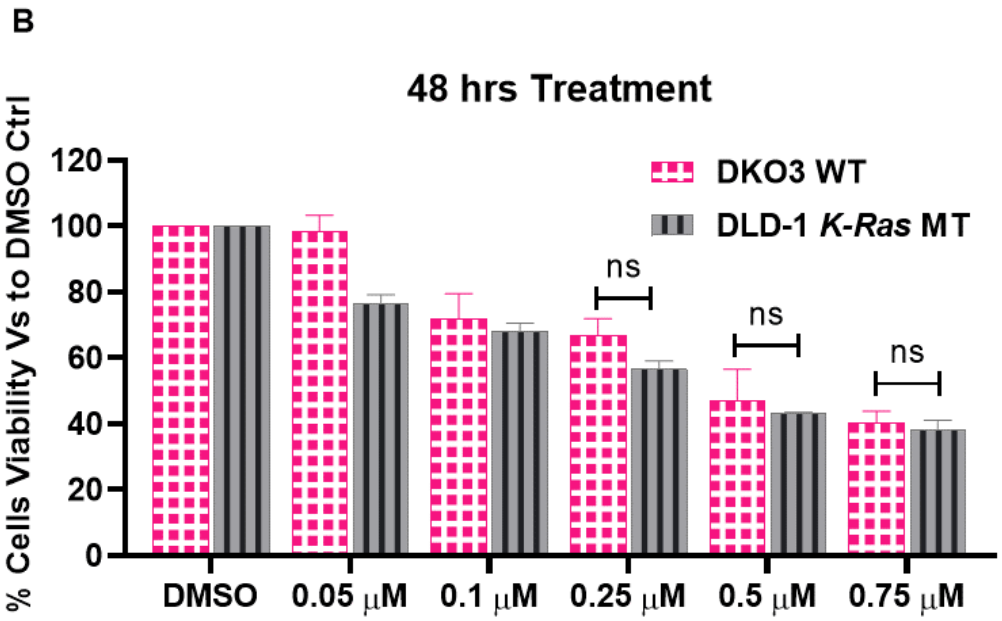
After 48 h of drug exposure at different concentration 0.05, 0.1, 0.25, 0.5, 0.75 µM, the percentage of survival was 56, 43 and 38% on Mt. and 66, 47 and 40% for Wt. The respective IC_{50} values for the cell lines were 0.456 µM±0.15 and 0.52 µM ± 0.9 (mean SD)though the *p*-value was not significant (Figure 4.2).

After 72 h of cell exposure the absorbance values from treated cells decreased significantly when compared to the cells exposed to the MBZ for 48 h. Furthermore, after 72 h exposure, the Mt. cell survival was 63, 48 and 25%, for drug concentrations 0.25, 0.5, 0.75 µM but Wt. had similar survival of 69, 51 and 33%. IC_{50}, values were 0.524 µM ± 0.09 and 0.562 µM± 0.6 respectively (mean SD) The *p*-value was not significant (Figure 4.2).

An MBZ effect was observed on DLD-1 *K-Ras* Mt. and DKO-3 Wt. with a significant effect only on Mt. When compared with wild type cell line after 24 h of exposure to drug concentrations of 0.25, 0.5 and 0.75 µM the cell survival was 61, 59 and 45% of the DLD-1 *K-Ras* Mt. while on and 87, 78 and 68% of the DKO-3 Wt. after 24 h. A significant *p* value was found (p< 0.01) for these three concentrations. The IC_{50} values were 0.630 µM ± 0.5 1.112 µM ± 1.2 SD, respectively (mean SD), (Figure 4.2).

On the other hand, a less cytotoxic effect was observed in DKO-3 Wt. compared to the mutant cell line DLD-1 *K-Ras* Mt. 72%, (*p<0.001*) and 27%, (*P<0.001*), respectively.


A
24hrs Treatment
% Cells Viability Vs to DMSO Ctrl
120
100
80
60
40
20
0
DKO3 WT
DLD-1 K-Ras MT
**
**
*
DMSO
0.05 μM
0.1 μM
0.25 μM
0.5 μM
0.75 μM


B
48 hrs Treatment
% Cells Viability Vs to DMSO Ctrl
120
100
80
60
40
20
0
DKO3 WT
DLD-1 K-Ras MT
ns
ns
ns
DMSO
0.05 μM
0.1 μM
0.25 μM
0.5 μM
0.75 μM

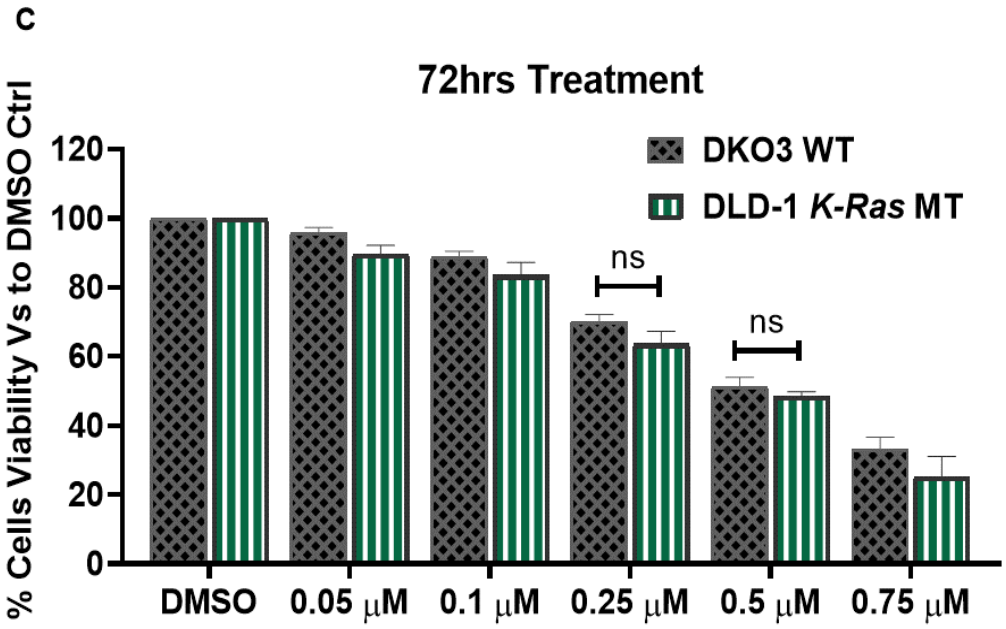


Figure 4.2: Cytotoxic effects of MBZ on DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells at (A) 24, (B) 48 and (C)72 h exposure to the drug.

1×10^4 DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells in a 96-well plate were treated with different concentration (0.05 – 0.75 μM) of MBZ and with vehicle (DMSO) for (A) 24, (B) 48 and for (C) 72 h. On day five MTT solutions were added, and absorbance was measured at 540 nm using a colourimetric plate reader. The percentage viability is calculated by the formula as described in methods (Section 2.2.1.9). The data represent results from at least three independent experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.01* and P **p<0.05).

4.3.1.3 Effect of MBZ on HCT-116 *Pten* null Mt. and HCT-116 *K-Ras* Wt. cells using MTT.

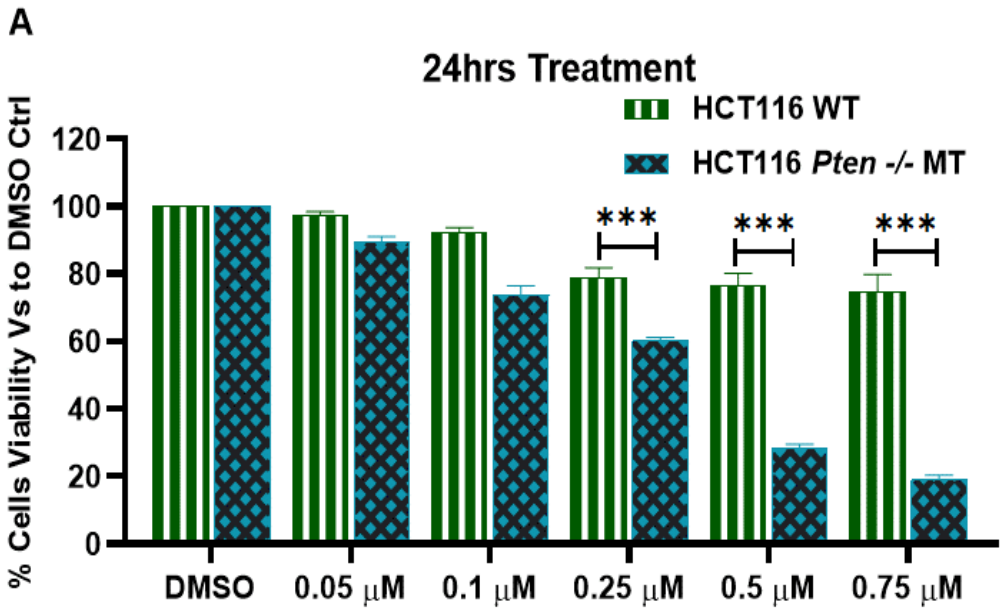
The MTT assay showed that 24 h exposure of isogenic pairs of human colorectal HCT-116 *Pten* null Mt. and HCT-116 *K-Ras* Wt. cells to MBZ produced a marked reduction in cell viability compared to control (DMSO). However, this exposure to MBZ did not produce reduction of cell viability more than 50% when compared to control. After 48 h of exposure the absorbance values from treated cells decreased significantly when compared to the cells that were exposed to the MBZ for 24 h (p=0.003),

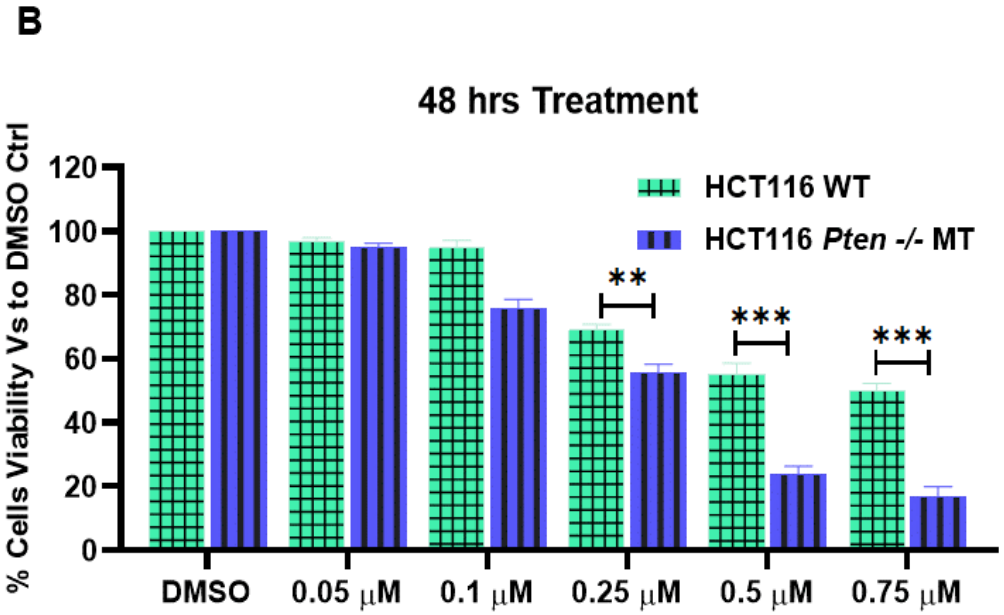
After 72 h of cell exposure, the absorbance values for treated cells decreased significantly when compared to the cells that were exposed to the MBZ for 48 h (p= 0.001).

We observed that the cells sensitivity to the MBZ increased and the cell survival reduced when the drug concentration increased from 0.05 to 0.75 µM more sigificantly on the mutant cell line than wild type, at 24 h the survival was 73% at the lowest concentration to 18% at the highest concentration for HCT-116 *Pten* null Mt. and 92 to 74% for HCT-116 Wt., respectively. The IC_{50} was 0.39 µM ± 1.5 and 2.4 ± 1.9 for the mutant and wild type cell lines,(mean±SD, Figure 4.3).

After 48 h of drug exposure at different concentration 0.05, 0.1, 0.25, 0.5, 0.75 µM, the % of survival was 75, 55, 23 and 16% on HCT-116 *Pten* null Mt. and 94, 68, 55 and 50% for HCT-116 Wt. respectively. The *p*-value was significant at $p<0.05$ for concentrations of 0.05, 0.1, 0.25, 0.5 µM and $p<0.001$ at 0.75 µM concentration. The IC_{50} was 0.256 µM ± 0.15 and 0.629 ± 0.9 for the mutant and wild type cell lines (Figure 4.3).

After 72 h exposure, the % of HCT-116 *Pten* null Mt. cell survival was 63, 48 and 25% at MBZ concentrations of 0.25, 0.5, 0.75 µM but HCT-116 Wt. had similar % of survival 69, 51 and 33%. The IC_{50}, values were 0.486 ± 0.09 SD and 0.791 µM ± 0.6 SD, respectively (mean±SD). The *p* value was not significant (Figure 4.3).


A
24hrs Treatment
HCT116 WT
HCT116 Pten -/- MT
% Cells Viability Vs to DMSO Ctrl
120
100
80
60
40
20
0
***
***
***
DMSO
0.05 μM
0.1 μM
0.25 μM
0.5 μM
0.75 μM


B
48 hrs Treatment
HCT116 WT
HCT116 Pten -/- MT
% Cells Viability Vs to DMSO Ctrl
120
100
80
60
40
20
0
**
***
***
DMSO
0.05 μM
0.1 μM
0.25 μM
0.5 μM
0.75 μM

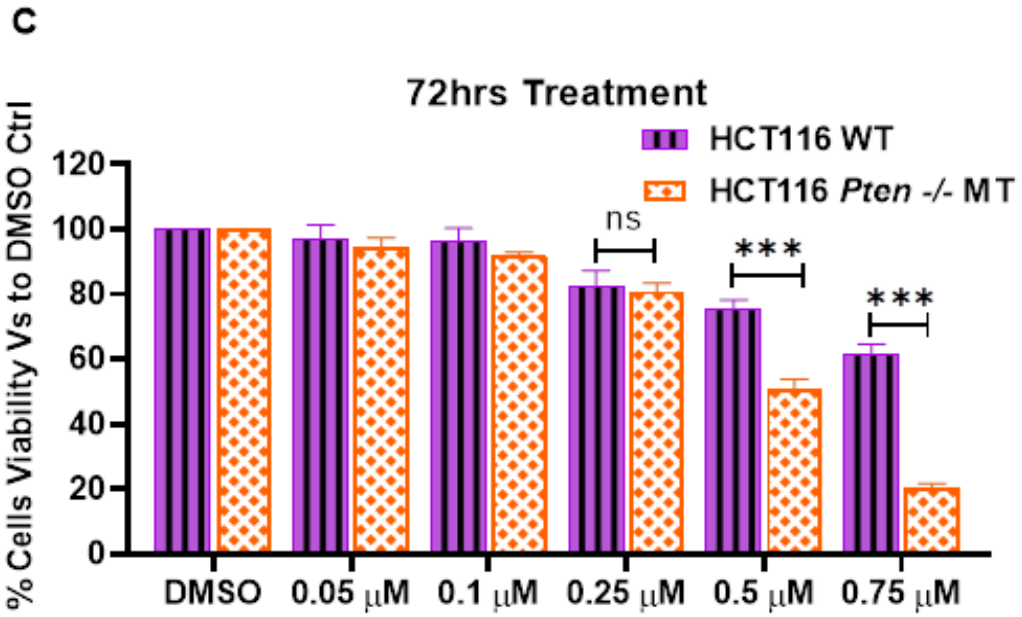


Figure 4.3: Cytotoxic effects of MBZ on HCT-116 *Pten* null and HCT-116 Wt. cells, after treatment for (A) 24, (B) 48 and (C) 72 h.

1×10^4 HCT-116 *Pten* null and HCT-116 Wt. cells in a 96-well plate were treated with different concentrations (0.05 – 0.75 µM) of MBZ or with control (DMSO) for (A) 24, (B) 48 and for (C) 72 h. On day five MTT solutions were added, and absorbance was measured at 540 nm using a colourimetric plate reader. The percentage viability is calculated by the formula as described in methods (Section 2.2.1.9). Data represent results from at least three independent experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* and P ***p<0.01*). IC_{50} were calculated from graphs and are shown in the following (Table 4. 1). These are mean IC_{50} values ±SD in µM for three independent experiments.

Table 4.2: Summary of *in vitro* cytotoxic results following different exposure time interval to MBZ.

Cell line	*IC_{50}*, ± SD (µM) 24 h	*IC_{50}*, ± SD (µM) 48 h	*IC_{50}*, ± SD (µM) 72 h
HCT-116 *K-Ras* Mt.	0.486± 0.25	0.335± 0.015	0.436± 0.09
HCT-116 *K-Ras* Wt.	3.7± 1.2	0.679± 0.23	0.88± 0.36
HCT-116 *pten -/-*	0.39± 1.9	0.27± 0.15	0.486± 0.08
DLD-1 *K-Ras* Mt.	0.65± 0.08	0.45± 0.15	0.524± 0.06
DKO-3 Wt.	0.894± 0.08	0.52± 0.90	0.562± 0.06

4.3.2 Effect of Ran inhibitor-MBZ on expression of anti-apoptotic and apoptotic factors in HCT-116 K-Ras Mt. and Wt. cells.

4.3.2.1 MBZ effect on expression of Bcl-2 mRNA in HCT-116 *K-Ras* Mt. and Wt. cells analysed using qRT-PCR.

Bcl-2 expression was significantly down regulated in both isogenic cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. the significant were *p<0.001* and *p<0.01*, respectively (Figure 4.4).

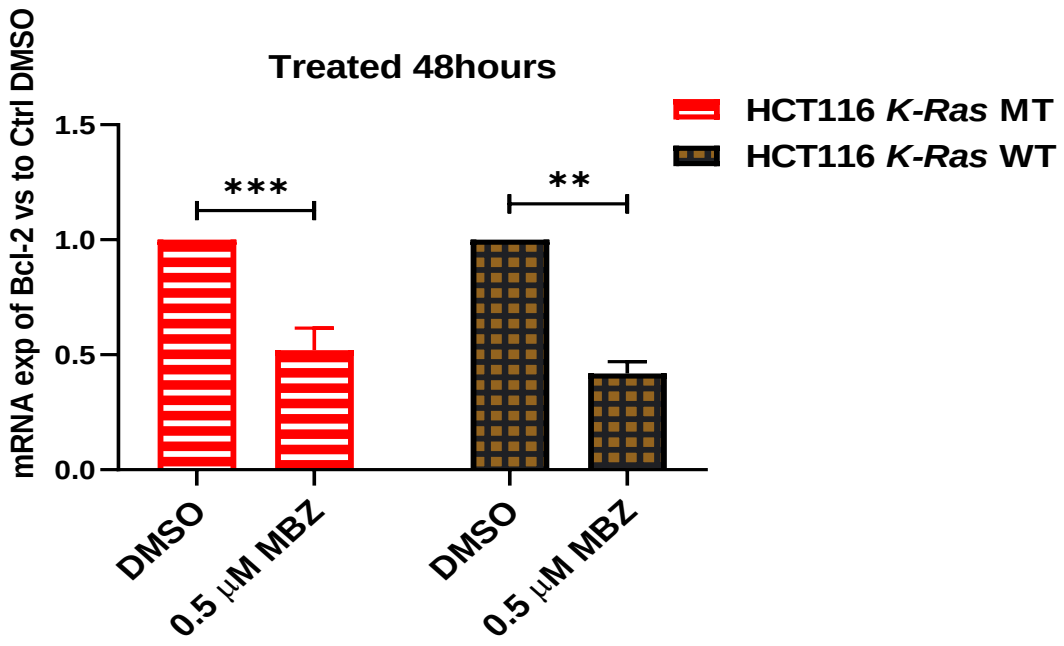


Figure 4.4: MBZ impact on relative mRNA expression level of Bcl-2 in HCT-116 *K-Ras* Mt. and Wt. cells using qRT-PCR.

HCT-116 *K-Ras* Mt. and Wt. cells were either un-treated or treated with 0.5 µM MBZ for 48 h. mRNA isolated and used for preparation of cDNA. The histogram (Figure 4.4) shows Bcl-2 relative expression in HCT-116 *K-Ras* Mt. and Wt. standardised to endogenous housekeeping gene B2M. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* and ***p<0.01* was statistically significant).

4.3.2.2 Effect of Ran inhibitor MBZ on expression of Mcl-1 gene in HCT-116 *K-Ras* Mt. and Wt. on level of mRNA by qRT-PCR.

Mcl-1 expression was significantly down regulated in both isogenic cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. to a similar extent the levels of significance were *p<0.01* and *p<0.001* respectively (Figure 4.5).

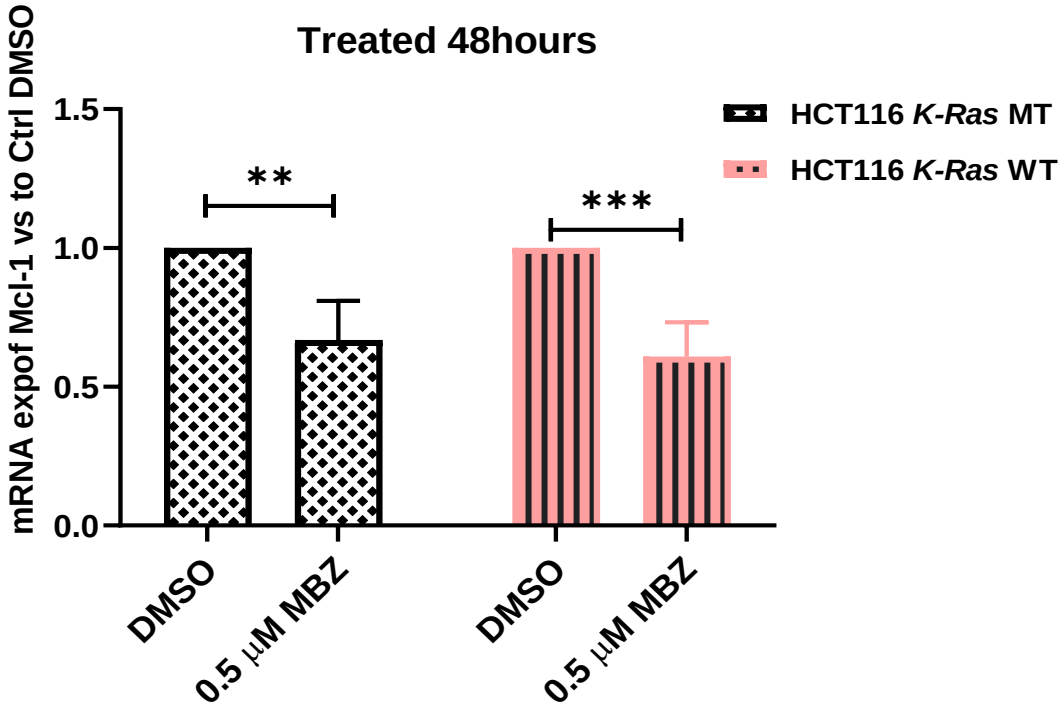


Figure 4.5: MBZ impact on relative mRNA expression level of Mcl-1 in HCT-116 *K-Ras* Mt. and Wt. cells using qRT-PCR.

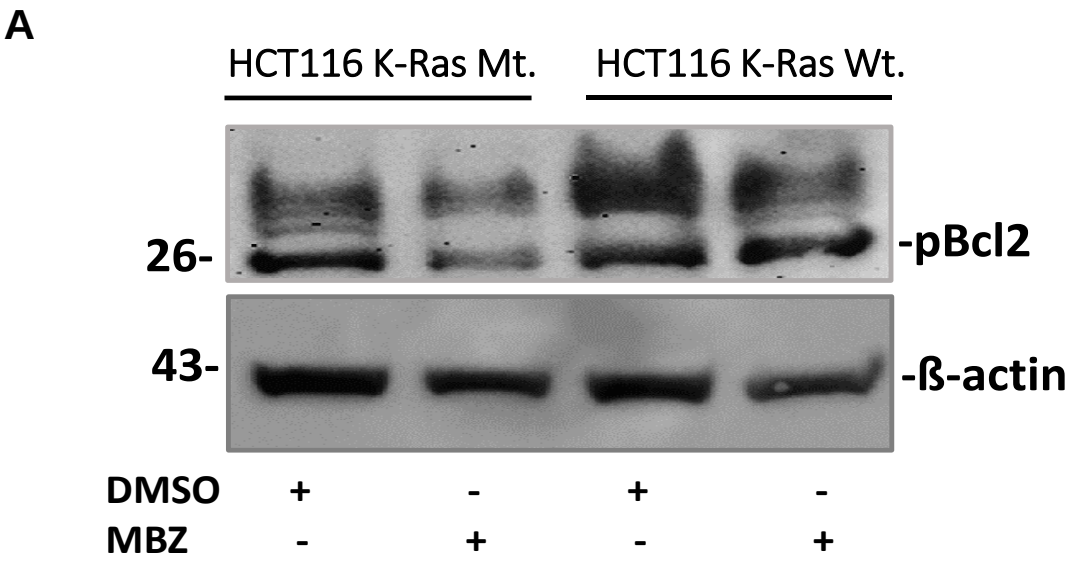
HCT-116 *K-Ras* Mt. and Wt. cells were either un-treated or treated with 0.5 µM MBZ for 48 h. mRNA was isolated and used for preparation of cDNA. The histogram shows Mcl-1 relative expression in HCT-116 *K-Ras* Mt. and Wt. standardised to endogenous housekeeping gene B2M. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* and ***p<0.01* was statistically significant).

4.3.3 Effect of MBZ on the level of anti-apoptotic and apoptotic proteins in isogenic colorectal cancer HCT-116 *K-Ras* Mt. and Wt. using western blot.

4.3.3.1 Effect of MBZ on Bcl-2 protein in HCT-116 *K-Ras* Mt. and Wt. cells.

Previous research have attributed the anti-tumour effect of MBZ and its action to induce apoptosis was prevented by the Bcl-2 phosphorylation, which avoids the interaction between the pro-survival Bcl-2 and the pro-apoptotic (Bax), thereby promoting apoptosis (Doudican et al. 2008; Williamson et al. 2016).

The expression of Bcl-2 protein level was measured after the cells were treated with 0.5 µM MBZ or with 0.1% DMSO as control for 48 h. Protein was extracted, and proteins concentration assessed by a Bradford assay and an equal amount of protein was subjected to SDS-page and western blotting. Western blot analyses revealed that MBZ shows an inhibitory effect on Bcl-2 protein so inducing apoptosis. The inhibitory effect was observed in (lane 2) HCT-116 *K-Ras* Mt. cells treated with MBZ 0.5 µM $p<0.001$, but not in (lane 1) untreated cells. When comparing HCT-116 *K-Ras* Wt. treated with 0.5 µM MBZ (lane 4) with untreated cells in (lane 3) we observed a significant difference $p<0.001$ (Figure 4.6).



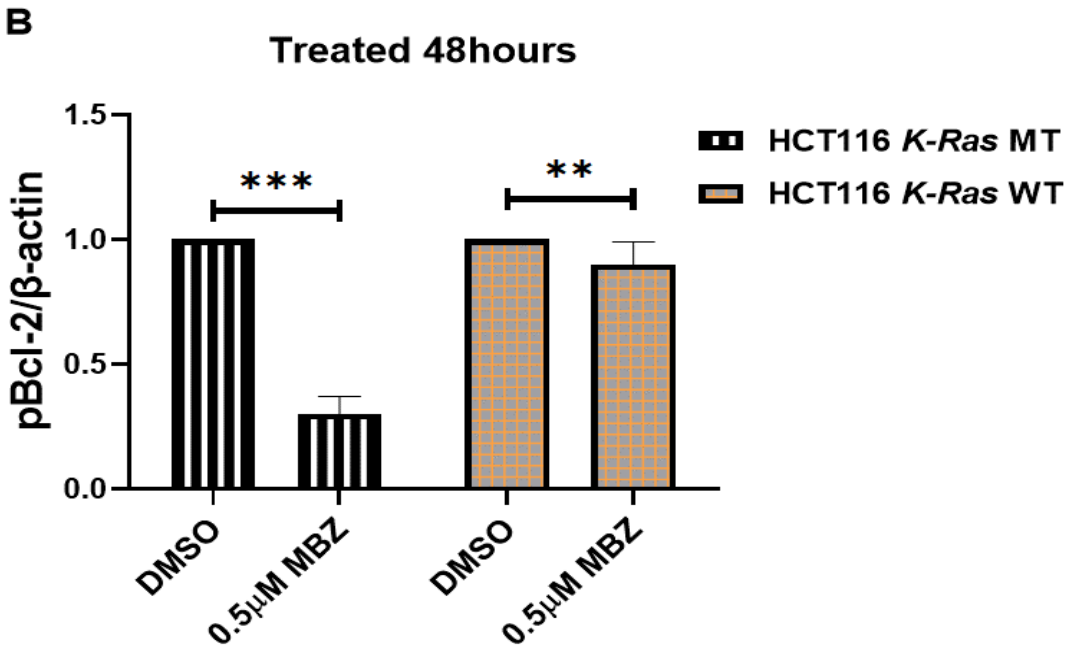


Figure 4.6: Protein levels of Bcl-2 when treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 µM MBZ for 48 h. Cellular proteins were isolated and western blotting performed. Blots were probed with the Bcl-2 and β-actin antibodies as indicated. (B) The histogram indicates Bcl-2 protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. The Bcl-2 levels were normalised with β-actin protein levels. Error bars representSD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (***$p<0.001$).

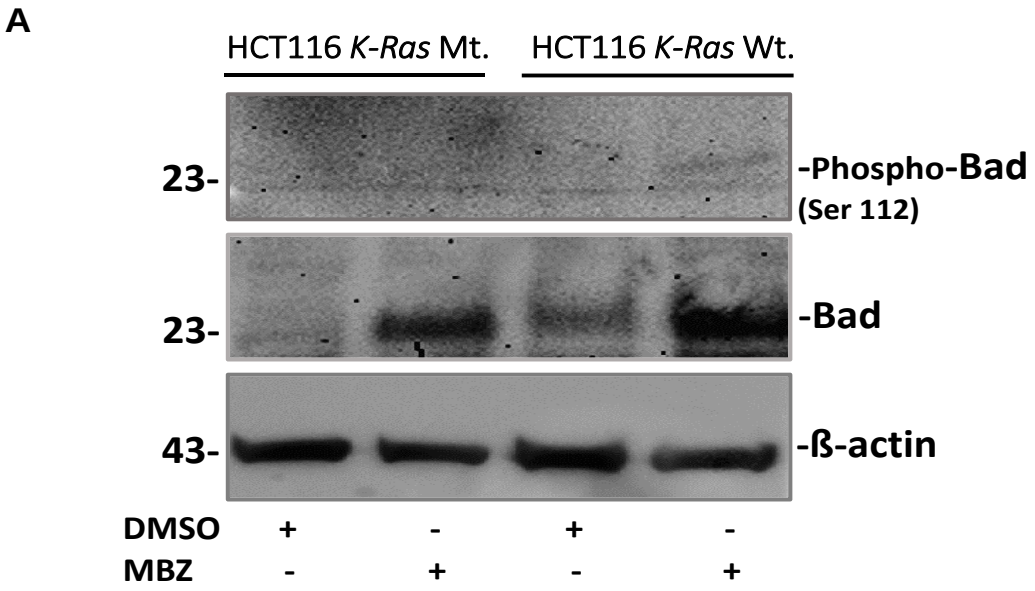
4.3.3.2 Effects of MBZ on pro-apoptotic family proteins such as BH3 only proteins (Bad) and p-Bad in isogenic CRC cell lines such as HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt., 48 h using a western blot.

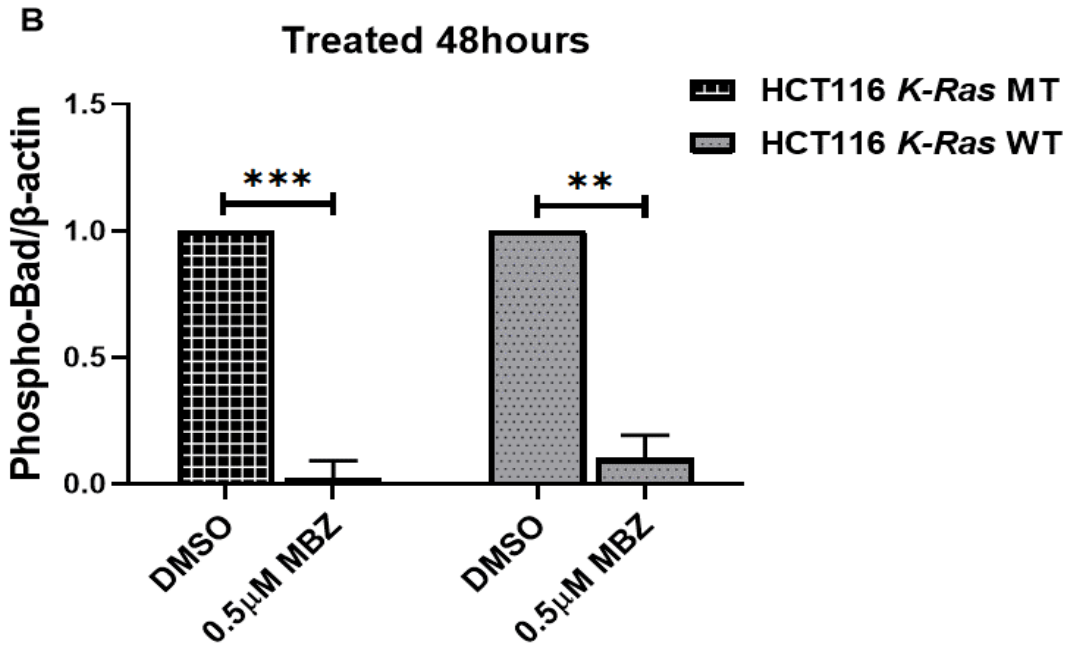
A western blot was carried out to assess the effect of MBZ on the levels of pro-apoptotic gene family proteins such as BH3 only proteins (Bad). The expression of phosphorylated Bad (Ser112) (40A9) protein level was measured, so the cell lines were treated with 0.5 µM MBZ or 0.1% DMSO as a control for 48 h then cells were extracted, and protein concentration assessed by a Bradford assay. A similar amount of proteins was subjected to SDS-page gel. The total protein levels were assayed using immunoblot analysis and normalised to vehicle untreated control.

After treatment with 0.5 µM MBZ in both the HCT-116 *K-Ras* Mt. and Wt. Bad was upregulated significantly ($p<0.001$, compared to control), Phosphorylated Bad (Ser112) (40A9) was downregulated significantly ($p<0.001$, compared to control) in both cell lines. P-Bad (Ser112) detects endogenous amounts of Bad proteins only at Ser112 when it is phosphorylated. BH3 only protein Bad is a pro-apoptotic member that encourages cell to undergo apoptosis by shifting Bax from binding to Bcl-2 and Bcl-xL (Zha et al. 1996).

Pro-survival factors Bcl-2 proteins were down-regulated in HCT-116 *K-Ras* Mt. ($p<0.001$, compared to control) and less significantly with HCT-116 *K-Ras* Wt. (Figure 4.7) suggesting its dependence on the mitochondrial apoptotic pathway.

Previous research have found MBZ induces apoptosis by preventing Bcl-2 phosphorylation, which prevents the interaction between the pro-survival Bcl-2 and the pro-apoptotic (Bax), thereby promoting apoptosis (Doudican et al. 2008; Williamson et al. 2016).


A
HCT116 K-Ras Mt.
HCT116 K-Ras Wt.
23-
-Phospho-Bad
(Ser 112)
23-
-Bad
43-
-ß-actin
DMSO + - + -
MBZ - + - +


B
Treated 48hours
HCT116 K-Ras MT
HCT116 K-Ras WT
Phospho-Bad/β-actin
1.5
1.0
0.5
0.0
***
**
DMSO
0.5μM MBZ
DMSO
0.5μM MBZ

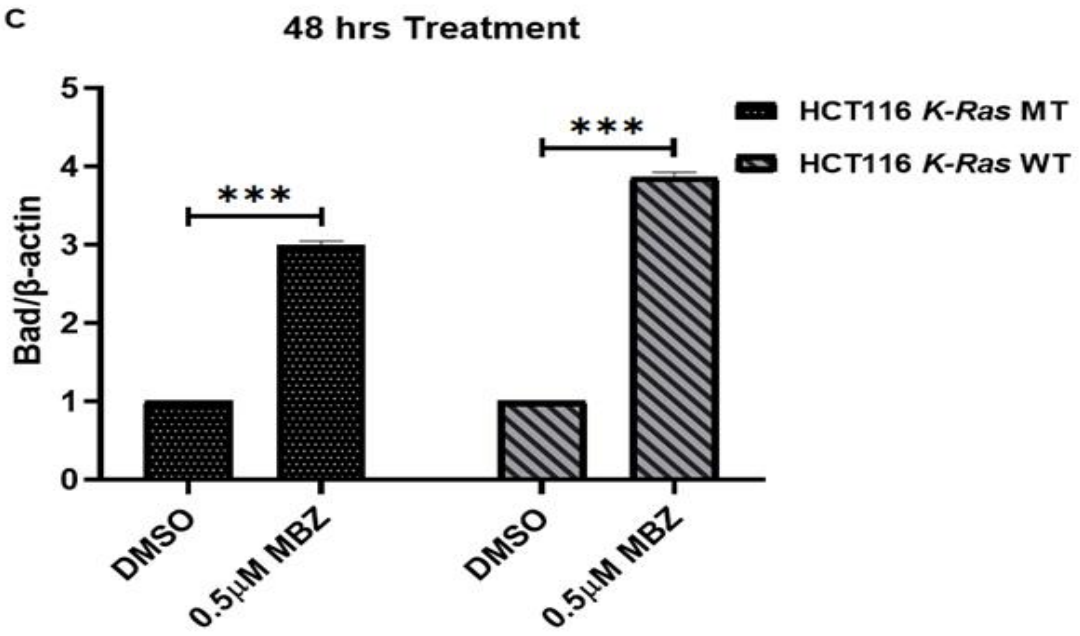


Figure 4.7: Protein levels of pro-apoptotic family member BH3-only proteins Bad and Phospho-Bad when treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 μM MBZ for 48 h. Cellular proteins were isolated and western blotting was performed. Blots were probed with the pro-apoptotic family member BH3-only proteins Bad and Phospho-Bad and β-actin antibodies as indicated. The histogram shows Bad protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. (B) The Phospho-Bad and (C) Bad levels were normalised with β-actin protein levels. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001*, P ***p<0.01*).

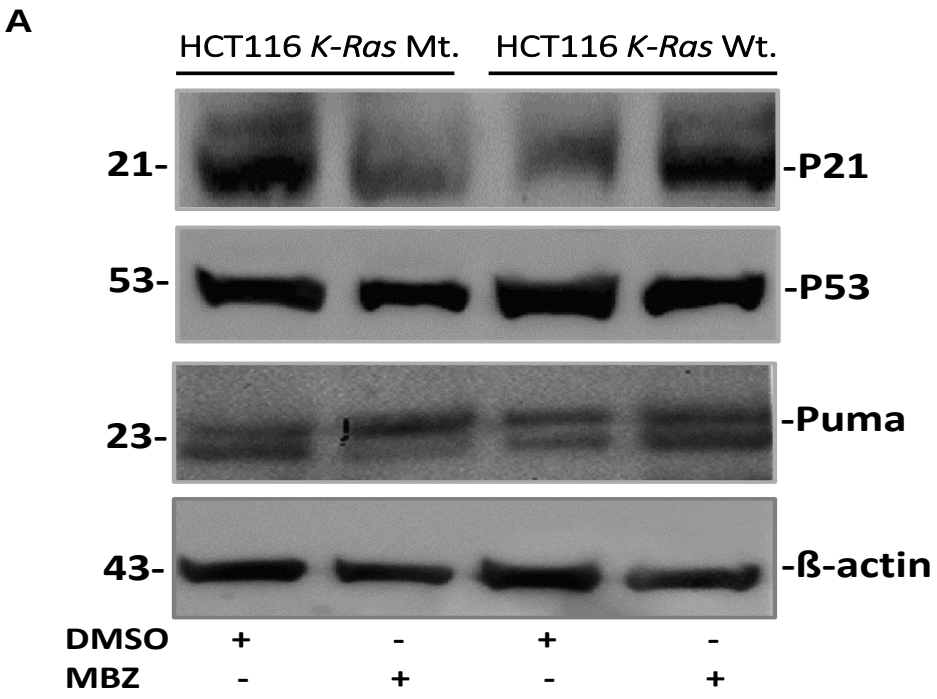
4.3.3.3 Effects of MBZ on levels of pro-apoptotic family proteins such as BH3 only proteins puma and p53 Tumour suppressor and p21 protein in HCT-116 K-Ras Mt. and HCT-116 K-Ras Wt.

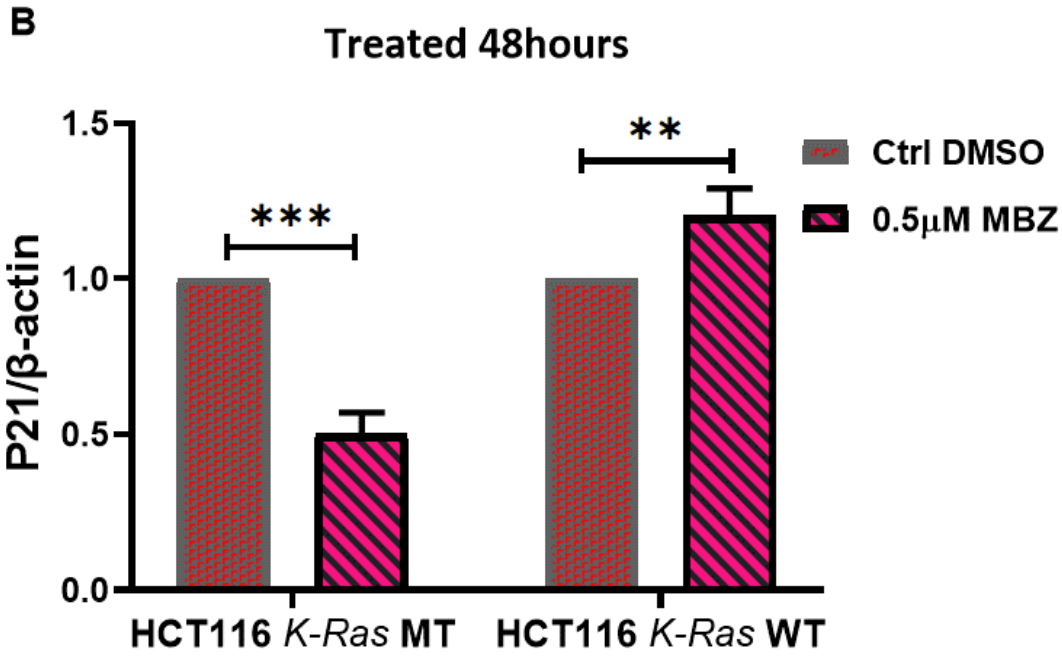
A western blot was carried out to assess the pro-apoptotic family proteins such as BH3 only proteins puma and p53 Tumor suppressor and p21 proteins. The cell lines were treated with 0.5 µM MBZ or with 0.1% DMSO as control for 48 h then extracted and the protein concentration assessed by a Bradford assay. A similar amount of proteins was subjected to an SDS-page gel. The total protein levels were assayed by using immunoblot analysis and normalised to control.

In addition, several pro-apoptotic family proteins were measured via western blot analysis. p53 protein was elevated in both the colorectal isogenic cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. treated with 0.5 µM MBZ, signifying its participation in the apoptosis induction (Martarelli et al. 2008). Furthermore, MBZ significantly increased release of cytochrome c in a dose 0.5 µM of MBZ (Martarelli et al. 2008).

To establish the potential effect of MBZ on cell growth and apoptosis, puma protein expression was assessed by the western blot as described in materials and methods section 2.1.4. Thus, the potential effect of MBZ to induce apoptosis on isogenic CRC cell lines such as HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. was assessed. The pro-apoptotic puma proteins expression levels associated to the mitochondrial death pathway were significantly up-regulated ($p<0.001$, compared to control in both cell line), after 48 h of 0.5 µM MBZ.

p21 protein was upregulated $p<0.01$ after 48 h, with accumulation of p53. MBZ induced p53 and p21 protein expression in both HCT-116 *K-Ras* Mt. ($p<0.05$ *and* $p<0.001$) and HCT-116 *K-Ras* Wt. ($p<0.05$ and $p<0.01$). However, HCT-116 *K-Ras* Mt. was upregulated to half in case of P12 $p<0.001$ after 48 h treatment (Figure 4.8). p53 protein up-regulation by MBZ may have been caused by protein stabilisation similar to that brought about by paclitaxel (Abbas and Dutta 2009).


A
HCT116 K-Ras Mt.
HCT116 K-Ras Wt.
21-
-P21
53-
-P53
23-
-Puma
43-
-ß-actin
DMSO + - + -
MBZ - + - +


B
Treated 48hours
P21/β-actin
1.5
1.0
0.5
0.0
***
**
Ctrl DMSO
0.5μM MBZ
HCT116 K-Ras MT
HCT116 K-Ras WT

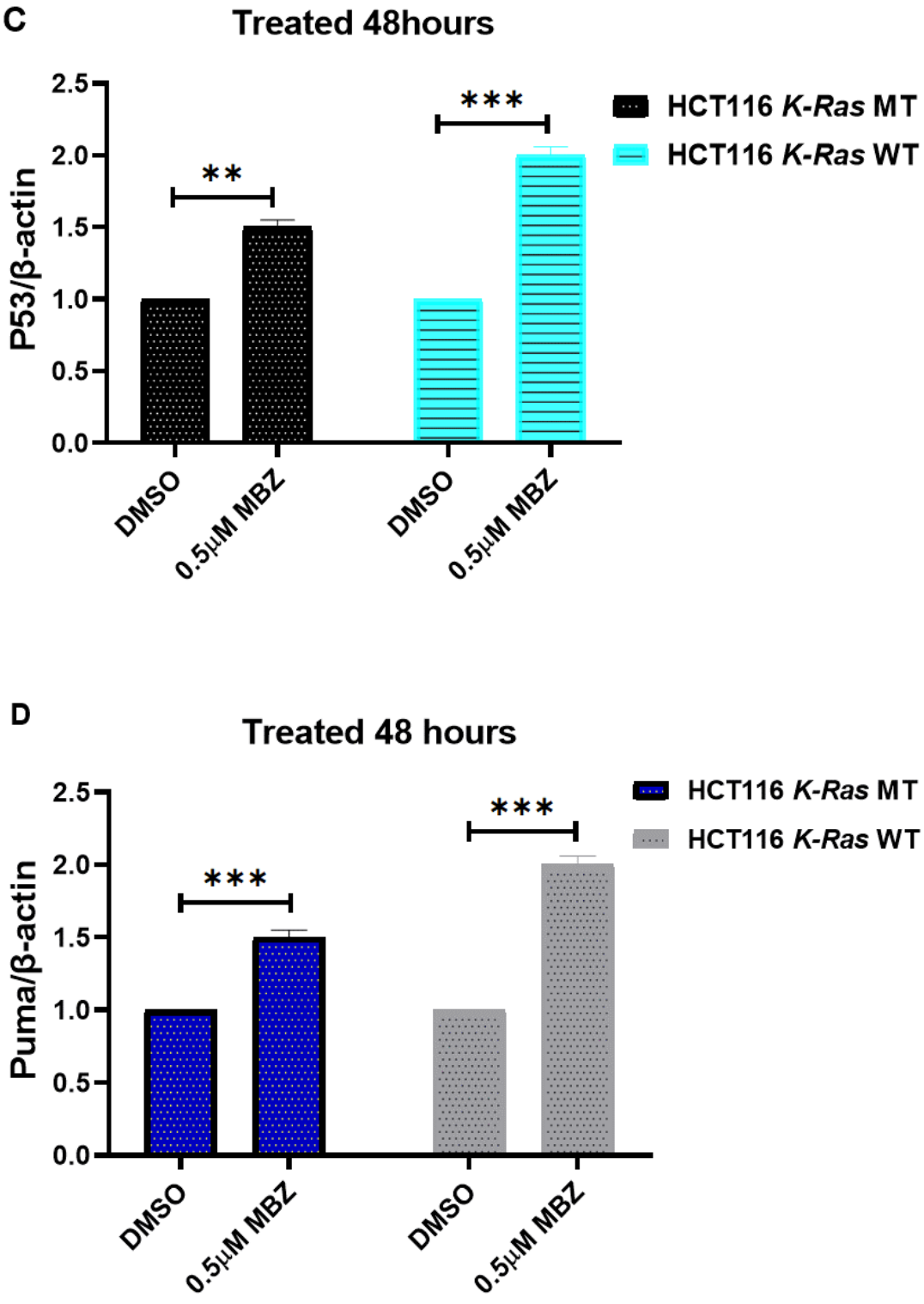


Figure 4.8: Protein levels of pro-apoptotic family member BH3 only puma, p53 tumour suppressor and p21 protein when treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

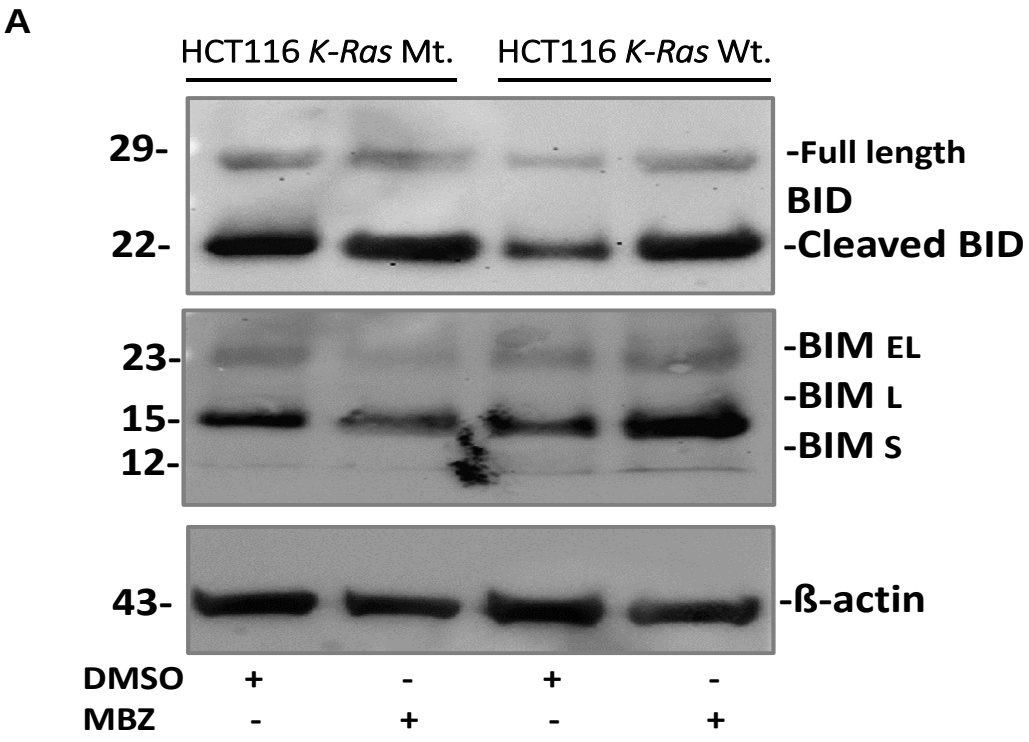
(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 µM MBZ for 48 h. Cellular proteins were isolated and western blotting was performed. Blots were probed with p21, p53 tumour suppressor and the pro-apoptotic family member BH3 only puma protein and β-actin antibodies as indicated. Histograms

indicate protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. (B)The p21, (C) p53 tumour suppressor and (D) puma protein levels were normalised with β-actin protein levels. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* and P ***p<0.01*).

4.3.3.4 Effects of MBZ treatment on levels of pro-apoptotic gene family proteins such as BH3 only proteins Bid and Bim in the isogenic CRC cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt.

A western blot was carried out to assess levels of the pro-apoptotic family proteins such as BH3 only proteins Bid and Bim proteins. so, the cell lines were treated with 0.5 µM MBZ or with 0.1% DMSO as control for 48 h then the cells were extracted, and protein concentrations assessed by a Bradford assay. A similar amount of proteins was subjected to SDS-page gel. The total protein levels were assayed by using immunoblot analysis and normalised to vehicle untreated control (DMSO). In addition, several pro-apoptotic family proteins were measured via western blot analysis. Bid and Bim proteins were elevated in both HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells treated with 0.5 µM MBZ, suggesting a role for the drug in apoptosis induction in these cell lines.

This study found that MBZ significantly induced Bid activation, as evaluated through cleavage of Bid into their respective p29 active forms in HCT-116 *K-Ras* Mt. and Wt. colorectal cell lines ($p < 0.01$ in both cell lines, (Figure 4.9). Bim proteins which induce apoptosis were significantly activated ($p < 0.01$) as we observed elevated levels of three isoforms BimS, BimL and BimEL in both colorectal cell lines (Figure 4.9).



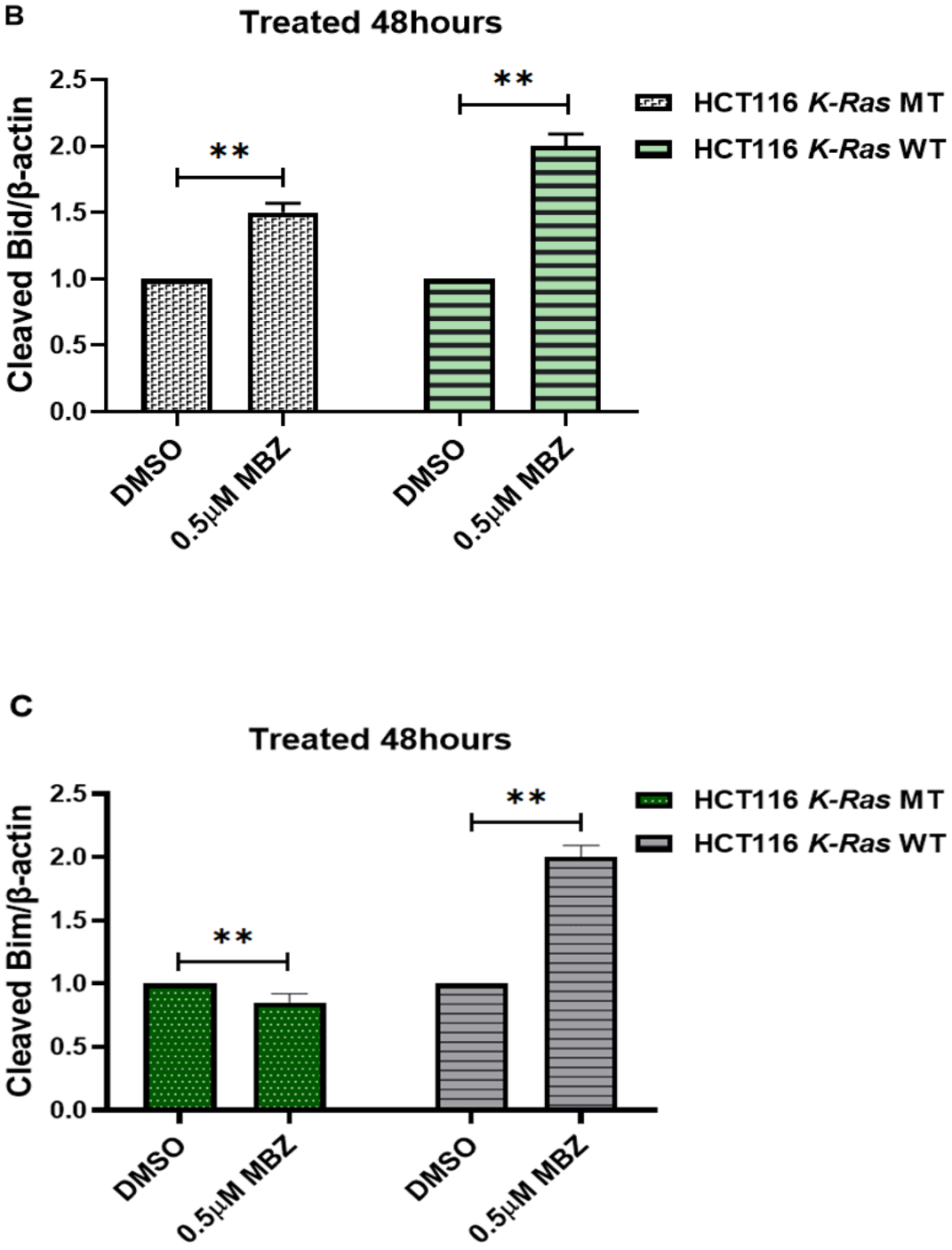


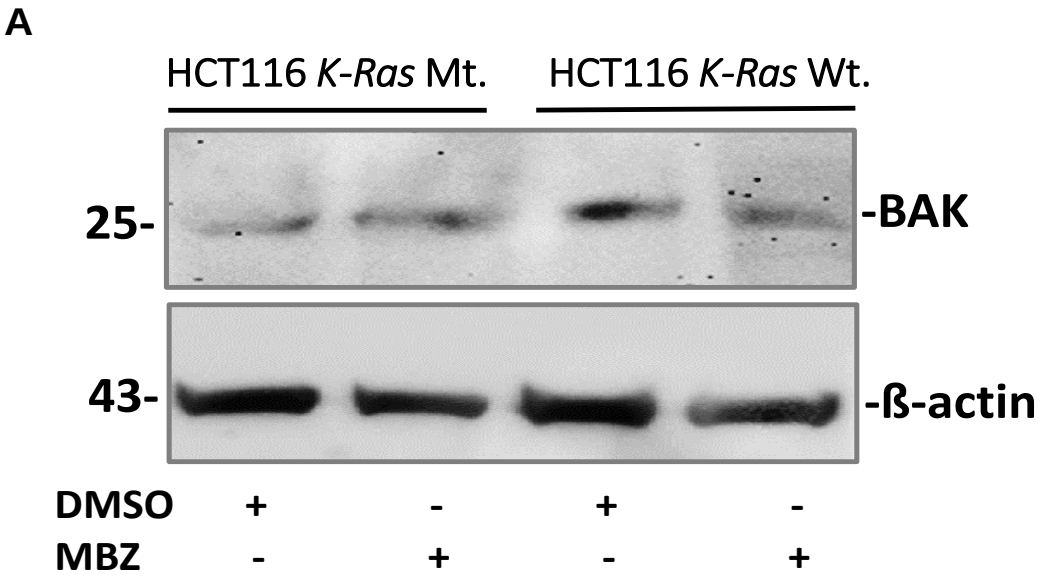
Figure 4.9: Protein levels of pro-apoptotic family member BH3 only Bim and Bid protein when treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 μM MBZ for 48 h. Cellular proteins were isolated and western blotting performed. Blots were probed with the Bid and Bim protein and β-actin antibodies as indicated. The histogram indicates (B) Bid and (C) Bim protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. The pro-apoptotic family member BH3 only Bid and Bim protein levels were normalised with β-actin protein levels. Error bars

represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (***p<0.01* was statistically significant).

4.3.3.5 Effects of MBZ treatment on levels of pro-apoptotic gene family proteins such as BH3 only proteins Bak in isogenic CRC cell lines such as HCT-116 K-Ras Mt. and HCT-116 K-Ras Wt., 48 h using a western blot.

The results illustrate that treatment with 0.5 µM MBZ after 48 h significantly inhibits Bak protein expression in HCT-116 *K-Ras* Mt. (*p<0.01*, Figure 4.10). However, when compared to the HCT-116 *K-Ras* Wt., 0.5 µM MBZ appears to induce Bak but to a less extent (*p<0.05,* Figure 4.10). Lastly, both isogenic cell lines when treated with MBZ showed a significant inhibition in the levels of Bak protein after 48 h of treatment when compared with that of the un-treated cells.



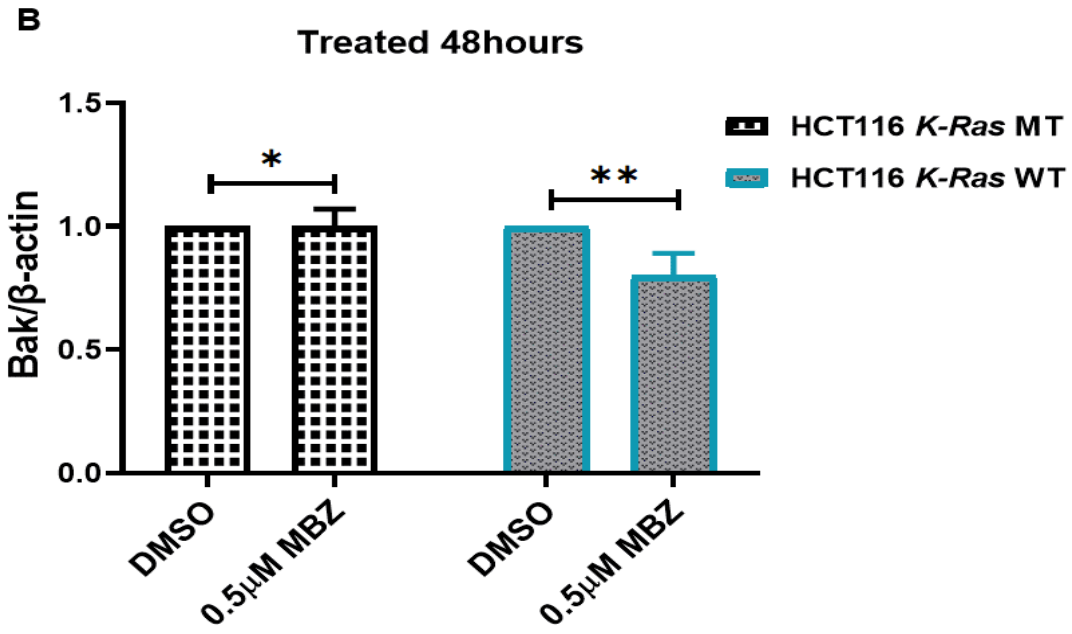
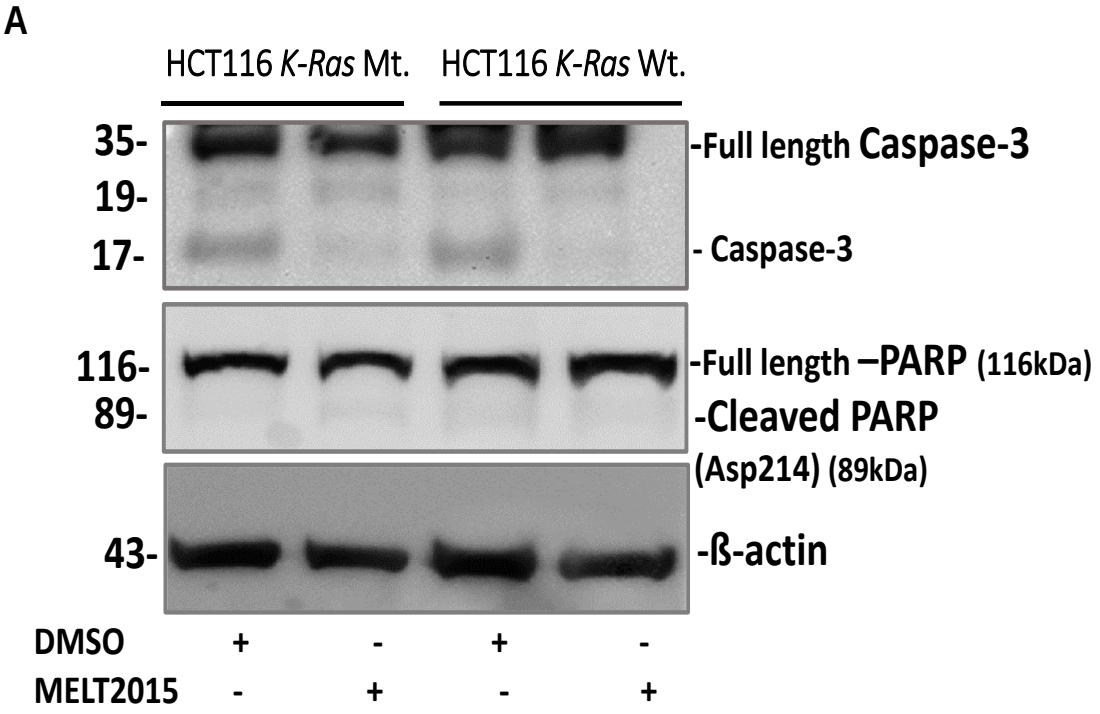


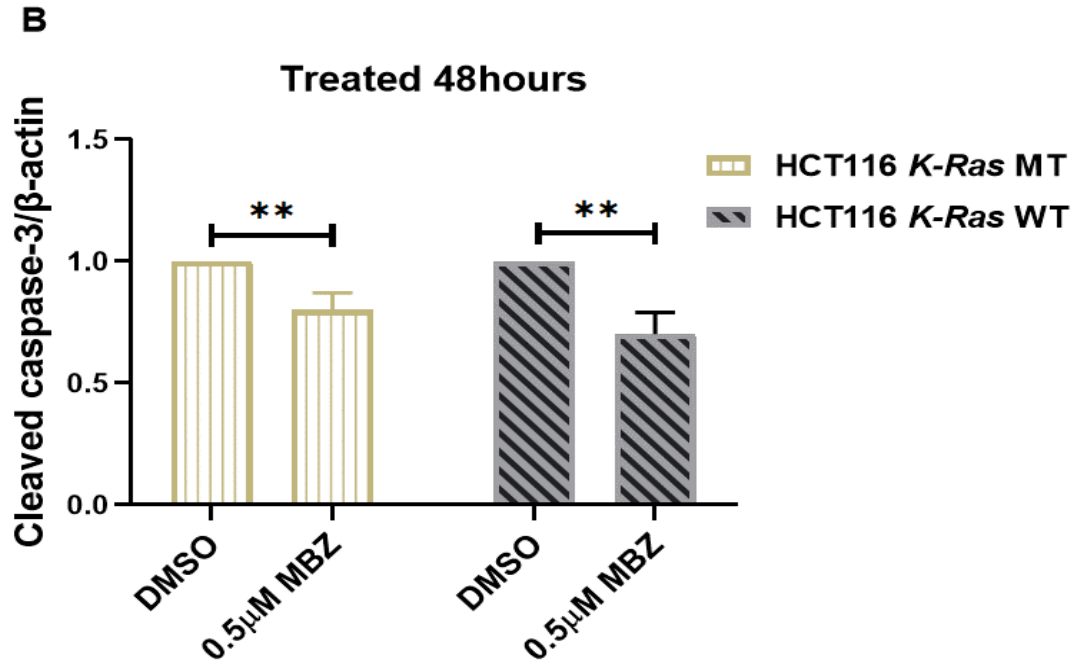
Figure 4.10: Protein levels of pro-apoptotic family member BH3 only Bak protein when treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

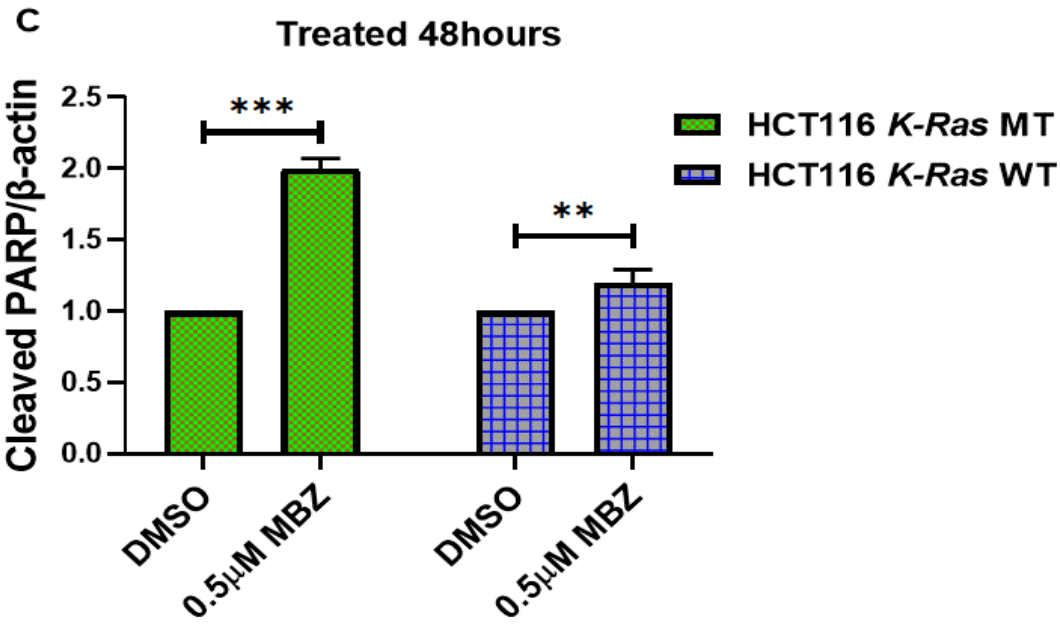
(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 µM MBZ for 48 h. Cellular proteins were isolated and western blotting performed. Blots were probed with the pro-apoptotic family member BH3 only Bak protein and β-actin antibodies as indicated. (B) The histogram indicates Bak protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. The pro-apoptotic family member BH3 only Bak protein levels were normalised with β-actin protein levels. Error bars representSD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (***p<0.01* and * indicates *p<0.05* was statistically significant).

4.3.3.6 Effects of MBZ treatment on initiator and effector apoptotic family proteins.

In this study MBZ induced caspase -3 activation, as evaluated through cleavage of procaspase -3 into their respective p20 active forms in HCT-116 *K-Ras* Mt. and Wt. colorectal cell lines ($p < 0.01$, Figure 4. 11). MBZ also induced significant proteolysis of the caspase -3 substrate 116 kDa-(PARP) into the 86 kDa cleaved form of PARP in HCT-116 *K-Ras* Mt. and Wt. colorectal cell lines ($p<0.001$ and $p< 0.01$ respectively, Figure 4. 11).




B
Treated 48hours
Cleaved caspase-3/β-actin
1.5
1.0
0.5
0.0
**
**
HCT116 K-Ras MT
HCT116 K-Ras WT
DMSO
0.5μM MBZ
DMSO
0.5μM MBZ


C
Treated 48hours
Cleaved PARP/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
***
**
HCT116 K-Ras MT
HCT116 K-Ras WT
DMSO
0.5μM MBZ
DMSO
0.5μM MBZ

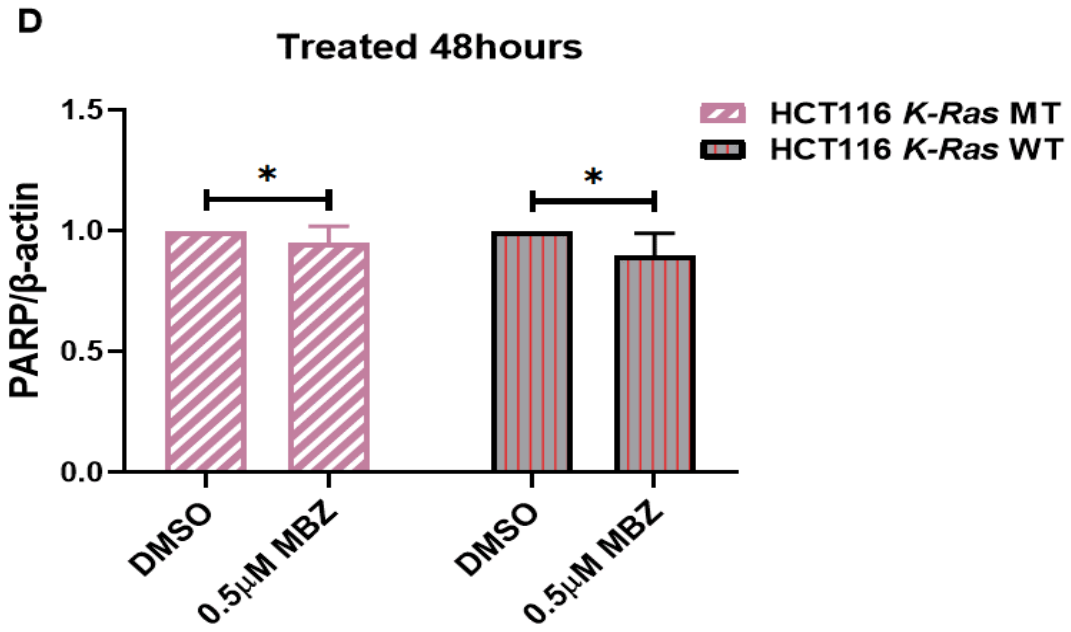
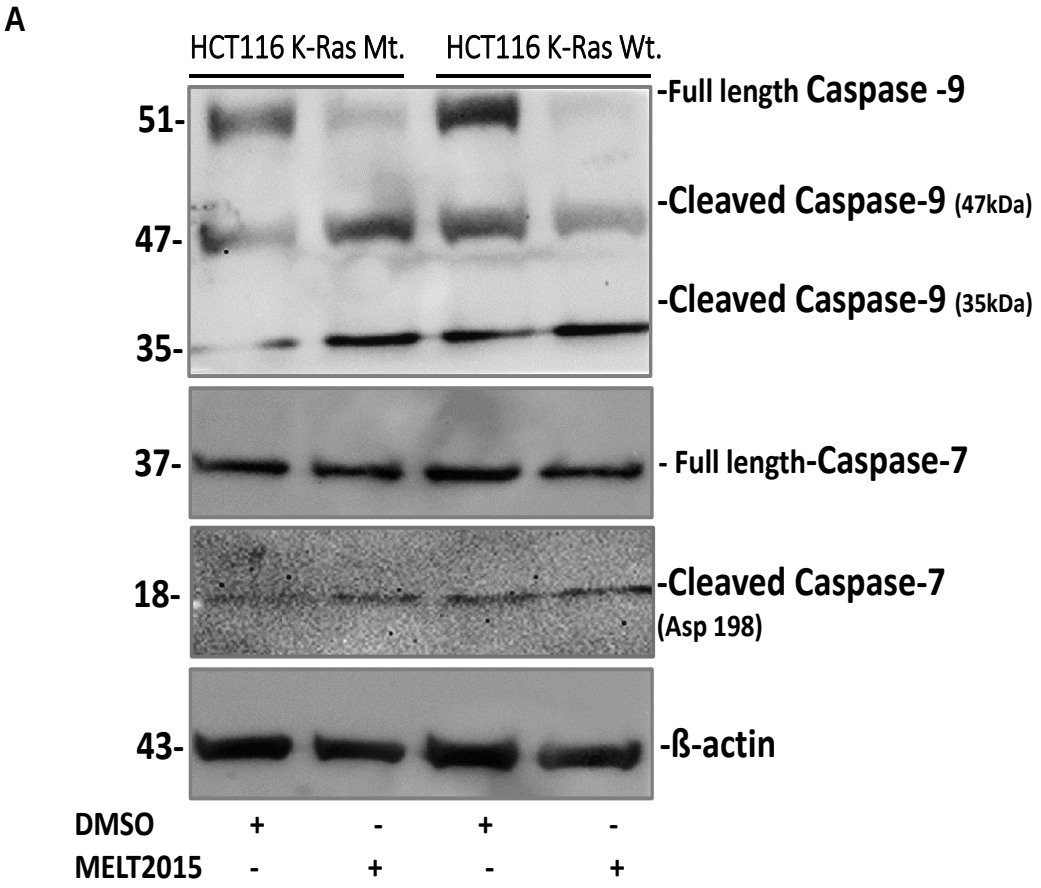


Figure 4. 11: Protein levels of caspase and cleaved caspase family caspase-3, PARP, cleaved caspase -3 and cleaved PARP treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 µM MBZ for 48 h. Cellular proteins were isolated and western blotting performed. Blots were probed with the caspase -3, PARP, cleaved caspase -3 and cleaved PARP and β-actin antibodies as indicated. The histogram indicates caspase -3 protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. (B) The cleaved caspase -3 and (C) cleaved PARP and (D) total PARP levels were normalised with β-actin protein levels. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* were statistically high significant ***p<0.01* and **p<0.05* was statistically significant).

4.3.3.7 Effects of MBZ on initiator and effector apoptotic family proteins of caspases: caspase -7, caspase -9 in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras.*

This study found that MBZ induced caspase -7 activation, shown through cleavage of procaspase -7 into their respective p18 active forms in both cell lines HCT-116 *K-Ras* Mt. and Wt. (Figure 4.12). The drug also induced caspase -9 activation shown through cleavage of procaspase -9 into their respective p47 and 35 active forms in HCT-116 *K-Ras* Mt. and Wt. colorectal cell lines which was revealed by a western blot (Figure 4.12).



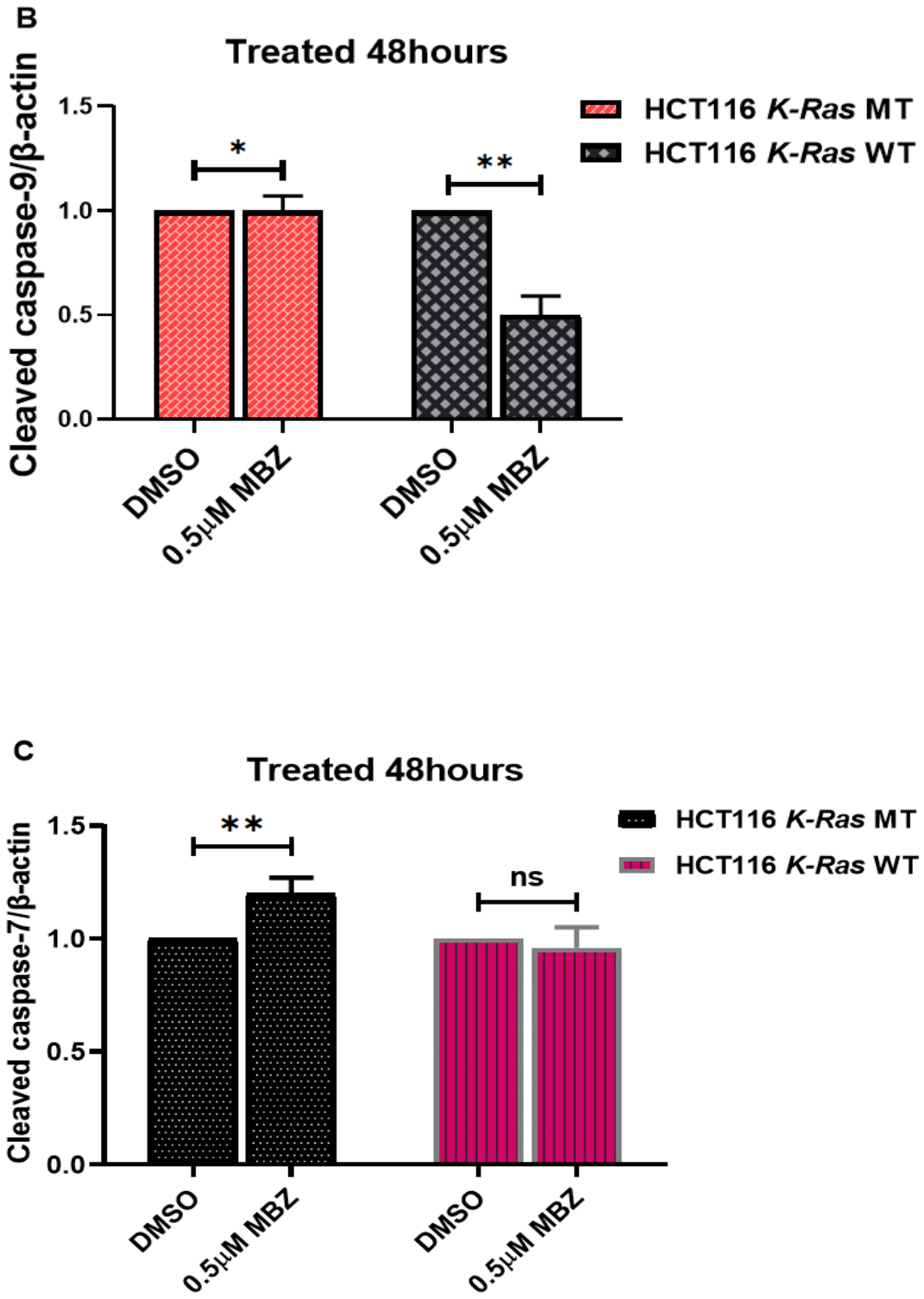


Figure 4.12: Protein levels of caspase and cleaved caspase family caspase -7 initiator,-9, and cleavage caspase -7 and caspase -9 protein when treated with MBZ on HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. were either un-treated or treated with 0.5 μM MBZ for 48 h. Cellular proteins were isolated and western blotting performed. Blots were probed with the caspase -7 initiator, -9 and cleavage caspase -7 and caspase -9 protein and β-actin antibodies as indicated. The histogram indicates protein expression levels in HCT-116 *K-Ras* Mt. and Wt. cells. (B) The cleavage

caspase -9 and (C) cleavage caspase -7 protein levels were normalised with β-actin protein levels. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (***p<0.01* and **p<0.05* were statistically significant).

4.4 Discussion

The effect of MBZ on cell viability was measured using an MTT assay on isogenic pairs of colorectal cancer cell lines such as HCT-116 *K-Ras* Mt., DLD-1 *K-Ras* Mt., HCT-116 *Pten -/-* Mt., HCT-116 *K-Ras* Wt. and DKO-3 Wt. The results indicate reduction in cell viability in a dose and time dependent manner. MBZ treatment also down-regulated the expression of p-Bcl-2 and Mcl-1 mRNA and the levels of protein related to pro-apoptotic and apoptotic markers such as caspases and phosphorylation of Bad, Bak, Bim, Bid, p53, puma and p21 in HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt.

Additionally, MBZ increased caspase -3, -7, -9 and PARP activity, which was evaluated through analysis of cleaved products of procaspase -3, -7, -9 and PARP. MBZ decreased signals, Ran, p-Bcl-2 and Mcl-1mRNA and Ran and pBcl-2 protein expression in HCT-116 *K-Ras* Mt. and Wt. cell lines. MBZ induced Bad and pBad as well as Bak, Bim and Bid. The drug also induced p53, puma and p21. MBZ reduced cell viability in HCT-116 *K-Ras* Mt. and Wt. cell lines in the same concentration.

The MTT assay is a technique that evaluates the cytotoxic effect of MBZ at different concentrations and a range of exposure times on isogenic pairs of human colorectal cell lines. The assay revealed that 2 µM of MBZ was cytotoxic to HCT-1166 *K-Ras* Mt. producing 27% survival but, 72% of HCT-116 Wt. cells survived after 24 h incubation. The IC_{50} values were0.486 ± 0.25 µM and 3.743 ± 1.2 µM (mean±SD). At 48 h HCT-116 *K-Ras* Mt. the absorbance values for treated cells decreased significantly compared cells exposed to MBZ for 24 h. In addition we observed that the cells sensitivity to the compound increased and the cell survival reduced sigificantly for the mutant cell line more than wild type when the concentration increasedas the IC_{50} was 0.335 µM ± 1.5 and IC_{50} = 0.679 ± 0.39 respectively (mean±SD). After 72 h of cell, exposure the absorbance values from treated cells decreased significantly compared to the cells that were exposed to the MBZ for 48 hr. with the IC_{50} values 0.436 ± 0.09 and 0.886 µM± 0.36, respectively (mean±SD).

MBZ cytotoxicity effect was observed on DLD-1 *K-Ras* Mt. and DKO-3 Wt. but with the significant effect only on Mt. However, it first appears when compared with wild type cell line after 24 h of exposure and become obvious after 48 and 72 h at different MBZ concentration when the IC_{50} values were 0.340 ± 0.5 µM

and 1.012 ± 1.2 ± µM, respectively (mean±SD). On the other hand, little cytotoxic effect was again observed on DKO-3 Wt. compared to the mutant cell lines DLD-1 *K-Ras* Mt. until an MBZ concentration of 1.2 µM, but this was not statistically significant.

MBZ in this study has revealed p-Bcl-2 inhibition on HCT-116 *K-Ras* Mt. compared to HCT-116 *K-Ras* Wt. which is consistent with a previous study that showed antitumor activity of MBZ by Bcl-2 inactivation and caspase activation (Doudican et al. 2008; Bai et al. 2011; Doudican et al. 2013; Larsen et al. 2015; Wang et al. 2020c). In addition, caspases -3, -7 and -9 were activated by MBZ in H460 cells (Ferrara 2001; Sasaki et al. 2002; Ferreira et al. 2010)

Previous research has shown that the activation of pro-survival factors Bcl-2 protein signaling encourages cell survival (Cory et al. 2003; Montero and Letai 2018) while MBZ induces inhibition which leads to the expression of multiple targets called pro-apoptotic gene family proteins e.g. BH3 only proteins such as Bad and BH3 123 proteins such as Bax.

Earlier work indicates that down-regulation of Bcl-2 protein level coincide with increased apoptosis in treated cell lines. Treatment with MBZ induced apoptosis in liver tissue HCC, through augmented hepatic expression level of caspase -3 and caspase -9 and reduced level of hepatic Bcl-2 (Younis et al. 2019).

Cancer cell lines with *K-Ras* mutation show Ran silencing–induced apoptosis compared to their wild-type (Yuen et al. 2012). Silencing Ran furthermore affects nucleocytoplasmic transport of transcription factors as Mcl-1 expression is down-regulated through the PI3K/Akt/mTORC1 and Mek/ Erk pathway (Yuen et al. 2012).

In a study MBZ induced caspase -7 activation which was analysed through cleavage of procaspase -7 into their respective p20 active forms in HCT-116 *K-Ras* Mt. and Wt. colorectal cell lines and also by proteolysis of the caspase -3 substrate 116 kDa-(PARP) into the 86 kDa cleaved form of PARP (Dakir et al. 2018).

These results suggest that MBZ-induced cell death requires cytochrome c release from mitochondria. To investigate whether caspases -3, caspases -7 and caspases -9 were stimulated throughout MBZ, the proteolysis of procaspase -3, procaspase -7 and procaspase -9 was measured by a western blot. After 48 h of treatment with 0.5 µM of MBZ the cleavage of procaspase -3, procaspase -7 and

procaspase -9 was induced in isogenic colorectal cell lines HCT-116 *K-Ras* Mt. and Wt. This result agrees with other work on different cancer cell lines H295R and SW-13 (Martarelli et al. 2008; Banovic et al. 2018; Ölgen 2020).

Bad can also form an active pro-apoptotic complex with Bcl-2 when translocated to mitochondria and trigger the apoptosis by release of cytochrome c. This step is inhibited by survival factors that are responsible for Bad phosphorylation, e.g., protein kinase B (Akt), which leads to its cellular isolation**.** In our work, MBZ is shown to down regulate protein level of P-Bad with both treated HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. isogenic colorectal cancer cell lines. These results suggest that MBZ may play a key role in apoptosis in isogenic colorectal cancer cell lines. Earlier work showed MBZ caused mitotic arrest followed by programmed cell death pathway with the activation of caspases and release of cytochrome c due to inhibition of Bcl-2 phosphorylation (Sasaki et al. 2002; Shashaani et al. 2016).

This study has the shown induction of p53, puma and p21 in isogenic colorectal cell lines HCT-116 *K-Ras* Mt. and Wt. which is consistent with a previous study showing increased p53 protein in H295R and SW-13 cell lines treated with MBZ. This suggests the drug contributes to apoptosis initiation (Martarelli et al. 2008; Banovic et al. 2018; Ölgen 2020).

Bim proteins encourage apoptosis through Bcl-2, and the short form BimS is constitutively proapoptotic. This Bim isoform protein expression was increased in HCT-116 *K-Ras* Mt. and Wt. cells treated with MBZ (O'Connor et al. 1998). It remains uncertain whether Bim induces apoptosis by directly suppressing the pro-survival roles of Bcl-2 or by secretion of the pro-apoptotic functions of Bax-like proteins from suppression of Bcl-2 (Zong et al. 2001).

Our work regarding Bcl-2 and Bak expression in CRC cell lines is consistent with PCR and western blot data generated previously on the DLD-1 cell line (Ishikawa et al. 2019). Overall, our results show that MBZ induces greater levels of intrinsic apoptosis on HCT-116 *K-Ras* Mt. compared to HCT-116 *K-Ras* Wt.

4.5 Conclusion

To conclude. exposure to MBZ inhibited Bcl-2 phosphorylation in HCT-116 *K-Ras* Mt. and Wt. cell lines. These results also demonstrate that most of the isogenic colorectal cancer cell lines were sensitive to Ran inhibitor MBZ and there was no

association between this effect and their effect on Akt phosphorylation in HCT-116 *K-Ras* Mt. cells or their programmed cell death. Previously, MBZ suppressed cells invasion in H295R and SW-13 cell lines with an IC_{50} of 0.085 μM as well as inducing apoptosis mediated through caspase -9, and caspase -3 (Guerini et al. 2019). The activity of MBZ as an antitumor drug is established as it inactivates Bcl-2 phosphorylation and induces caspase activation (Doudican et al. 2008; Bai et al. 2011; Doudican et al. 2013; Larsen et al. 2015; Wang et al. 2020c). In addition, caspases -3, caspases -7 and caspases -9 were activated by MBZ in H460 cells (Sasaki et al. 2002; Ferreira et al. 2010). which is consistent with our study.

Chapter 5

Results

Investigate the effect of MBZ on association of *K-Ras* activation and *Pten* deletion mutation with Ran expression as well as cell invasion and metastasis in isogenic CRC cell lines

5. Effect of MBZ on signalling pathways and functional assays such as migration in wound healing, invasion and colony formation in isogenic colorectal cancer cell lines.

5.1 Introduction

(MAPK) mitogen-activated protein kinase is a cascade complex interaction signal associated with tumour formation, development, and drug resistance. MAPK pathway is a bridge from extracellular signals to intracellular responses. Alterations of signaling cascades are observed in several diseases, including cancer, as a consequence of genetic and epigenetic changes, and many treatments have been developed against them (Braicu et al. 2019).

The Ras superfamily of GTPases which includes G-proteins, such as *K-Ras*, *H-Ras* and *N-Ras* (Lim and Leprivier 2019), is considered the initial cytosolic intermediates to activate the phosphorylation cascade of the MAPK pathway (Johnson and Chen 2012; Zhang et al. 2012). This signal cascade activates due to the interaction between growth factors (GFs) with their specific growth factor receptors (GFRs). Ras GTPase is activated following EGFR activation mediated by an EGFR-associated nucleotide exchange factor Son of Sevenless 1 (SOS1) and engaging adaptor proteins (GRB-2) (Vo et al. 2016; Liu et al. 2018). SOS1 regulates the prompt conversion of GTP to GDP, which is the rate limiting step for the formation of Ras-GTP, the active Ras (Vo et al. 2016). There are a family of three serine/threonine-specific protein kinases which called RAF kinases that are associated to retroviral oncogenes. RAF is the ultimate responder of RAS, and is thus, dependent on the interaction with activated RAS. The RAF family includes many variants (such as A-Raf, B-Raf, C-Raf) (Matallanas et al. 2011; McCain 2013), consisting of serine/threonine kinases that are responsible for initiating the pathway by activating Mek (MAP kinase-Erk kinase) and Erk1 / 2 (Extracellular signal-regulated kinases).

Both Mek and Erk 1/2 are implicated with a wide variety of processes such as cell survival, proliferation, and differentiation, all of which are determined by the phosphorylated targets of Mek and Erk 1/2, respectively. Erk 1/2 specifically exhibits variability in its phosphorylation targets, independent of cellular location and compartment. Erk 1/2 can activate transcription factors in the nucleus e.g. CREB (cAMP response element-binding protein), to c-Myc (transcriptional

regulator Myc-like) and NF-κB (nuclear factor kappa B). This makes Erk 1/2 an essential anti-tumour target (McCain 2013; Lavoie and Therrien 2015).

Akt (protein kinase B) or PKB has been demonstrated to play an essential role in the relation of cell survival and apoptosis (Franke et al. 1997; Song et al. 2019).The Akt or PI3K-Akt signaling pathway is a signal transduction pathway which encourages survival and growth in response to extracellular signals (Xie et al. 2019). Vital proteins comprise phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) (Hoxhaj and Manning 2020).

The P13K/Akt/mTOR cascades can be triggered as well as via RTK and Ras, and their main effects are metabolic signaling and protein synthesis that supports cell growth (Braicu et al. 2019). By enhancing phosphorylation of pro-apoptotic protein Bad (Pandey et al. 2018) and inhibiting various targets such as caspase -9 significant negative regulators of their P13K/Akt/ mTOR signaling pathways (Xie et al. 2019) include *Pten* phosphatase and forkhead transcription factor (Chen et al. 2018a).

Overexpression or activation of Akt (protein kinase B) has been detected in several cancers, comprising colorectal, pancreatic, lung and ovarian cancers and is linked with augmented cancer cell proliferation and survival. Consequently, down regulation of Akt (protein kinase B) could provide a treatment strategy for cancer cells (Song et al. 2019). Akt or PKB has been shown plays an essential role in the relation of cell survival and apoptosis (Chen et al. 2020c).

The Mitogen-activated protein kinase (MAPK) pathway, often known as the Ras/Raf/Mek/Erk signalling cascade, is activated in several of human cancer (Jia et al. 2016). MAPK pathway sends signals upstream to its effectors downs cancer stream to control physiological developments such as angiogenesis, migration, cell proliferation, differentiation, survival and apoptosis (Sparmann and Bar-Sagi 2004; Dhillon et al. 2007; Montor et al. 2018). The MAPK pathway activation causes binding of the tyrosine kinase receptor (TKR) to growth factors which stimulates Raf serine/threonine kinase. Consequently, Raf activation phosphorylates and activates Mek, then directly initiates the phosphorylation of (Erk) which is considered as the main signal effector in the kinase pathway (Gollob et al. 2006), (p-Erk1/ p-Erk2) proteins are responsible for phosphorylating several transcription factors that regulate the expression of several proteins involved in angiogenesis, proliferation, extracellular matrix production and

programmed cell death resistance (Montagut and Settleman 2009; El-Adl et al. 2020)

The Raf/Mek/Erk cascade furthermore has different actions in programmed cell death signalling, e.g Bcl-2 is an anti-apoptotic regulator while caspase -3 and caspase-9 are apoptotic regulator (Huynh et al. 2003; McCubrey et al. 2007; Dwivedi et al. 2020), and has various effects on the expression of proteins in cell cycle regulation (Huang et al. 2005; Zagouri et al. 2017). The activation of cell cycle progression and regulation of programmed cell death or survival is due to this signalling pathway. Furthermore, activated Ras/Raf/Mek/Erk is a common downstream pathway for various growth factor such as VEGF, (Chapnick et al. 2011; Kudo et al. 2012). Moreover, the enhanced transcription of MMPs that are responsible for cell invasion and metastasis are due to continuous activation of Erk in malignant cells (Song et al. 2013).

A western blot was carried out to investigate the impact of MBZ on phosphorylation of Akt protein expression on isogenic human CRC cell lines HCT-116 *K-Ras* Mt. and HCT-116*K-Ras* Wt. Previously, it has been shown that apoptosis is promoted when Ran was knocked down using potent shRNA (Yuen et al. 2012).

Inhibited PI3K/Akt/mTORC1 and Ras/Mek/Erk pathways in cancer cells have exhibited less response to Ran silencing–induced programmed cell death (Yuen et al. 2012). Cancer cells with *K-Ras* mutated and *Pten* deleted are highly susceptible to Ran-silencing–induced programmed cell death rather than their Wt. counterparts and this influence is decreased via inhibitors of both pathways PI3K/Akt/mTORC1 and Mek/Erk (Yuen et al. 2012). Ran overexpression in clinical samples is significantly linked with poor patient consequence in cancers (Yuen et al. 2012).

This activation in the PI3K/Akt/mTORC1 and/or Ras/Mek/Erk pathways is potentially correlated with increase in oncogenic mutations such as *K-Ras* (Yuen et al. 2012).

There are two signaling pathways hyper activation which are very common in cancer development, namely (PI3K)/Akt/mTORC1) phosphoinositide 3-kinase and (Ras/Mek/Erk) mitogen-activated protein/extracellular signal-regulated kinase (Erk; Mek) (Yuen et al. 2012).

5.1.1 Scratch assay

Tumour cell migration can be investigated *in vitro* via the scratch assay, a straightforward, affordable and real-time documentation technique (Liang et al. 2007; Gnerucci et al. 2020) that avoids disturbance of cell-cell and cell-ECM interactions by seeding cells on a coated plate as monolayer instead of in suspension (Liang et al. 2007; Goetsch and Niesler 2011; Gnerucci et al. 2020). It involves an assessment of the degree of tumour cell migration in a scratch of the confluent cell monolayer (Gnerucci et al. 2020).

Here. a scratch assay investigated the impact of MBZ on the migration pf isogenic human CRC cell lines such as HCT-116 *K-Ras* Mt., HCT-116 *K-Ras* Wt., DLD-1 *K-Ras* Mt., DKO-3 Wt. and HCT-116 *Pten null* Mt., HCT-116 *K-Ras* Wt. Previously, it has been shown that the MBZ has inhibitory effect dependent on its dose manner and the type of cancer cells.

MBZ on role of Ran in the metastasis of isogenic pairs colorectal cancer cell lines was compared to controls, assuming that cell motility is essential for cancer metastasis.

5.1.2 Matrigel invasion assay

Cancer cell lines move in a similar way to normal cells. Movement can be observed throughout embryonic morphogenesis, wound healing and immune-cell trafficking (Eccles et al. 2005; Kramer et al. 2013).

Movement in cancer cells is of two types, migration, and invasion. Migration typically describes non-pathological movement of cells through the body and invasion defines a pathological condition which involves cancer cell movement (Pijuan et al. 2019).

Malignant carcinomas are well-described by the capability of tumour cells to invade and infiltrate through tissues and basement membranes to underlying interstitial tissues. For instance, colonic cancers invade to the basal membrane and arrive at the layer of sub-mucosal muscle. Obviously, although assessing migration is valuable in detecting metastatic potential, assessment of invasion is more significant (Eccles et al. 2005; Pijuan et al. 2019).

In this section of Chapter 5 we have carried out *in vitro* assays that inform migration and invasion of isogenic pairs of colorectal cancer cells.

5.1.3 Colony formation assay (CFA)

5.1.3.1 Isogenic human CRC cell lines

We wish to investigate the impact of MBZ on the ability of isogenic human CRC cell lines such as HCT-116 *K-Ras* Mt., HCT-116 *K-Ras* Wt., DLD-1 *K-Ras* Mt., DKO-3 Wt. and HCT-116 *Pten null* Mt., HCT-116 *K-Ras* Wt. to form colonies. Previously, it has been shown that the MBZ has an inhibitory effect dependent on its dose and type of cancer cells. Tumour cells have a capacity for unlimited proliferation, and the effects of agents on cell proliferation are studied through colonogenic assays.

On this basis we assess Ran inhibitor MBZ on function of Ran in the metastasis of isogenic pairs colorectal cancer cell lines compared to controls. If cell proliferation is vital for cancer metastasis, then colony formation assay (CFA) should measure any inhibitory effect of MBZ on cancer cell colonies forming through Ran-GTPase.

5.1.3.2 Breast cancer MDA-MB-231 cell lines

The expression of Ran in invasive breast cancer cell lines, MDA-MB-231 was established to be significantly correlated with proliferation, invasion and metastasis (Sheng et al. 2018).

Thus, we assessed Ran inhibitor MBZ on role of Ran in the proliferation and metastasis of breast cancer and compared with isogenic pairs colorectal cancer cell lines.

Ran inhibition leads to a significant reduction in the proportion of treated cells, and suppresses cell proliferation (Sheng et al. 2018). In a dose response manner in the case of CRC cell lines. Colony formation should reduce on both cell lines of breast, lung, and CRC when Ran is inhibited with MBZ.

Ran protein was highly expressed in tissues of breast cancer and that knock-down of its expression inhibited *in vitro* the proliferation and migration of breast cancer cells (Sheng et al. 2018). Ran over expression was associated with poor prognosis of breast cancer patients (Sheng et al. 2018).The finding in this study was shown when Ran was inhibited by Ran inhibitor MBZ in both breast and colorectal cell lines in colony formation assays.

5.1.3.3. Human non-small-cell lung cancer NSCLC (A549) cell lines

Human non-small-cell lung cancer NSCLC (A549) cell lines are an adenocarcinoma of human alveolar basal epithelial cells. An anti-proliferative effect of MBZ on A549 assessed by colony formation assay was found in a earlier study (Guerini et al. 2019).

In xenograft model of A549, MBZ was also capable to prevent the formation of lung metastatic colonies by approximately 80% without any toxicity effect (Guerini et al. 2019). Another study showed that IC_{50} for the drug was about 0.16 µM, whereas no influence was exposed on normal Human umbilical vein endothelial cells (HUVECs) and fibroblasts (Mukhopadhyay et al. 2002).

MBZ also highly repressed the growth of breast, colon carcinoma, ovary and osteosarcoma cell lines, generating IC_{50} values of 0.1 to 0.8 µM (Guerini et al. 2019).

This chapter aims to determine the effect of MBZ on the functional role of some genes which are involved in cell survival, migration, invasion, metastasis, and colony formation in isogenic colorectal cancer cell lines using techniques such as western blots, qRT-PCR, migration, invasion, and colony formation assays. The reason for selection of such cell lines HCT-116 *K-Ras* and *Pten* Mt. and Wt. as well as DLD-1 *K-Ras* Mt. and its Wt. DKO-3 from among other isogenic CRC cells are due to a one-point mutation that performs an important role in the cell cycle. These mutations are the oncogene (*K-Ras*) and another tumour suppressive gene (*Pten* deletion).

5.2 Materials and Methods

5.2.1 Materials

Materials that used in this chapter to generate data were described earlier in chapter 2 section 2.1.

5.2.2 Methods

Methods that used in this chapter to generate data were described earlier chapter 2 in section 2.2.

Scratch assay:

Methods that used in this chapter to generate data for scratch assay were described earlier in details in chapter 2 in section 2.2.10.

Matrigel invasion assay:

Methods that used in this chapter to generate data for Matrigel invasion assay were described earlier in details in chapter 2 in section 2.2.11.

Boyden chamber:

Methods that used in this chapter to generate data for Boyden chamber were described earlier in details in chapter 2 in section 2.2.11.

Colony formation:

Methods that used in this chapter to generate data for CFA were described earlier in details in chapter 2 in section 2.2.12.

5.3 Results

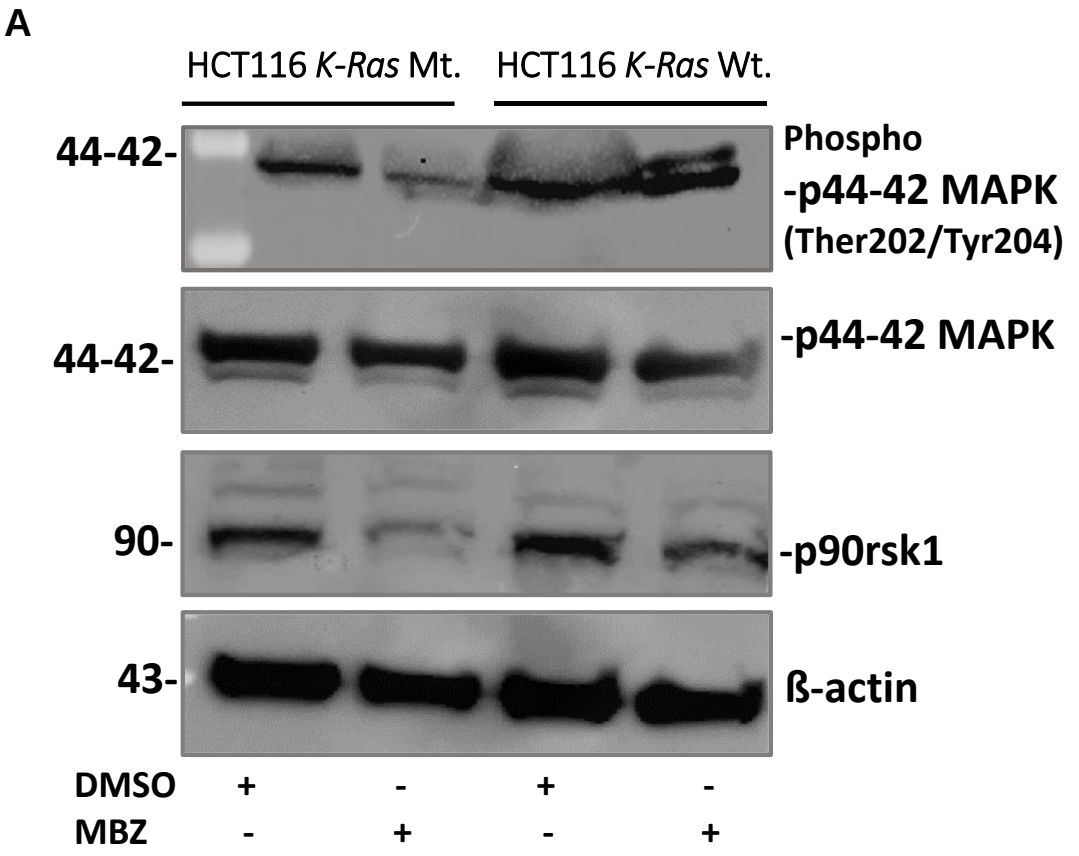
5.3.1 Effect of MBZ on signaling pathways

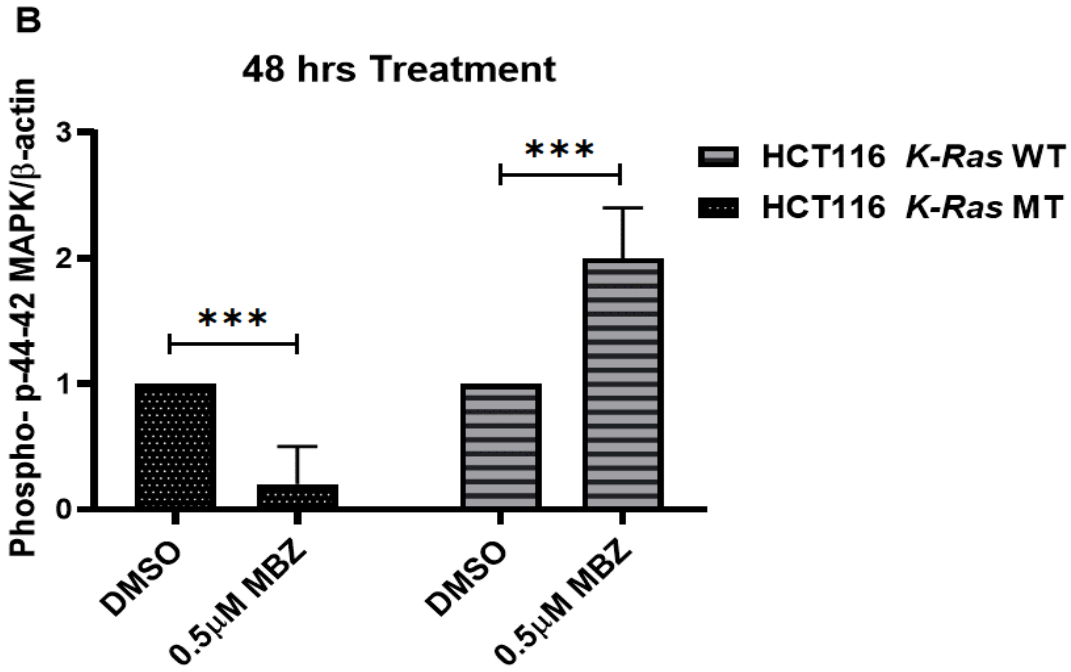
5.3.1.1 Effect of MBZ on expression of Erk 1/2 and p90rsk1α proteins in HCT-116 *K-Ras* Mt. and Wt. cells using western blot.

A western blot was carried out to investigate the impact of MBZ on phosphorylated protein level of Erk 1 Thr202 and Erk 2 Tyr204 expression in isogenic human CRC cell lines HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. Previously, it has been shown that Ran knockdown using shRNA promoted apoptosis (Yuen et al. 2012),Therefore, the cell lines were treated for 48 h with 0.5 µM MBZ while 0.1% DMSO was used as a control. Cells were extracted, and protein concentrations were assessed by a Bradford assay. Then a similar amount of proteins was subjected to SDS-page gel. The protein levels and densitometry of Erk 1/2 proteins expression shown in (Figure 5.1) were assayed using immunoblotting analysis.

The results illustrate that 0.5 µM MBZ treatment after 48 h significantly down regulated p- Erk 1/2 protein expression in HCT-116 *K-Ras* Mt. *p<0.001* (Figure 5.1). However, when compared to the HCT-116 *K-Ras* Wt., 0.5 µM MBZ appears to up regulate p- Erk 1/2 expression (*p<0.001).* Isogenic cell lines treated with MBZ showed different effects. A Significant increase in the levels of Erk 1/2 protein in HCT-116 *K-Ras* Wt. was found compared with that of the un-treated cells at 48 h ($p<0.001$), (Figure 5.1).

Inhibition of phosphorylation of p90rsk1α was detectable after 48 h drug treatment in HCT-116 *K-Ras* Mt. (*p<0.001)* and less so in the case of the HCT-116 *K-Ras* Wt., (*p>0.05)*, (Figure 5.1). Intensity of the target proteins is compared to that for un-treated after normalisation to β-actin.


A
HCT116 K-Ras Mt.
HCT116 K-Ras Wt.
44-42-
Phospho
-p44-42 MAPK
(Ther202/Tyr204)
44-42-
-p44-42 MAPK
90-
-p90rsk1
43-
ß-actin
DMSO + - + -
MBZ - + - +


B
48 hrs Treatment
Phospho- p-44-42 MAPK/β-actin
3
2
1
0
***
***
HCT116 K-Ras WT
HCT116 K-Ras MT
DMSO
0.5μM MBZ
DMSO
0.5μM MBZ

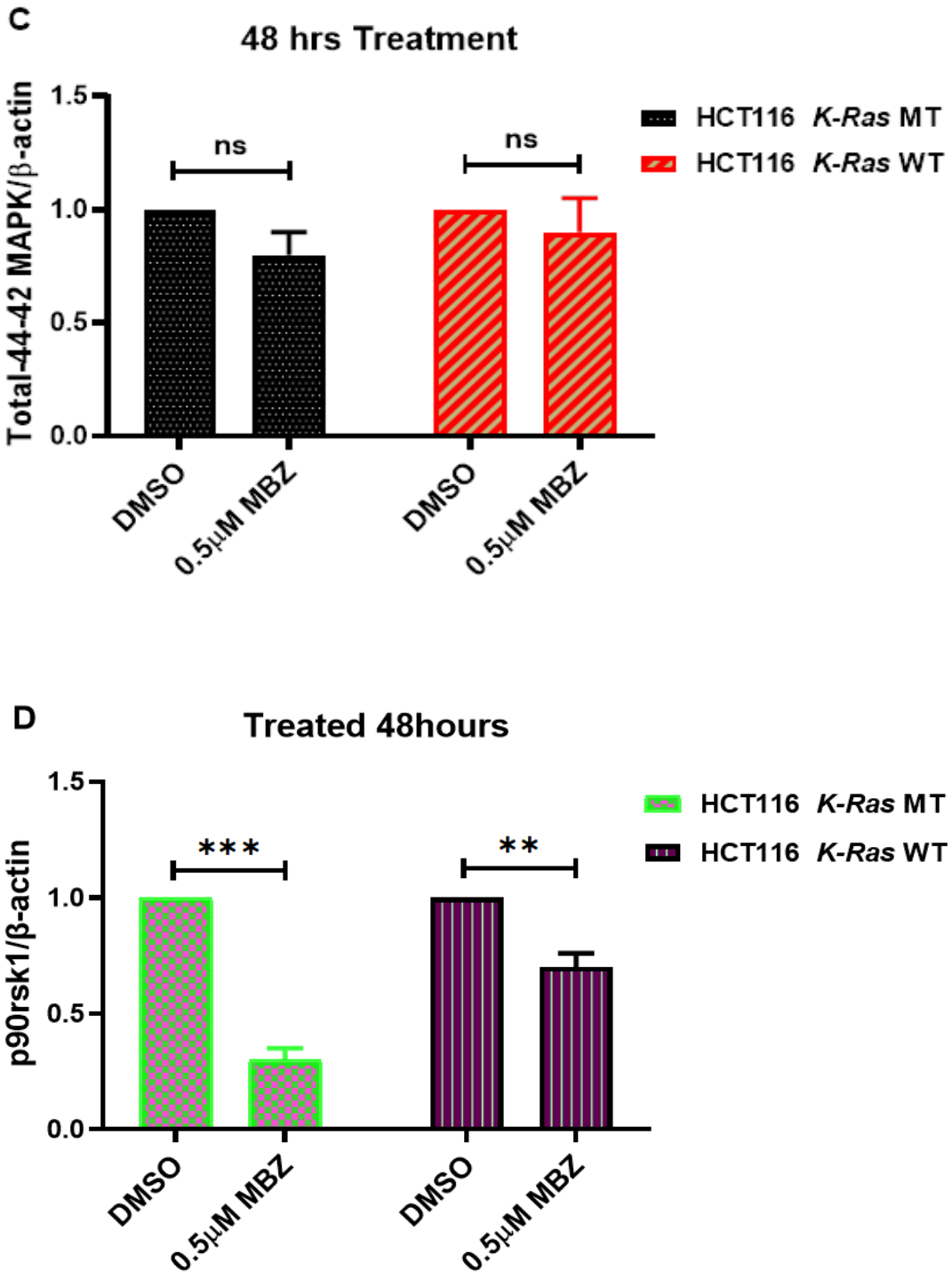


Figure 5.1: Effect of MBZ on protein expression of MAPK pathway components in HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. cells were either treated with 0.5 µM MBZ or DMSO for 48 h. Cellular proteins were isolated and western blotting was performed. Blots were probed with the Erk1, Erk2, p90rsk1α and β-actin antibodies as indicated. The histograms represent protein expression levels (B) phospho- Erk1, Erk2 and (C) total Erk1, Erk2, (D) p90rsk1α in HCT-116 *K-Ras* Mt. and Wt. cells. The Erk1, Erk2, p90rsk1α levels were normalised with β-actin protein levels. Error bars represent SD from three separate experiments.

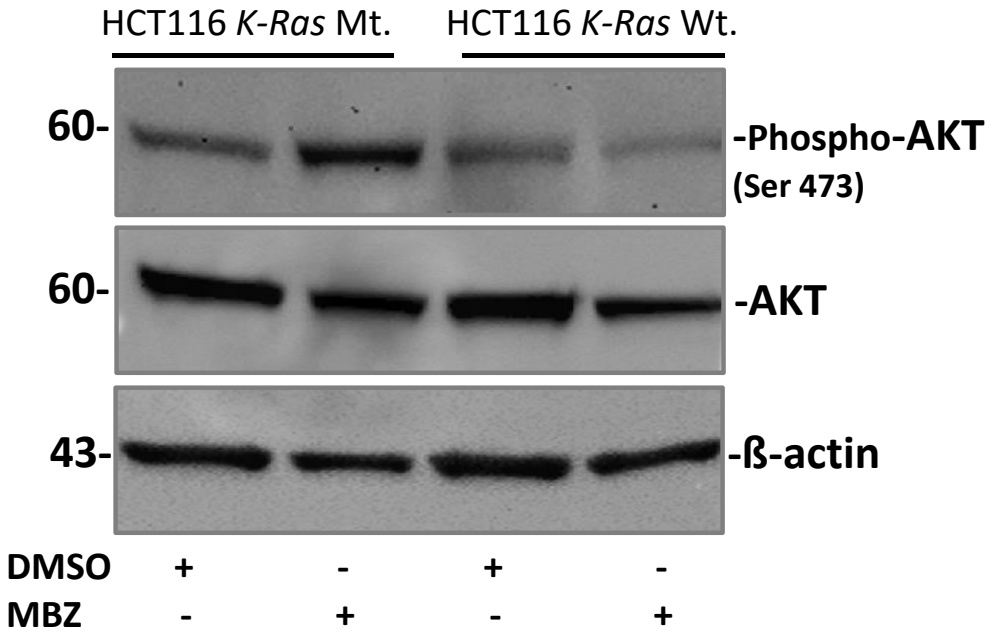
Statistical analysis was performed by unpaired *Student t*-test (****p<0.001*, P ***p<0.01,* ns means *p>0.05*).

5.3.1.2 Investigation if Ran inhibition by MBZ contributes to a reduction in Akt /p-Akt protein expression, in human isogenic CRC cell lines by western blot.

The protein levels and densitometry of p-Akt proteins expression shown in (Figure 5.2), were assayed using immunoblotting analysis and the bands intensity of the target proteins (after treatment with 0.5 µmol/L MBZ) compared to that of un-treated control 0.1% DMSO sample after normalisation with β-actin.

The results illustrate that 0.5 µM MBZ after 48 h significantly up regulated p-Akt protein expression in HCT-116 *K-Ras* Mt. ns means *p>0.05* (Figure 5.2). However, when compared to the HCT-116 *K-Ras* Wt., MBZ appears to inhibit p-Akt (*p<0.001)*. Isogenic cell lines when treated with MBZ showed a different effect, namely a significant decrease in the levels of p-Akt protein in HCT-116 *K-Ras* Wt. after 48 h of treatment when compared with that of the un-treated cells at 48 h (Figure 5.2). However, the two-fold increase (up regulation) in the HCT-116 *K-Ras* Mt. cells was not significant.

A



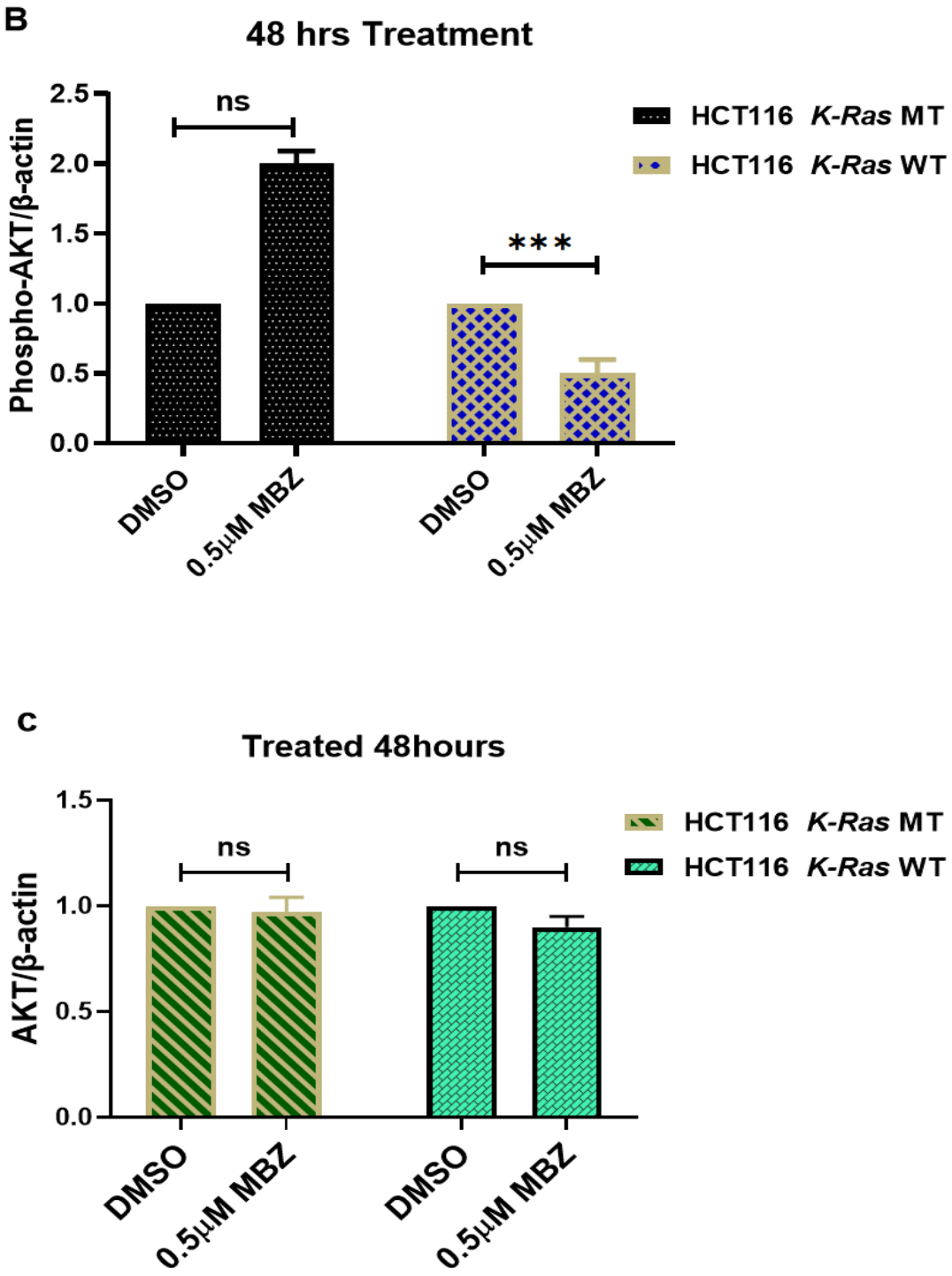
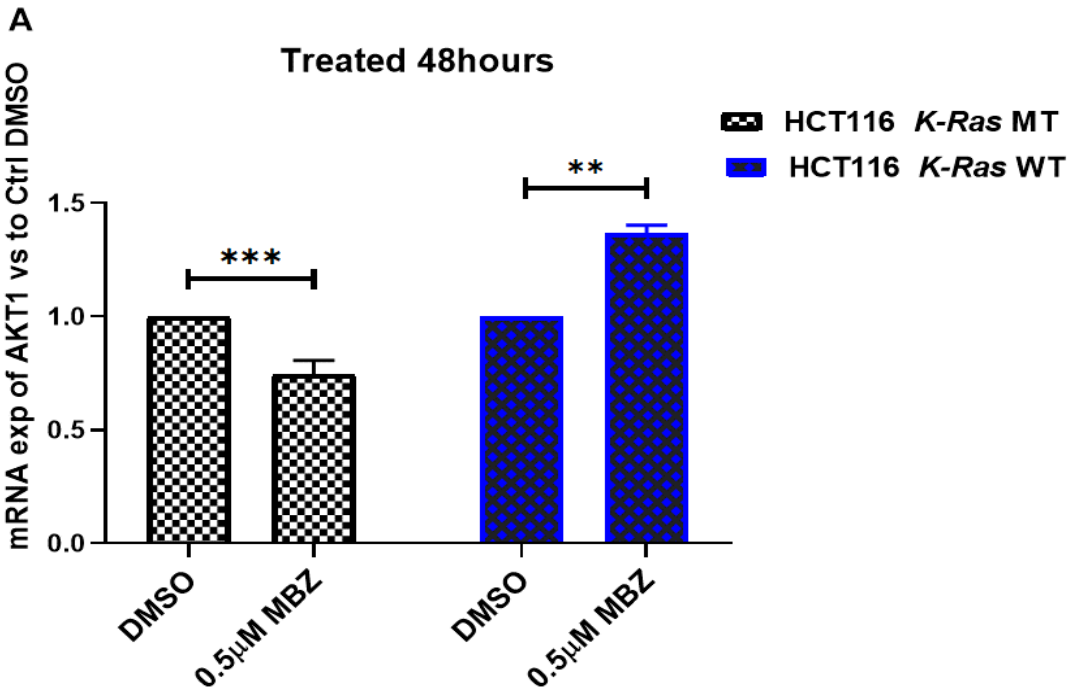


Figure 5.2: Effect of MBZ on protein expression of Akt /p-Akt pathway components in HCT-116 *K-Ras* Mt. and Wt. cells.

(A) HCT-116 *K-Ras* Mt. and Wt. cells were either treated with 0.5 µM MBZ or DMSO for 48 h. Cellular proteins were isolated and western blotting was performed. Blots were probed with the Akt /p-Akt and β-actin antibodies as indicated. The histograms represent protein expression levels of (B) p- Akt / (C) total Akt in HCT-116 *K-Ras* Mt. and Wt. cells. The Akt /p-Akt levels were normalised with β-actin protein levels. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (ns means *p>0.05* and *** *p<0.001*).

5.3.1.3 Effect of MBZ on expression of Akt 1, Akt 2 and Akt 3 genes in HCT-116 K-Ras Mt. and Wt. quantified by qRT-PCR.

Akt 1/2/3 expression was significantly down regulated in HCT-116 *K-Ras* Mt. in a different manner but greatest downregulation was shown with Akt 3. The significance levels were *p<0.001*, *p<0.01* and *p<0.001* respectively while in HCT-116 *K-Ras* Wt. the expression was significantly up regulated in Akt 1/2 (*p<0.01)* for cells treated with 0.5 µM MBZ, while was significantly down regulated in case of Akt 3 (*p<0.01)* when comparing each cell with their un-treated control as shown in (Figure 5.3).



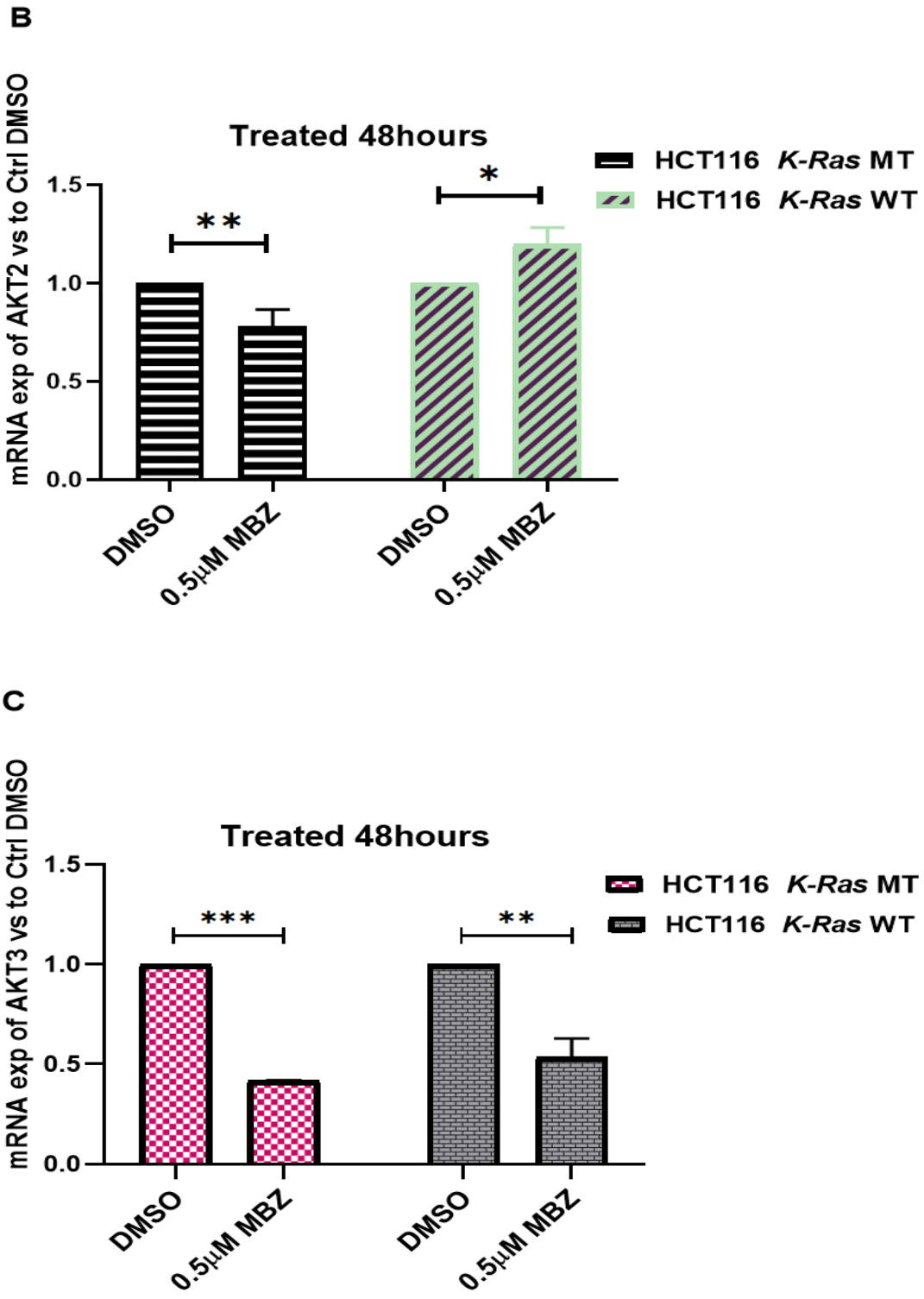


Figure 5.3: MBZ impact on relative mRNA expression level of Akt 1/2/3 in HCT-116 *K-Ras* Mt. and Wt. cells using qRT-PCR.

HCT-116 *K-Ras* Mt. and Wt. cells were either un-treated or treated with 0.5 µM MBZ for 48 h. The mRNA was isolated and used for preparation of cDNA. The histogram represents (A) Akt 1, (B) Akt 2, (C) Akt 3 relative expression in HCT-116 *K-Ras* Mt. and Wt. standardised to endogenous housekeeping gene B2M. Error bars represent SD from three separate experiments. Statistical analysis was performed by unpaired *Student t*-test (****p<0.001* and ***p<0.01* was statistically significant).

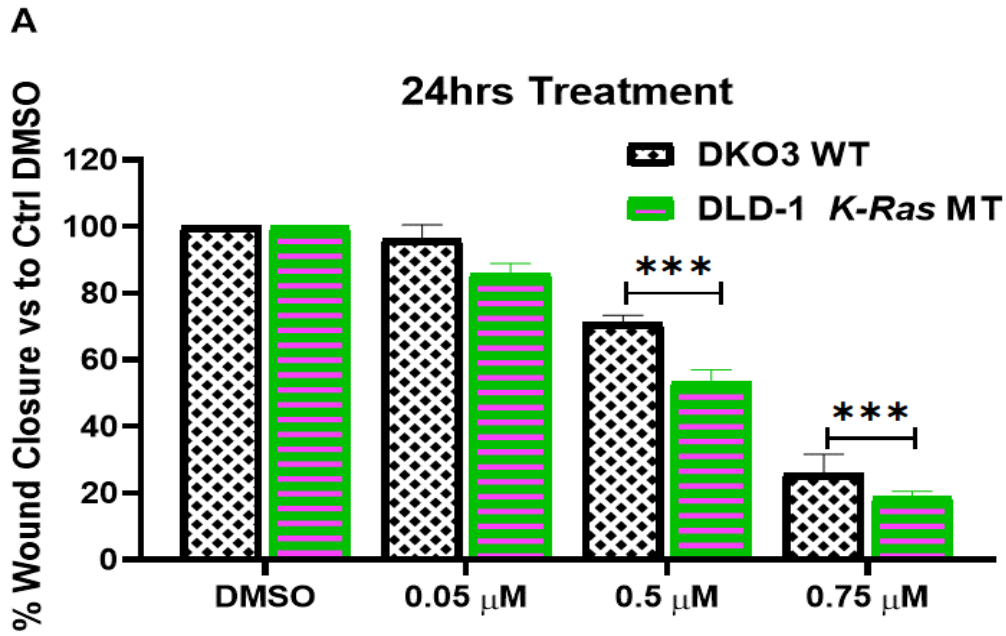
5.3.2 Scratch assay

5.3.2.1 The effect of MBZ on DKO-3 Wt. and DLD-1 *K-Ras* Mt. cell lines analysed by Scratch assay.

After 24 h of exposure to 0.5 µM MBZ, cells migration of DLD-1 *K-Ras* Mt. and DKO-3 Wt. was inhibited by 45% and 30%, respectively, which was significant as *p<0.01*. At a concentration of 0.75 µM MBZ cell migration was inhibited by 82% and 73%, respectively, which was again significant as p<0.05. and the respective IC_{50} valueswere 0.543 µM and 0.608 µM.

After 48 h drug treatment (Figure 5.4), cell migration reduced by 56% and 34% in the case of DLD-1 *K-Ras* Mt. and DKO-3 Wt., which was again significant (*p<0.01*). The inhibitory effect at 0.75 µM of MBZ on DLD-1 *K-Ras* Mt. and DKO-3 Wt. cells line of 85% and 75% was significant (p<0.05) and IC_{50} values were 0.491 and 0.588 µM respectively.The outcomes also demonstrated that DKO-3 Wt. cells line had significantly higher migration than DLD-1 *K-Ras* Mt. cells line at both exposure times (24 and 48 h) (Figure 5.4).

Furthermore, the inhibitory effect of MBZ at 0.05 µM on wound closure was observed but it was very low on Mt. cell line with no effect on the Wt. cells line.



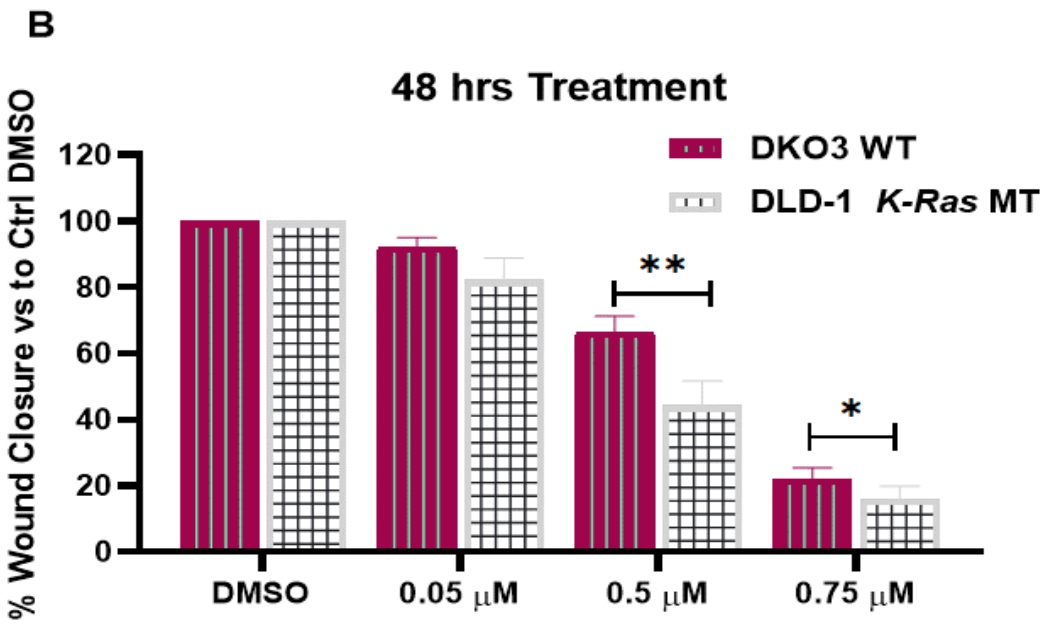


Figure 5.4: Effects of MBZ on the migration of DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells analysed by wound healing assay at different times and concentration.

1.5×106 cells/well of DKO-3 Wt. and DLD-1 K-Ras Mt. cells were treated with different concentration of MBZ (0.05- 0.75 µM) or DMSO for 24 and 48 h. Images of the entire wound area in each well were taken before 0 h and after migration (A) 24 and (B) 48 h and wound closure was measured. Results are presented as the mean and ±SD for three independent experiments. *P* *** indicates p<0.001, ** indicates p<0.01 and * indicates p<0.05.

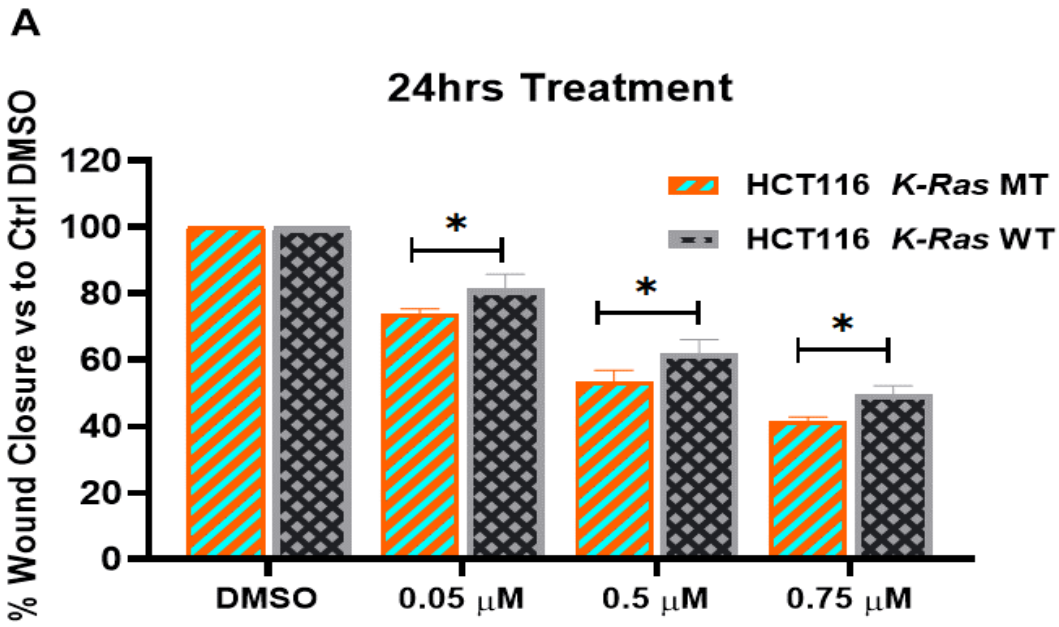
5.3.2.2 Effect of MBZ on HCT-116 *K-Ras* Wt. and HCT-116 *K-Ras* Mt. cell lines on cell migration.

After 24 h of drug treatment (Figure 5.5), cells migration of HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. were inhibited by 45% and 40%, respectively and, significantly ($p< 0.05$). The cell migration inhibition was 61% and 55% at concentration 0.75 µM respectively, which was again significant ($p<0.05$). the respective IC_{50} values were 0.481 µM and 0.933 µM.

After 48 h exposure to 0.5 µM MBZ (Figure 5.5), cell migration of HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt., reduced by 52% and 42% respectively which was due to, significant ($p< 0.05$). The respective inhibitory effect at 0.75 µM of MBZ on HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. was significant at 80% and 49% (*p<0.001*) while the IC_{50} values were 0.210 and 0.973 µM.

This study found that HCT-116 Wt. cells line had significantly higher migration than HCT-116 *K-Ras* Mt. cells line at both exposure times (24 and 48 h) (Figure 5.5).

Furthermore, the inhibitory effect of MBZ at 0.05 µM on wound closure was also seen but it was 27% on HCT-116 *K-Ras* Mt. and about 20% on the HCT-116 Wt. cells line.



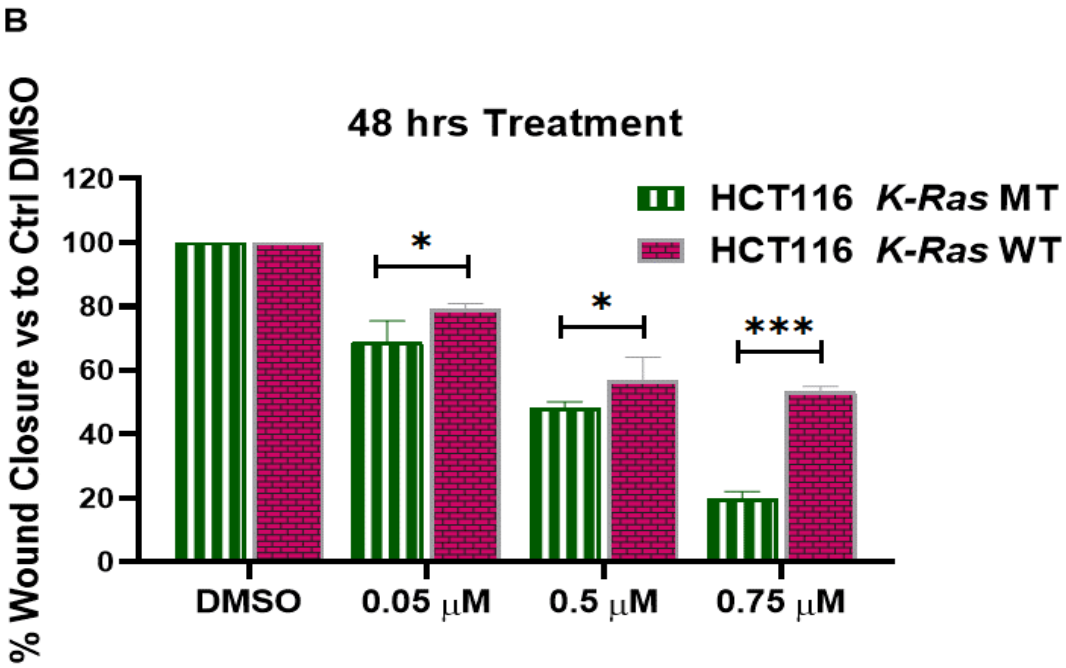


Figure 5.5: Effects of MBZ on the migration of HCT-116 Wt. and HCT-116 *K-Ras* Mt. cells analysed by wound healing assay at different times and concentration.

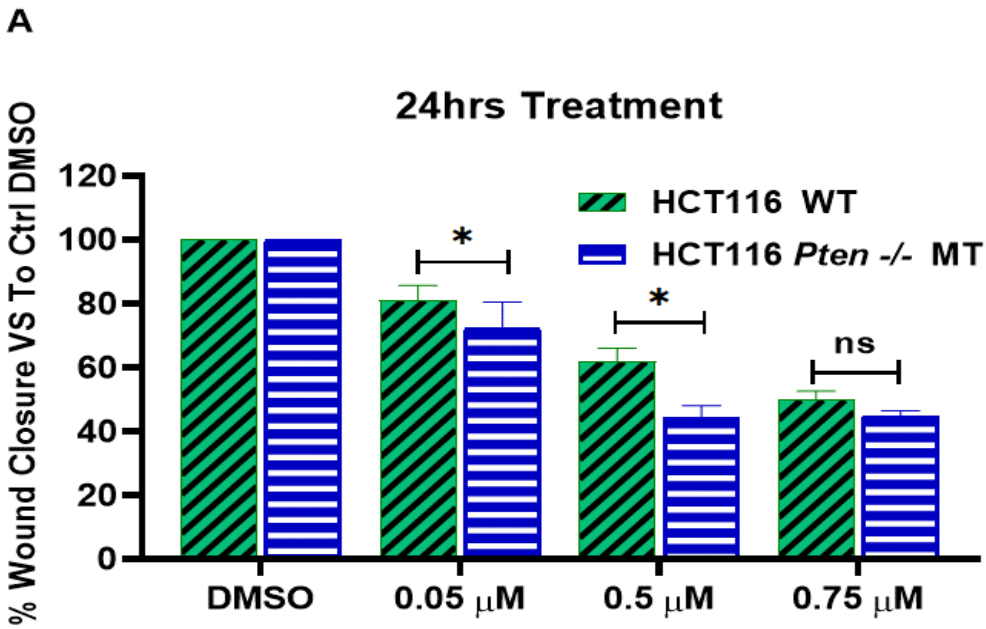
1.5×10^6 cells/well of HCT-116 Wt. and HCT-116 *K-Ras* Mt. cells were treated with different concentration of MBZ (0.05- 0.75 µM) or DMSO for 24 and 48 h. Images of the entire wound area in each well were taken before 0 h and after migration (A) 24 and (B) 48 h and wound closure was measured. Results are presented as the mean and ±SD for three independent experiments. (*** indicates $p<0.001$ and * indicates $p<0.05$).

5.3.2.3 Effect of MBZ on HCT-116 *K-Ras* Wt. and HCT-116 *Pten-/-* Mt. cell lines assessed using the scratch assay.

After 24 h exposure to 0.5 µM MBZ (Figure 5.6), cell migration of HCT-116 *Pten* deletion and HCT-116 Wt. was inhibited by 57% and 38%, respectively, which was significant ($p< 0.05$). At drug concentration of 0.75 µM the migration inhibition was 57% and 46% respectively which was again significant (*$p<0.01$*) The respective IC_{50} values were 0.389 µM and 0.953 µM.

However, after 48 h exposure to 0.5 µM MBZ (Figure 5.6), cell migration reduced by 52% and 41% for HCT-116 *Pten* deletion and HCT-116 Wt. cells which due to was significant ($p< 0.05$). The inhibitory effect at 0.75 µM of MBZ on HCT-116 *Pten* deletion and HCT-116 Wt. cells line was 59% and 47%, which was also significant (*$p<0.001$).* The respective IC_{50} values were 0.409 and 0.973 µM

This study found that HCT-116 Wt. cell lines had significantly higher migration than HCT-116 *Pten* deletion cells line at both exposure times (24 and 48 h) (Figure 5.6).



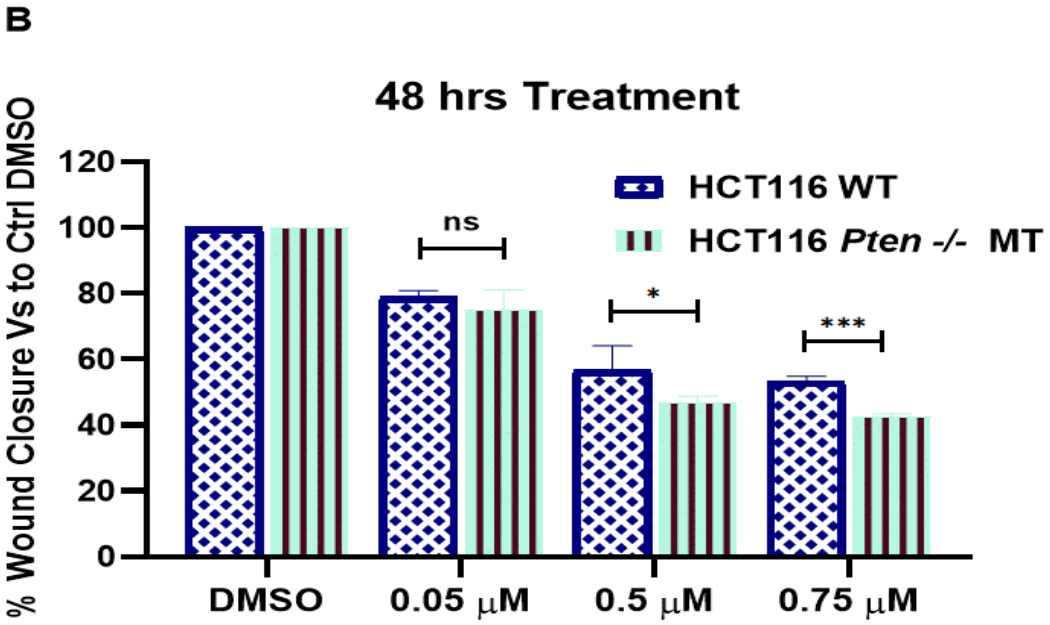


Figure 5.6: Effects of MBZ on the migration of HCT-116 Wt. and HCT-116 *Pten* -/- Mt. cells analysed by wound healing assay at different times and concentration.

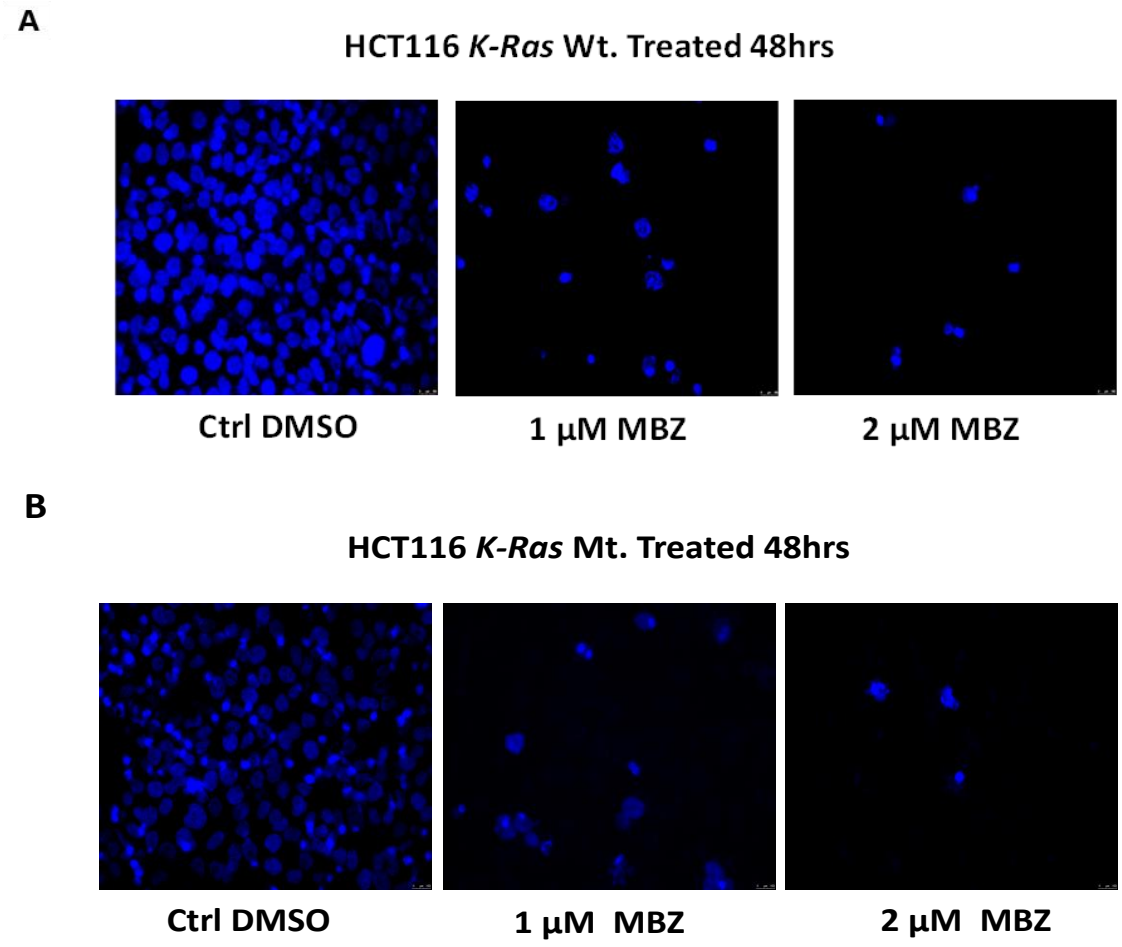
1.5×10^6 cells/well of HCT-116 Wt. and HCT-116 *Pten* -/- Mt. cells were treated with different concentration of MBZ (0.05- 0.75 µM) or DMSO for 24 and 48 h. Images of the entire wound area in each well were taken before (0 h) and after migration (A) 24 and (B) 48 h and wound closure was measured. Results are presented as the mean and ±SD for three independent experiments *P* (*** indicates $p<0.001$, and * indicates $p<0.05$).

5.3.3 Matrigel invasion assay

5.3.3.1 Effect of MBZ on invasion of HCT-116 *K-Ras* Wt. and HCT-116 *K-Ras* Mt. cells using transwell assay.

The data presented in (Figure 5.7) indicate that MBZ significantly reduced cell invasion in a dose dependent manner. The IC_{50} was 0.85 µM which is non-cytotoxic dose as established through the *in vitro* proliferation assay MTT. The full repressing effects achieved at 1 and 2 µM are attributable also to MBZ cytotoxicity respectively. MBZ stimulated apoptosis in H295R and SW-13 cells *in vitro*.

This research shows that MBZ significantly reduced the invasion ability of HCT-116 *K-Ras* Wt. more so than HCT-116 *K-Ras* Mt. cells through a collagen coated transwell membrane in a dose dependent manner, compared to control DMSO with free serum (Figure 5.7). Invading cells numbers were highest in the DMSO control, whereas lowest numbers were shown with high concentration of MBZ at 1 µM and 2 µM (Figure 5.7).



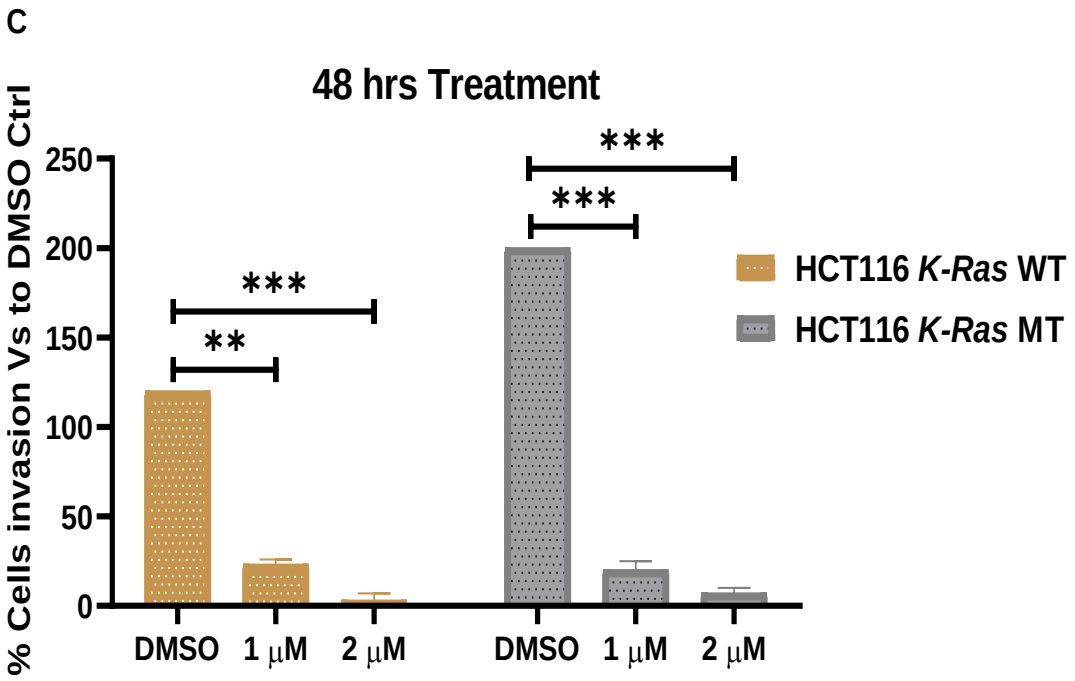


Figure 5.7: MBZ impact on invasion of HCT-116 *K-Ras* Mt. and Wt. cells lines.

HCT-116 *K-Ras* Wt. and Mt. cells were seeded at 6.7×10^5 density with 0% FBS-RPMI-1640 inside the insert and 10% FBS-RPMI-1640 medium and then incubated with either 1 µM, 2 µM of MBZ or DMSO as control for 48 h. The assessment of invasion was analysed from photos taken by a fluorescent microscope. Images are representative of three independent experiments (A) HCT-116 *K-Ras* Wt. and (B) HCT-116 *K-Ras* Mt. The histogram (C) indicates the percentage of cells invaded. Error bars represent SEM from three independent experiments. Statistical analysis was performed by unpaired *Student's t*-test (ns indicates not significant *$p>0.05$, ** $p<0.01$ and *** $p<0.001$).

5.3.3.2 Effect of MBZ on invasion of isogenic pairs of colorectal cancer HCT-116 *K-Ras* Wt. and HCT-116 *K-Ras* Mt. cells lines using a Boyden chamber.

Untreated CRC cells demonstrated a significant increase in their migratory behavior (Figure 5.8). MBZ antagonised the invasive ability of the isogenic colorectal cell lines such as HCT116 *K-Ras* Mt and Wt., cells as investigated by fluorimetric invasion assay. The fluorimetric invasion assay method permits a quantitative evaluation of any effect of MBZ and thus a statistical analysis.

MBZ significantly inhibited invasion more strongly on HCT116 *K-Ras* Wt. at concentration of 1 µM rather than HCT116 *K-Ras* Mt., at a concentration of 2 µM it highly significantly inhibited invasion of both HCT116 *K-Ras* Mt and Wt. (Figure 5.8).

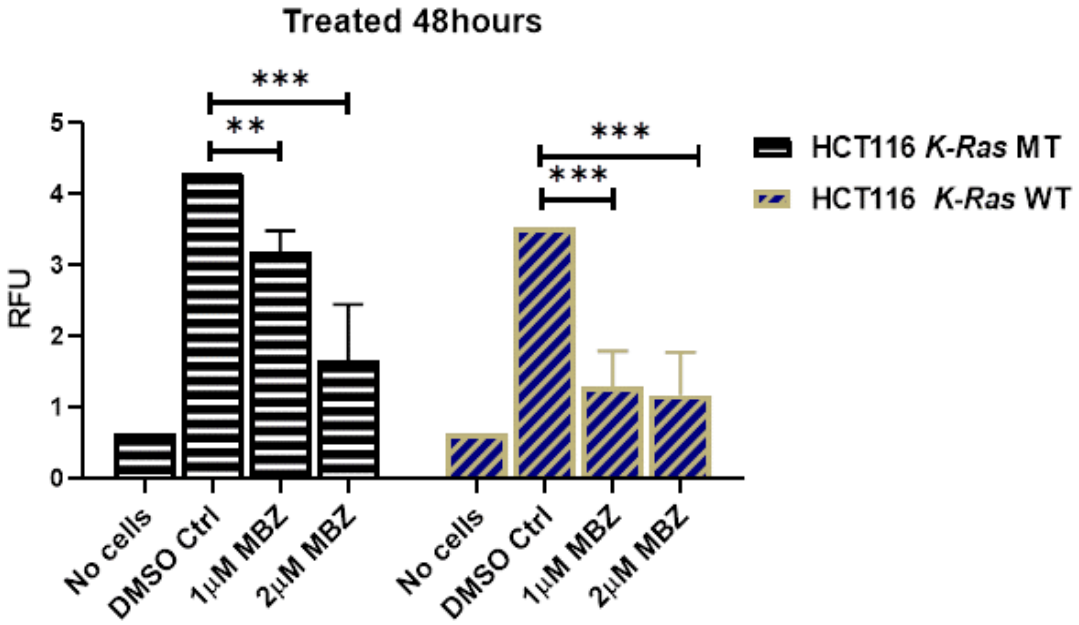


Figure 5.8: MBZ impact on invasion of HCT-116 *K-Ras* Mt. and Wt cell lines.

HCT-116 *K-Ras* Wt. and Mt. cells were seeded at 6.7×10^5 density with 0% FBS-RPMI-1640 inside the insert with 10% FBS-RPMI-1640 medium and then incubated with either 1 µM, 2 µM of MBZ or DMSO as control for 48 h. The assessment of invasion was analysed from photos taken by fluorescent microscope. The data here representative of three independent experiments. The histogram is indicates the percentage of cells invaded. The Fluorescence measurements were reported as RFU (relative fluorescence unit) values. Error bars represent SEM from three independent experiments. Statistical analysis was performed by unpaired *Student's t*-test (** *p<0.01* and *** *p<0.001)*.

5.3.4 Colony formation (CFA)

A clonogenic assay was carried out to test each cell in a given population for the ability to endure infinite division and form colonies (on cell proliferation).

5.3.4.1 The effect of MBZ on colony formation of HCT-116 *K-Ras* Wt. and Mt. cells.

A colony formation assay was presented to assess the effect of MBZ on proliferation of the cells HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. As shown in (Figure 5.9), MBZ indicated a significant inhibition on colony formation in a dose-dependent manner when compared with control. The drug also significantly inhibited colony formation when comparing isogenic pairs which were exposed to MBZ concentrations of 0.05, 0.1, 0.25, 0.5 and 0.75 µM., Very small numbers of colonies were detected on HCT-116 *K-Ras* Mt at 0.75 µM. On the other hand, we observed 51% reduction of the colonies formation of HCT-116 *K-Ras* Wt. and the IC_{50} values were 0.158 µM for Mt. and 0.919 µM for Wt.

HCT-116 *K-Ras* Mt. cell line compared to HCT-116 *K-Ras* Wt.**,** showed statistical significance in the percentage of cells colonies formation, which was 51, 47 and 33% when compared with HCT-116 *K-Ras* Wt. 73, 66 and 57% at concentrations of 0.1, 0.25 and 0.5 µM of MBZ respectively.

The outcomes from colony assay showed that MBZ could significantly inhibit the CRC with mutation compared with the wild type as control, which were consistent with the outcome from MTT assay.

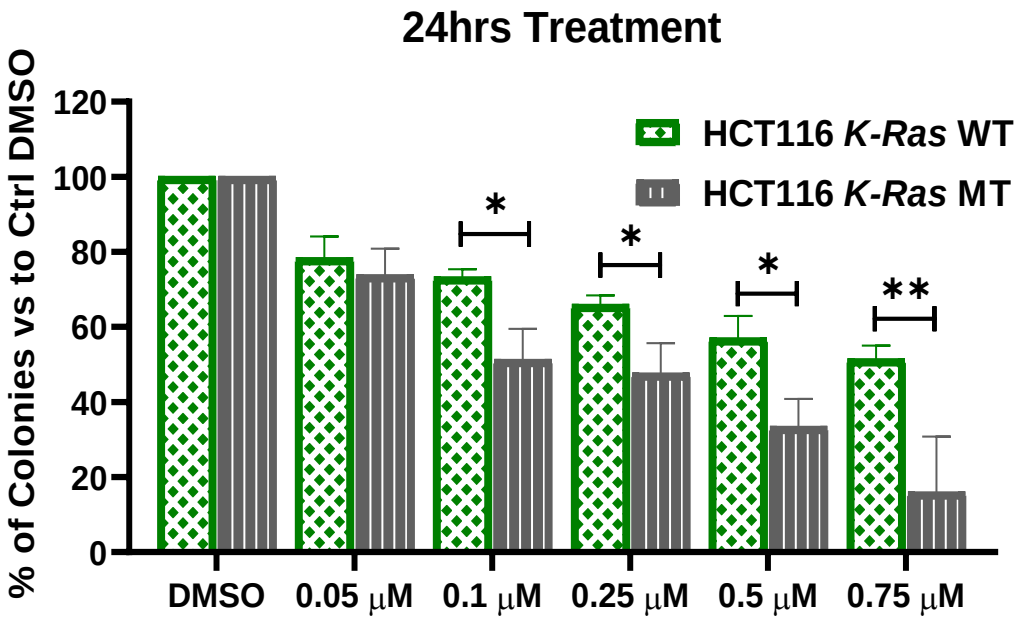


Figure 5.9: Effect of MBZ on colony formation of HCT-116 *K-Ras* Wt. and Mt. cells.

300 cells /well of HCT-116 *K-Ras* Wt. and Mt., cell lines were plated in 6 well plates. The colony formation was assessed after treatments with different MBZ concentration (0.05 - 0.75 µM) and DMSO for 24 h. The graph shows the percentage of colonies. The results are presented as the mean and ±SD of three independent experiments (**$p<0.01$and *$p<0.05$).

5.3.4.2 The effect of Ran inhibitor (MBZ) on colony formation on isogenic pairs HCT-116 Wt. and HCT-116 *Pten* null cells lines.

The colony formation assay was performed on the cells HCT-116 *Pten* deletion and HCT-116 Wt. after treatment with MBZ. As shown in (Figure 5.10), MBZ demonstrated a significant inhibition on colony formation in a dose-dependent manner when compared to control. The drug also gave significant inhibition of colony formation when comparing isogenic pairs which were exposed to MBZ at concentrations of 0.05, 0.1, 0.25, 0.5 and 0.75 µM. Very small numbers of colonies were detected on HCT-116 *Pten* deletion 11% at 0.75 µM. On the other hand, we observed 51% of the colonies formation of HCT-116 Wt. The IC_{50} values were 0.273 µM for Mt. and 0.919 µM for Wt.

HCT-116 *Pten* deletion cell line compared to HCT-116 Wt.**,** exhibited statistical significance in the percentage reduction of cells colonies formation 37% when compared with HCT-116 Wt. 57% at similar concentration of 0.5 µM of MBZ. There was no significant colony reduction between the isogenic pairs of cells HCT-116 *Pten* deletion and HCT-116 Wt. at drug concentrations of 0.05, 0.1 and 0.25 µM of MBZ as the colony formation % were 80, 67 and 63% for Mt. and 78, 73 and 66% of Wt. respectively.

The results from colony assay showed that MBZ could significantly inhibit the CRC with mutation compared with the Wt. as control, which was consistent with the outcome from MTT assay.

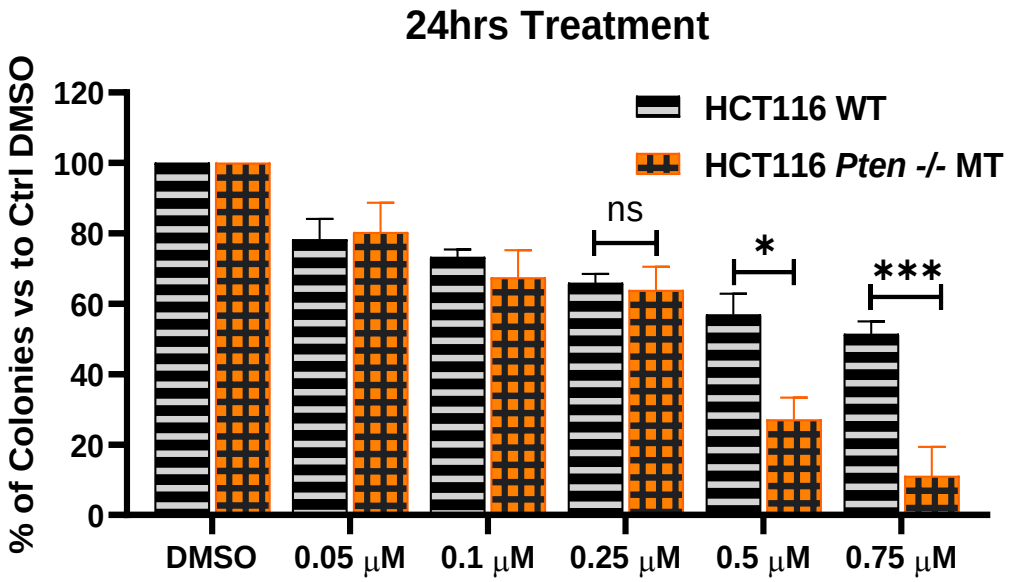


Figure 5.10: Effect of MBZ on colony formation of HCT-116 Wt. and HCT-116 *Pten* null cells.

300 cells /well of HCT-116 Wt. and HCT-116 *Pten* null cells, cell lines were plated in 6 well plates. The colony formation was assessed after treatments with different MBZ concentration (0.05 - 0.75 µM) and DMSO for 24 h. The graph shows the percentage of colonies. The results are presented as the mean and ±SD of three independent experiments (****p<0.001*, **p*<0.05 and ns means *p>0.05*).

5.3.4.3 The effect of Ran inhibitor (MBZ) on colony formation on isogenic pairs DKO-3 Wt. and DLD-1 *K-RAS* Mt. of CRC cells lines.

A colony formation assay was performed then on the cells DLD-1 *K-Ras* Mt. and DKO-3 Wt. after treatment with MBZ. As shown in (Figure 5.11), MBZ showed a significant inhibition on colony formation in a dose-dependent manner when compared with control. Significant inhibition of colony formation was also found when comparing isogenic pairs which were exposed to MBZ at concentrations of 0.05, 0.1, 0.25, 0.5 and 0.75 µM. Smaller numbers of colonies were detected on DLD-1 *K-Ras* Mt. at 0.25 , 0.5 and 0.75 µM as the % of colonies were 55, 52 and 42%. On the other hand, we observed 80, 80 and 64% of the colonies formation of DKO-3 Wt. at those MBZ concentrations. The IC_{50} values were 0.472 µM for Mt. and 4.7 µM for Wt. respectively.

When comparing DLD-1 *K-Ras* Mt. cell line to DKO-3 Wt.**,** no statistical significance was observed in cells colony formation which was 77 and 72% but in DKO-3 Wt. it was 86 and 84% at concentrations of 0.05 and 0.1 µM of MBZ respectively.

The results from the colony assay shown that MBZ could significantly inhibit the CRC with mutation compared with the Wt. as control, which was consistent with the result from MTT assay.

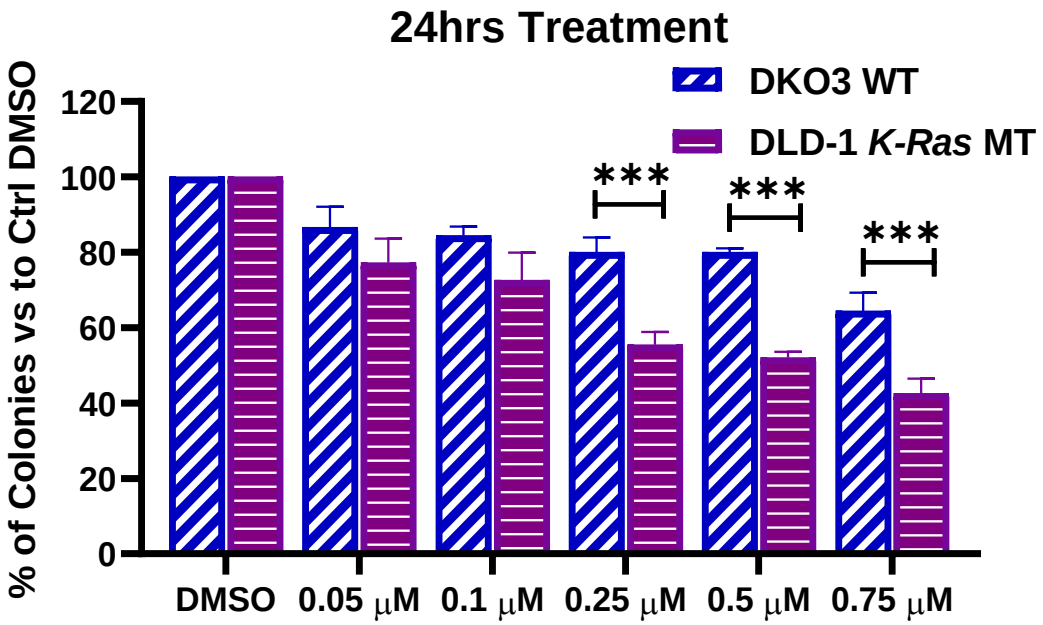


Figure 5.11: Effect of MBZ on colony formation of DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells.

300 cells /well of DKO-3 Wt. and DLD-1 *K-Ras* Mt. cells, cell lines were plated in 6 well plates. The colony formation was assessed after treatments with different MBZ concentration (0.05 - 0.75 µM) and DMSO for 24 h. The graph shows the percentage of colonies formed. The results are presented as the mean and ±SD of three independent experiments (***$p<0.001$).

5.3.4.4 The effect of Ran inhibitor (MBZ) on invasive breast cancer MDA-MB-231 and and non-small cell lung carcinoma A549 cells lines by colony formation.

We tested the effect of MBZ on other kinds of cancers including breast and lung. We performed a colony formation assay to find out the effect of MBZ on breast cancer cell line MBA MD 231 and non-small cell lung carcinoma A549. We have found that the MBZ inhibits colony formation on both cell lines at IC_{50} values of 0.11 µM and 0.2 µM, respectively.

Therefore, our future plan will investigate the effect of MBZ on other *in vitro* and *in vivo* biological properties both breast and lung cancer cell lines.

5.3.4.4.1 The effect of Ran inhibitor (MBZ) on colony formation on invasive breast cancer MDA-MB-231 cells lines.

The colony formation assay was performed to evaluate the effect of MBZ on MDA-MB-231 cell proliferation, so these cells were seeded and treated with MBZ. As shown in (Figure 5.12), MBZ demonstrated a significant inhibition on colony formation in a dose-dependent manner when compared with control. group as well as when compared to isogenic pairs which exposed to similar condition of MBZ at different concentration of 0.05, 0.1, 0.25, 0.5 µM, whereas no colonies were detected on invasive breast cancer cell lines, MDA-MB-231 at 0.5 µM, on the other hand, the result had showed 25% inhibition of the colonies formation at 0.05 µM $p<0.05$, 52% at 0.1 µM $p<0.001$ and 75% at 0.25 µM $p<0.001$ and the IC_{50} value was 0.11 µM.

The results from colony assay demonstrated that MBZ significantly exhibits anti-proliferative effect on the MDA-MB-231 at IC_{50} = 0.11 µM and when compared with isogenic colorectal cancer with mutation compared with the wild type as control, which were consistent with the outcome from MTT assay and shown the highest inhibition at low concentration.

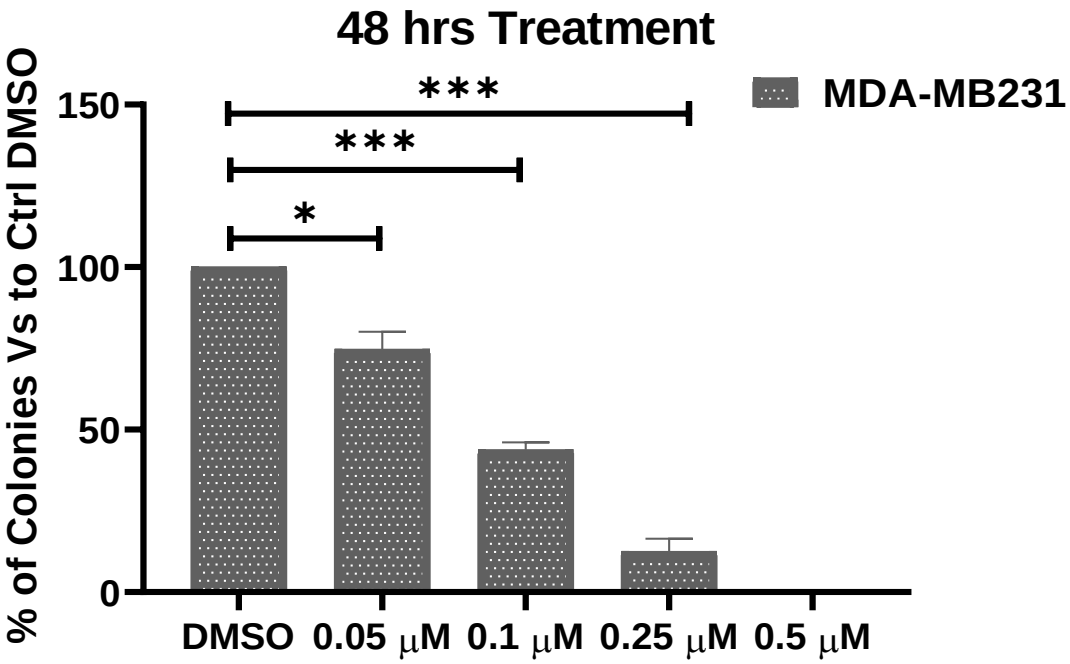


Figure 5.12: Effect of MBZ on colony formation of invasive breast cancer MDA-MB-231 cells.

300 cells /well of MDA-MB-231 cells, cell lines were plated in 6 well plates. The colony formation was assessed after treatments with different MBZ concentration (0.05 - 0.75 μM) and DMSO for 24 hr. The graph shows the percentage of colonies formed. The results are presented as the mean and ±SD of three independent experiments (****p<0.001* and *$p<0.05$).

5.3.4.4.2 The effect of Ran inhibitor (MBZ) on colony formation on non-small lung cancer (A549) cells lines.

A colony formation assay was performed to assess the effect of MBZ on proliferation of human non-small-cell lung cancer NSCLC (A549) cell lines. As shown in (Figure 5.13), MBZ showed a significant inhibition on colony formation in a dose-dependent manner when compared with control. group as well as when compared to isogenic pairs which exposed to similar condition of MBZ at different concentration of 0.05, 0.1, 0.25, 0.5 µM, whereas around 10% of colonies were detected of human non-small-cell lung cancer NSCLC (A549) cell lines at 0.5 µM *p<0.001* and 90% of inhibition, on the other hand, we had showed 5% inhibition of the colonies formation at 0.05 µM, 15% at 0.1 µM and 65% at 0.25 µM *p<0.001* and the IC_{50}= 0.2 µM. The results from colony assay demonstrated that MBZ significantly exhibits an anti-proliferative effect on the human non-small-cell lung cancer NSCLC (A549) at IC_{50} value of 0.2 µM and when compared with isogenic colorectal cancer with mutation compared with the wild type as control, which were consistent with the outcome from MTT assay and shown the highest inhibition at 0.5 µM concentration (Figure 5.13).

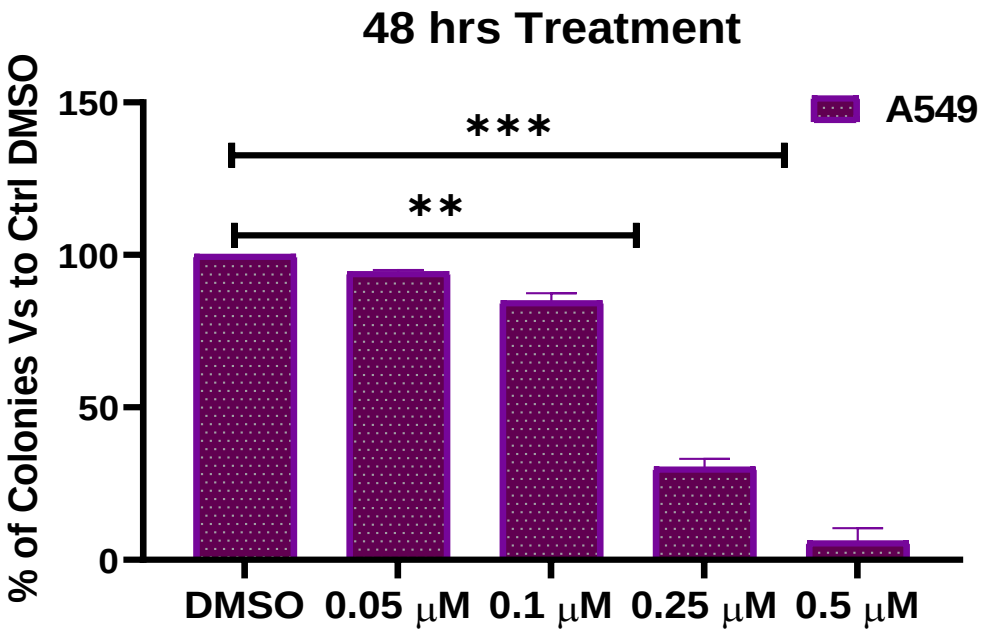


Figure 5.13: Effect of MBZ on colony formation of A549 cells.
300 cells /well of A549 cells, cell lines were plated in 6 well plates. The colony formation was assessed after treatments with different MBZ concentration (0.05 - 0.75 µM) and DMSO for 24 h. The graph shows the percentage of colonies formed. The results are presented as the mean and ±SD of three independent experiments (****p<0.001* and *$p<0.01$).

5.4 Discussion

The effect of MBZ on expression levels of several mRNA and proteins that belong to cell survival, migration, and metastasis in isogenic pairs of colorectal cancer, invasive breast cancer and Human non-small-cell lung cancer NSCLC (A549) cell lines was assessed in this chapter. The hypothesis was that the Ran inhibitor MBZ has an anti-cancer effect in colorectal cancer.

The MBZ was shown to downregulate the expression levels of several signalling proteins Akt 1, Akt 2 and Akt 3 and proteins such as Erk 1, Erk 2, p90rsk1 and up-regulated Akt on HCT-116 *K-Ras* Mt. and Wt. with same effect on migration. HCT-116 *K-Ras* Mt. and Wt., DLD-1*K-Ras* Mt., and DKO-3 Wt. and HCT-116 *Pten -/-* Mt. and Wt. were inhibited in a dose-dependent manner by MBZ.

Moreover,MBZ has exhibited an inhibitory effect on invasion of colorectal cell lines such HCT-116 *K-Ras* Mt. and Wt. as well as on colony formation. The drug has shown the same effect in HCT-116 *K-Ras* Mt. and Wt., DLD-1 *K-Ras* Mt. and DKO-3 Wt. and HCT-116 *Pten -/-* Mt. and Wt.

The first *in vitro* and *in vivo* studies which exhibit the antitumor and anti-angiogenetic effects of MBZ were carried out in different type of cancer cell lines (Mukhopadhyay et al. 2002). The results of this research on isogenic colorectal cell lines agreed with this study with regard to anti-tumour and anti-angiogenic properties.

The results showed that MAPK (Erk 1/2) protein expression was down regulated significantly by MBZ when treating both HCT-116 *K-Ras* mutant and wild type. The effect went on to down-regulate its downstream p90rsk1α when analysed by western blotting. This data are consistent with a previous study confirming that the downstream MAPK pathway is inhibited through Erk 1/2 phosphorylation in Hepatocellular carcinoma cells (HCC) by MBZ (Younis et al. 2019). So, the results in this project show that MBZ has the same effect on the downstream MAPK pathway by 1/2 phosphorylation in HCT-116 *K-Ras* Mt. and Wt. colorectal cell lines.

Both gene Akt 1/2/3 (Xie et al. 2019) and protein p-Akt expression in isogenic pairs of colorectal cancer HCT-116 *K-Ras* Mt. and Wt. cells was assessed and shown to be significantly down regulated in mRNA level Akt 3 in HCT-116 *K-Ras* Mt., while up-regulation was observed in Akt 1/2 in Wt. The levels of p-Akt were up-regulated in HCT-116 *K-Ras* Mt. and down-regulated in HCT-116 *K-Ras* Wt.

by comparison each cell with their un-treated control as well as when compared to each other. However, the observed reduction was significant in the HCT-116 *K-Ras* Wt. while in the HCT-116 *K-Ras* Mt. it was insignificant.

MBZ significantly inhibits Ran protein expression leading to reduction of downstream pathways of phosphorylation of Erk 1 and Erk 2 in HCT-116 *K-Ras* Mt. rather HCT-116 *K-Ras* Wt. It has also shown significant up regulation of phospho- Akt, in HCT-116 *K-Ras* Mt. and less effect in HCT-116 *K-Ras* Wt. phosphorylation. Moreover, Akt activation is correlated to amplified invasiveness and metastasis (Shukla and Singh 2007; Hara et al. 2008; Xie et al. 2019). One consequence of RAN overexpression is to increase the phosphorylation of Akt (Bischoff et al. 1995; Elsheikh et al. 2020). So, MBZ antagonizes the effect of Akt by Ran inhibition but has not shown any inhibitory effect through this mechanism. MBZ potentially will not prevent the cells migration and invasion through the Akt pathway.

MBZ also prevented the mRNAs that code for several of proteins that are important in cancer proliferation. These proteins are all considered as markers of programmed cell death e.g., Akt 1, Akt 2, p53 and Bcl-2. Akt 1 and Akt 2 are associates of the PI3K/mTOR pathway (Xie et al. 2019) which is hyperactive in common types of cancers and in about 90% of TK-resistant cancers (El-Tanani 2017).

RAN silencing has also encouraged programmed cell death in cancer cells which have mutations that correlated with activation of the PI3K/Akt/mTORC1 (Xie et al. 2019) and Ras/Mek/Erk signaling pathways (Yuen et al. 2012) RAN was proposed as a potential target for cancer treatments which activate protein such as the PI3K/Akt/mTORC1 and Ras/Mek/Erk pathways.

Earlier studies demonstrated that suppression of Ran enhanced the potency of the apoptotic response in cancer cells with activated phosphoinositide 3-kinase PI3K/Akt/mTORC1 (Yuen et al. 2012; Xie et al. 2019) and Ras/Mek/Erk [mitogen-activated protein/extracellular signal-regulated kinase] (Erk; Mek) pathways (Yuen et al. 2012). These pathways support not only cell survival, but also cell proliferation, angiogenesis, metastasis, and other key cellular processes (Sebolt-Leopold and Herrera 2004; Engelman 2009; Yuen et al. 2012).

K-Ras protein transmits signals from outside the cell to the nucleus which trigger cell proliferation and then differentiation. The *K*-Ras protein is a GTPase and has

an intrinsic enzymatic activity which is responsible for cleaving the terminal phosphate of GTP into GDP molecule. In this way, the *K-Ras* protein works similar to a switch turning on and off through converting the GTP active form and GDP inactive form (Taya et al. 1984; Tsuchida et al. 2016). This system will be damaged when a mutation occurs to *K-Ras*. Subsequently it becomes active due to activation of the RAS/MAPK pathway and does not follow the homeostasis role, leading to tumour growth and metastasis in distant organs. This is one reason for selecting the isogenic colorectal cancer cells in this research project.

Furthermore, MBZ has been shown to prevent the growth of different type of cancer cells *in vitro* and *in vivo* (Mukhopadhyay et al. 2002; Doudican et al. 2008; Martarelli et al. 2008; Bai et al. 2011). MBZ inhibits Ran-GTPase leading to reduction in the phosphorylation of Erk 1 and Erk 2 in HCT-116 *K-Ras* Mt. rather HCT-116 *K-Ras* Wt. It has also been shown to induce phosphorylation of Akt in HCT-116 *K-Ras* Mt. significantly and to slightly increase in HCT-116 *K-Ras* Wt. Previous studies have shown that *K-Ras* mutant (Morgan-Lappe et al. 2007; Yuen et al. 2012), *Pten-deleted* (Yuen et al. 2012), overexpressing cancer cells are more sensitive to Ran knockdown compared to their Wt. counterparts, suggesting that Ran may be a potential therapeutic target for cancers with these oncogenic mutations such as *K-Ras* and *Pten* null (Yuen et al. 2013). Ran expression was significantly higher in colon and rectum cancers than in adjacent non-cancerous tissue and Ran was positively correlated with depth of invasion and distant metastases (Yao and Liu 2013).

Gefitinib treatment has exhibited an inhibitor effect on both Akt and Erk1/2 phosphorylation pathways in both gefitinib highly sensitive cell lines HCC82 shScr and HCC827-shRan. In contrast, the phosphorylation of Akt and Erk1/2 in GR5shScr cells did not significantly alter in the presence of 1 g of gefitinib which is resistant to gefitinib. However, when Ran was Knocked down in GR5-shRan cells, a decrease in phosphorylation was observed in the presence of gefitinib in p-Akt and p-Erk1/2.

These results suggest that the inhibition of the PI3K / Akt and Mek / Erk pathways were mediated by knockdown of RAN to expand sensitivity of GR5 cells to gefitinib (Yuen et al. 2016a). MBZ significantly inhibits Ran protein expression which leads to reduction of the phosphorylation of Erk 1 and Erk 2 in HCT-116 *K-Ras* Mt. rather HCT-116 *K-Ras* Wt. It has also been demonstrated to significantly

induce phosphorylation of Akt in HCT-116 *K-Ras* Mt. and slightly increase it in HCT-116 *K-Ras* Wt.

Moreover, Akt activation is linked to amplified invasiveness and metastasis (Patel et al. 2007; Hara et al. 2008; Shukla et al. 2014).

The effect of MBZ on migration, invasion, and colony formation in isogenic pairs of colorectal cancer cell lines was assessed using scratch wound, invasion, and colony formation assays. In this study, we tested the hypothesis that the Ran inhibitor MBZ provides an anti-cancer effect in colon cancer and inhibits cancer cell migration and invasion. MBZ suppressed cell invasion in both H295R and SW-13 cell lines with an IC_{50} of 0.085 μM (Guerini et al. 2019)

The aim of the study in this chapter was to examine the impact of MBZ on Ran expression through cell biological properties. This research has shown that migration, invasion, and colonisation in the mutant cell lines DLD-1 *K-Ras*, HCT-116 *Pten* deletion and HCT-116 *K-Ras* Mt. were reduced by MBZ compared to wild-type cell lines.

The results showed that Ran inhibitor MBZ has activity on isogenic colorectal cancer cell line through inhibition of cell migration, invasion, and colony formation. Previous study on this medicine of low cost has shown reduced toxicity to normal cells, and MBZ was able to decrease the growth, migration and invasion of cancer cells *in vitro* (Pinto et al. 2015). Compounds that can alter of any stage of the metastatic cascade have some potential as an anticancer treatment (Lu et al. 2017). Previous work showed that MBZ repressed malignant ascites cells migration and invasion (Pinto et al. 2015) and inhibited adrenocortical carcinoma cells *in vitro* (Martarelli et al. 2008). All these studies corroborated our findings in this research project on levels of invasion, migration, growth, and proliferation in colony formation. With HCT-116 *K-Ras* Mt. and Wt. cell lines the results were confirmed with different experimental techniques such as scratch and invasion assays by two different techniques and a fluorimetric cell invasion assay.

Different tumour types (including colorectal, breast and kidney tumours) are associated with Ran overexpression leading to local invasion, metastasis, and increased patient mortality. Ran expression has also been found to be essential to tumours with oncogenic *K-Ras* mutations (Bertucci et al. 2004; Matchett et al. 2014). All reported that the cancer cells that have activation mutations in *K-Ras* rely more on the expression of Ran than their wild counterparts, *K-Ras* (Matchett

et al. 2014). Moreover, a range of cancer cell lines e.g. colon adenocarcinoma HCT-116, SW620 and COLO 201, breast adenocarcinoma MCF-7 and MDA-MB-231 are also associated with RAN up-regulation (Azuma et al. 2004; Xia et al. 2008; Rensen and Lavia 2010). Cells that are undergoing active proliferation or have transformed usually have high expression of Ran protein (Azuma et al. 2004; Xia et al. 2008; Rensen and Lavia 2010). The cell cycle is inhibited by a tumour suppressive *Pten* by disrupting the PI3K pathway (Chu and Tarnawski 2004).

Tumor invasion, and metastatic potential is linked to cell migration (Russo et al. 2018). We have assessed the effect of Ran inhibitor MBZ on isogenic pairs of CRC cell migration and invasion. The results establish that MBZ inhibited mutant CRC cells migration and invasion when compared to Wt. cells that were treated with the same drug concentration. Moreover, when isogenic pairs CRC cells were treated with DMSO, migration was not affected. Our findings were confirmed with multiple scratch assays. Therefore, MBZ significantly repressed invasion intensely on HCT-116 *K-Ras* Wt. at concentration of 1 μM rather than HCT-116 *K-Ras* Mt. In contrast, it has shown extremely significant repression of invasion on both HCT-116 *K-Ras* Mt. and Wt. at a concentration of 2 μM of MBZ.

Based on the scratch assay, the migration of cells in the control cells line would be likely to provide wound closure at 48 h. The assay normally measures migration of cells by measuring the wound closure with time.

At 24 h, cells migration of DLD-1 *K-Ras* Mt. and DKO-3 Wt. were inhibited in a dose-dependent manner of MBZ and had a significant effect on Mt. but less so on Wt. After 48 h cell migration was reduced further than at 24 h. Additionally, the inhibitory effect of MBZ at 0.05 μM on wound closure was also seen but it was very low on Mt. cell line but with no effect on the Wt. cells line in both time courses.

This study found that HCT-116 and DKO-3 Wt. cell lines had significantly higher migration than HCT-116 *K-Ras* Mt., HCT-116 *Pten* deletion and DLD-1 *K-Ras* Mt. cells line at both time periods and doses manners.

Treatment of HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt., HCT-116 *Pten* deletion, HCT-116 Wt., DLD-1 *K-Ras* Mt. and DKO-3 Wt. cells with MBZ resulted in decreased capability of the cells to migrate in wound healing and decreased capability of the HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells to migrate in

the Boyden chamber in migration assays and to invade through the matrigel in invasion assay, respectively.

The data presented here highlight that human isogenic pairs of colorectal cancer cell line can be regarded as a useful way to evaluate the impact of Ran inhibitor drugs against CRCs on proliferation.

Treated with MBZ versus non-treated cells of both HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. exhibited fewer colonies formed at 0.75 µM respectively, A significant difference between treated and non-treated cells was also exhibited by HCT-116 *Pten* deletion at 0.75 µM. However, there was significant variation between wild-type HCT-116 and mutant HCT-116 *K-Ras*. We observed a significant reduction in colonies formation ability of HCT-116 *Pten* deletion (>50%) at 24 h of treatment with 0.5 µM, MBZ, but not with control DMSO.

A significant difference between treated and non-treated cells was also shown by DLD-1 *K-Ras* Mt. significant, in contrast to DKO-3 Wt. Thus, in keeping with (Yuen et al. 2012), the findings of the scratch and colony formation assays all confirmed that the Ran inhibitor affected mutant cells but not wild type cells (Yuen et al. 2012). Furthermore, the ability to form colonies was impaired when HCT-116 *K-Ras* Mt., HCT-116 *Pten* and DLD-1 *K-Ras* Mt. cells were treated with MBZ at 0.5 µM.

A highly significant difference between treated and non-treated cells was also shown by MDA-MB231, in contrast to DKO-3 Wt. Thus, in keeping with (Yuen et al. 2012), the findings of the scratch and colony formation assays all confirmed that the Ran inhibitor affected mutant cells but not wild type cells (Yuen et al. 2012). Furthermore, the ability to form colonies was impaired when HCT-116 *K-Ras* Mt., HCT-116 *Pten* and DLD-1 *K-Ras* Mt. cells were treated with MBZ at 0.5 µM.

A significant difference between treated and non-treated cells was also shown by A549, in contrast to DKO-3 Wt. Thus, in keeping with (Yuen et al. 2012), the findings of the scratch and colony formation assays all confirmed that the Ran inhibitor affected mutant cells but not wild type cells (Yuen et al.2012). Furthermore, the ability to form colonies was impaired when HCT-116 *K-Ras* Mt., HCT-116 *Pten* and DLD-1 *K-Ras* Mt., cells were treated with MBZ at 0.5 µM.

5.5 Conclusion

The data confirmed by western blot show a significant Ran inhibition by MBZ in both HCT-116 *K-Ras* Mt. and HCT-116 *K-Ras* Wt. cells. In addition to inhibition in phosphorylation of Erk 1 and Erk 2 in HCT-116 *K-Ras* Mt. more so than HCT-116 *K-Ras* Wt. It has been shown MBZ induced phosphorylation of Akt in HCT-116 *K-Ras* Mt and slightly increased it in HCT-116 *K-Ras* Wt. as well as on downstream p90rsk1 protein. Furthermore, MBZ demonstrated effect on inhibition of cell proliferation, migration, invasion and finally on metastasis on colorectal and proliferation of breast and lung cancer cell lines.

In colorectal cancer Ran is significantly upregulated in both cell lines and tissues, especially in metastatic tissues. The upregulation of Ran is associated with poor colorectal cancer patient prognosis. Silencing Ran has been shown to reduce cell proliferation, induce apoptosis and inhibit the invasion and metastasis of colorectal cancer cells in *vitro* (Wang et al. 2020b).

In summary, these results and previous studies support the notion that MBZ is active alone in managing of progressive gastric cancer in vitro. Moreover, this low-cost medication with a satisfactory safety profile has less toxicity to normal cells, and can decrease cancer cells growth and prevent their migration and invasion *in vitro* (Pinto et al. 2015).

Chapter 6
General discussion and future work

Chapter 6: General discussion and future work

6.1 General discussion

The work presented in this thesis set out to test the hypothesis that the Ran inhibitor, mebendazole, has an anti-cancer effect in colon cancer. Ran is considerably upregulated in CRC cell lines and tissues, particularly in metastatic tissues, and its upregulation is linked with poor colorectal cancer prognosis. Ran silencing diminished proliferation and stimulated programmed cell death which as a consequence hindered the *in vitro* invasion and metastasis of colorectal cancer cell lines (Wang et al. 2020b). Mebendazole is a drug used to treat a number of parasitic worm infestations (Nygren et al. 2013). Here, we measured cell proliferation (MTT assay), colony formation and carried out scratch assays to determine the toxicity and the roles played by mebendazole, in isogenic pairs of human colorectal cell lines We also examined the drug's effect on immune system complement, angiogenesis and on cell death.

Results from chapter 3 indicated that in both HCT-116 K-*Ras* mutant and wild type cells treatment with MBZ for 48 hours caused down-regulation of Ran mRNA and protein expression and thus MBZ mimicked the effect of a specific knock down using Ran-shRNA. According to these studies, it has been demonstrated that silencing Ran induced programmed cell death (apoptosis) in both breast and lung cell cancer (Yuen et al. 2012). Recently, a group has shown that the anti-psychotic drug pimozide has reduced *in vitro* the proliferation and invasion of breast and lung cancer cells (Dakir et al. 2018). Moreover, it has been shown that pimozide reduced 60-70% of tumour volume and prevents breast cancer tumour metastasis *in vivo* (Dakir et al., 2018).

our results provide evidence that MBZ has shown a cytotoxic effect on mutant colorectal cell line rather than wild type and it prevents cell migration and invasion, colorectal cell lines colonisation and their effect on downstream signal of the MAPK pathway by preventing phosphorylation of Erk1/2 in chapter 5., Furthermore, it shown has effects on immune down-regulations in levels of mRNA and proteins for C5a and IL-1 α and IL-β and has an anti-angiogenesis effect on VEGFR1/2 down-regulations in a dose-dependent manner (see chapter 3). C5a is a protein that is cleaved by protease of complement C5 resulting in release of C5a and C5b. These results show that mebendazole may down-regulate C5a, IL-1 α and IL-β mRNA. Infiltration of inflammatory cell lead to increased pro-inflammatory cytokines C5a, IL1α and IL-1β promoting tumour development and

progression (Terzić et al. 2010). IL-1 is a cytokine that encourages CRC and colitis-associated tumour progress while several others were upregulated in these tumour kinds. On the other hand, IL-10 cytokine prevents CRC tumorigenesis (Terzić et al. 2010).

Ran is identified as overexpressed in several cancer cells which are activated through pathways such as Ras/Erk/ MEK and PI3K/Akt/ mTORC. In CRC cell lines such as HCT-116 *K-Ras* Mt. and Wt., mebendazole was found to act downstream of the MAPK pathway by preventing phosphorylation of Erk1/2 (Younis et al. 2019). Previous studies revealed that the cancer cells that have activation mutations in *K-Ras* depend on the expression of Ran rather than their wild counterparts, *K-Ras*. Furthermore, a variety of cancer cell lines, including colon adenocarcinoma HCT-116, SW620 and COLO 201 and breast adenocarcinoma MCF-7 and MDA-MB-231 are similarly linked with Ran upregulation (Azuma et al. 2004; Xia et al. 2008; Rensen and Lavia 2010).

This research provides evidence that mebendazole acts by inhibiting Ran transcription in the level of Ran-mRNA then subsequently inhibiting their downstream genes such as Akts (Akt1, Akt2 and Akt3) and MAPK. In addition to mebendazole acting to inhibit Ran protein then subsequently inhibits their functions such as Erk1 and Erk2 and other kinase pathways such as VEGF 1/2 and p90rsk1.

Ran inhibition with mebendazole caused significant effects on isogenic colorectal cell line such HCT-116 K-*Ras* Mt. and Wt. in both their levels of Ran-mRNA and Ran-protein. In addition, this work supports the effects of mebendazole on *K-Ras* activation cell lines through induce programmed cell death (apoptosis). Furthermore, effects are shown on *pten*-deletion cell line at the level of cell proliferation, migration, invasion, and colony formation. However, future work is needed to test this activity in PI3K/ Akt/ mTORC which is activated in *pten* -/- cell lines.

Mebendazole also inhibited other messenger-RNA that codes some proteins that are important in cancer and apoptosis such as Akt1, Akt2, Akt3, Bcl-2 and P53 (Elayapillai et al. 2020). This study showed obvious inhibition on Akt1/2/3 in the level of their mRNA but upregulated total p- Akt on level of protein which was not correlated with a previous study which showed p-Akt was deactivated through treated treating an AML xenograft mouse model with MBZ. Although in the case

of HCT-116 *K-Ras* Wt. cell line it has shown similar inhibition (He et al. 2018), as p-Erk1/p-Erk1 was inhibited through treating the cell lines with MBZ in a dose-dependent manner. This finding indicates that the inhibitory effect of MBZ on CRC cells growth might be facilitated through inhibition of the p-Erk1/ p-Erk2 pathway. These results were consistent with previous results from the same group (He et al. 2018).

In **Chapter 3** mRNA VEGFR1/ VEGFR2 expression was significantly down-regulated in both isogenic CRC cell lines HCT-116 K-*Ras* mutant although in the case of VEGFR1 mRNA HCT-116 K-*Ras* wild type this down regulation was less significant in contrast to VEGFR2 mRNA HCT-116 K-*Ras* wild type was highly significant for each cell against their un-treated control. These outcomes were consistent with a earlier study which proposed that MBZ in medulloblastoma preclinical mouse models produces antiangiogenic activity on VEGFR2, which is considered the main receptor facilitating the effects of VEGF (Bodhinayake et al. 2015).

Pro-apoptotic protein Bad when phosphorylated to p-Bad cannot ability to prevent anti-apoptotic Bcl-2 exerting its activity as anti-pro-apoptotic and thus will not produce apoptosis pathway. Bad encourages programmed cell death pathway by preventing Bcl-2 phosphorylation thereby a releasing BAK and then releasing cytochrome C to trigger the apoptotic pathway.

Mebendazole in **Chapter 4** was found to regulate p-Bcl-2 down-regulation and activation of caspase -3, caspase -7, caspase -9 and PARP in HCT-116 K-*Ras* Mt. and HCT-116 K-*Ras* Wt. This agrees with previous research that revealed the antitumor activity of mebendazole came about by Bcl-2 inactivation and caspase activation (Doudican et al. 2008; Bai et al. 2011; Doudican et al. 2013; Larsen et al. 2015; Wang et al. 2020c). Furthermore, caspases -3, caspase -7 and caspase -9 were triggered through mebendazole in H460 cells (Ferrara 2001; Sasaki et al. 2002; Ferreira et al. 2010).

Previous investigations have revealed that the activation of pro-survival factors Bcl-2 protein signaling encourage cell survival (Cory et al. 2003) while the drug encourages inhibition that leads to the expression of multiple targets which are called pro-apoptotic gene family proteins which induce programmed cell death, for example BH3 only proteins such as Bad.

Results in this thesis show that down-regulation of Bcl-2 protein levels coincide with treatment of HCT-116 *K-Ras* Mt. rather than Wt. whereas up-regulation of this protein will lead to proliferation of cells (Younis et al. 2019). Earlier work showed that treatment of liver tissue HCC with mebendazole induced apoptosis, as presented through increased hepatic expression level of caspase -3 and caspase -9 and decreased hepatic level of Bcl-2 (Younis et al. 2019).

MBZ down-regulated the expression levels of numerous genes mRNAs Akt1, Akt2 and Akt3 and proteins such as Erk1, Erk2, p90rsk1 and up-regulate Akt in HCT-116 *K-Ras* Mt. and Wt. at 0.5 µM of MBZ as well as exposed the similar effect on migration of human isogenic pairs CRC cell lines at dissimilar of HCT-116 *K-Ras* Mt. and Wt. DLD-1*K-Ras* Mt. and DKO-3 Wt. and HCT-116 *Pten -/-* Mt. and Wt. were repressed in a dose-dependent manner of MBZ more significantly on Mt. than on Wt.

Furthermore, MBZ inhibited invasion of colorectal cell lines such HCT-116 K-*Ras* Mt. and Wt. A study of colony formation revealed a similar effect on HCT-116 K-*Ras* Mt. and Wt., DLD-1*K-Ras* Mt. and DKO-3 Wt. and HCT-116 *Pten -/-* Mt. and Wt.

The results in this study on CRC cell lines mimicked previous study finding which were the first *in vitro* investigation which display the antitumor and anti-angiogenetic effects of MBZ were conducted on different type of cancer cell lines (Mukhopadhyay et al., 2002).

Tumour spread could be restricted by inhibiting Ran, which is understood to be involved in this process. Cell migration is a major tumour spread phase, which is triggered by cell polarisation and membrane protrusion development at the leading cell margin (Busch et al. 2002; Wang et al. 2005). Tumor invasion and metastatic potential is linked to cell migration (Russo et al.2018). This work on isogenic pairs of CRC cell migration has shown that MBZ inhibited mutant CRC cells migration when compared to Wt. cells that were treated with the same drug concentration. Moreover, when isogenic pairs CRC cells were treated with DMSO, migration was not affected. These findings were confirmed with multiple scratch assays.

The data presented here show that the isogenic pairs of colorectal human cancer cell line colony forming assay which is as a useful way to assess the effect of Ran inhibitor drugs against CRCs. There were consistent similarities in colonies

developed in isogenic pairs of colorectal cell lines, studies have demonstrated that the aggressively tumorigenic MDA-MB-231 are capable of robust colony formation (Korah et al. 2000; Zhang et al. 2002).

In the current study, the basic outcomes consist of the significant ability of MBZ in hindering phosphorylation of Erk and then decreasing expression gene such as VEGFR1/2 which is consistent with a previous study (Younis et al.,2019).

Similarly, Ras/Erk plays an essential role in programmed cell death through phosphorylating a variety of apoptosis-regulating factors and this study discovered that MBZ exhibited apoptosis in HCT-116 K-*Ras* Mt. and Wt. in a different way, as shown through augmented caspase -3, caspase -7 and caspase -9 levels and reduced Bcl-2 levels which is consistent with a previous study (Younis et al.,2019).

In conclusion, the body of work presented in this thesis demonstrates for the first time the effects of mebendazole on isogenic colorectal cell lines: HCT-116 K-*Ras* DLD-1 *K-Ras* Mt., HCT-116 *Pten -/-* and DKO-3 and HCT-116 *K-Ras* Wt. The inhibition of the migration, invasion and colony formation of CRC cell lines were shown using scratch, transwell Boyden chamber and colony formation assays. In addition, this study has evaluated the effects of mebendazole on levels of protein and mRNAs of HCT-116 K-*Ras* Mt. and HCT-116 K-*Ras* Wt. cells showing significant alterations suggesting that mebendazole has multicellular targets. Thus, the results in this study provide a strong proof that mebendazole can be used for clinical therapy to stop CRC invasion and metastasis.

6.2 Future work

6.2.1 Knockout studies in HCT-116 K-*Ras* Mt. and Wt. colorectal cell lines using Ran GTPase shRNA

A previous study showed that Ran silencing leads to initiation of apoptosis in cancer cell lines so it may be worthwhile to compare silencing Ran with shRNA to the effect of Ran inhibitor MBZ.

Silencing Ran results in significantly higher cell death in cancer cell lines such as breast (MDA-MB-231), lung (A549), prostate (DU-145), esophageal (EC109), and colon HCT-116 and DLD-1 (Yuen et al. 2012). Silence Ran in HCT-116 K-*Ras* Mt. and Wt. and test if the effects observed using mebendazole are via Ran mediated signaling pathway could be useful.

6.2.2 DNA fragmentation

To estimate if the inhibitory result of MBZ on the *in vitro* growth of HCT-116 K-*Ras* Mt. and Wt. cells is due to apoptosis, investigation of DNA fragmentation using electrophoresis could prove useful. To check if there are specific DNA cleavages a COMET assay can be used to compare Ran shRNA and mebendazole effects. In a previous study several apoptotic gene family proteins were analysed employing a western blot (Martarelli et al. 2008) and it will be worthwhile to look for these after treatments with mebendazole compared to shRNA mediated effects to seek specificity of the drug mediated effects.

6.2.3 Combination therapy

As mebendazole has shown effectiveness in preventing invasion and metastasis in HCT-116 K-*Ras* Mt. and Wt. cells the next steps are to test the IC_{50} of the existing anti-cancer drugs in combination with mebendazole. There is a possibility to achieve synergistic effect and in best case scenario the amount of drug used can be reduced, which reduces the side effects of the anticancer therapy. This can be achieved by performing MTT assays with various marketed drugs and choose a concentration to test in animal studies. Mebendazole is a safe drug and can be quickly repurposed if the preclinical results are promising.

6.2.4 Multicellular tumour spheroid model (MCTS)

Multicellular tumour spheroid (MCTS) model indicates the 3D cellular framework and therapeutically applicable pathophysiological gradients of *in vivo* tumours in contrast to classical monolayer-based models (Yu et al. 2021). Despite the convenience of a number of methods to examine the characteristic of the solid tumours microenvironment, involving of biopsy from patients, animal models, and 2D *in vitro* cell cultures, none accurately replicate *in vivo* cancer biology (Yu et al. 2021). The difficult micro-environments of a malignant tumour situation such as hypoxia or nutrient deficiency can be synthetically produced in cultures though the characteristic regional model of tumours will always be inadequate nevertheless (Friedrich et al. 2007; Smith et al. 2021). This allows a significant variation among *in vitro* cultures and *in vivo* tumours that can be partly simulated using 3D cell cultures (Nagelkerke et al. 2013). MCTS models have been applied to research CRC cancer biology. Expanding cell lines as 3D spheroids represents a solid tumour involving oxygen and nutrient diffusion better than monolayer cultures (Yu et al. 2021). Though, as there is no blood supply in spheroids *in vivo* preclinical models are still essential to accomplish an improved knowledge of how efficient the medicinal agents are in decreasing progression of cancer. Nevertheless, 3D spheroid culture is an *in vitro* technique that is better simulates the *in vivo* situation and improves cell created preclinical assessment of medications (Nagelkerke et al. 2013).

A future objective would be to create a 3D MCTS model from diverse colorectal cancer mutation cells such as HCT-116 K-*Ras* Mt. and Wt. as well as HCT-116 *Pten* -/- and optimise the method to establish these spheroids.

6.2.5 Preclinical animal study

MBZ is clinically used to treat a number of parasitic worm infestations (Pinto et al. 2015). Benefits include its satisfactory safety profile (i.e., not toxic) and its low cost. Given it has been well established that MBZ has anti-cancer activity and inhibits Ran, it may be repurposed for the treatments of colorectal cancer. As a first step this would require preclinical studies as proof of concept to establish if MBZ has any effect on Ran-GTPase and signalling (Ras/Akt/MAPK pathways) *in vivo*. It was previously published and reported that MBZ has shown anti-cancer activity (El-Tanani 2017). In addition, a previous study showed MBZ inhibited

Ran. Therefore, it may be repurposed for the treatment of colorectal cancer if promising results are achieved in preclinical studies.

Chapter 7

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