BZD9L1: ELUCIDATION OF ITS ANTI-ANGIOGENIC POTENTIAL IN *IN-VITRO* AND *IN-VIVO* COLORECTAL CANCER MODELS

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by

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5-FU	5- Fluorouracil
αvβ3	Alpha-v beta-3
ANG-2	Angiopoietin-2
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
bFGF	Basic fibroblast growth factor
CRC	Colorectal Cancer
FGF	Fibroblast Growth Factors
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HB-EGF	Heparin-binding epidermal growth factor
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factors
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cells
IL-6	Interleukin 6
mCD31	Murine cluster of differentiation 31
mCD34	Murine cluster of differentiation 34
MMP	Matrix metalloproteinase
MNCR	Malaysia National Cancer Registry
mRNA	Micro RNA
MySCan	Malaysian Study on Cancer Survival
NAD+	Nicotinamide adenine dinucleotide
PDGF-BB	Platelet-derived growth factor-BB
PI	Propidium Iodide
PlGF	Placental growth factor
RS	Relative survival
ROS	Reactive oxygen species
SIRT	Sirtuin

SOD 2	Superoxide dismutase 2
STAT 3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

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BZD9L1: PENJELASAN POTENSI ANTI-ANGIOGENESIS DALAM MODEL *IN-VITRO* DAN *IN-VIVO* KANSER KOLOREKTAL

ABSTRAK

Kanser kolorektal adalah kanser ketiga paling kerap di seluruh dunia. Kanser kolorektal bergantung pada angiogenesi untuk pertumbuhan dan metastasis. Pelbagai usaha telah dilakukan untuk menyasar laluan angiogenik secara selektif untuk menghalang pertumbuhan tumor. Walau bagaimanapun, sesetengah pesakit kanser kolorektal menunjukkan rintangan terhadap ubat anti-angiogenik dan rawatan standard. Keluarga protein sirtuin (SIRTs) histon deacetylase kelas III ditemui berkait rapat dengan kemajuan kanser tetapi tidak banyak yang diketahui tentang aktivitinya dalam mengawal angiogenesis tumor. Rawatan BZD9L1 dikenali sebagai perencat sirtuin yang novel dengan aktiviti anti-kanser yang terbukti. Kajian ini bertujuan untuk menyiasat potensi anti-angiogenik BZD9L1 terhadap sel endotelial EA.hy926 secara *in vitro* dan melalui model xenograft tumor tikus bogel secara *in vivo*. Eksperimen *in vitro* terdiri daripada ujian daya kehidupan sel, ujian penghijrahan sel, ujian pembentukan tiub, ujian pembiakan saluran sferoid sel, ujian pemblotting barat, ujian sitokin angiogenesi, analisis kitaran sel dan apoptosis sel melalui ujian sitometri aliran dan ujian kultur sel bersama secara tidak langsung. Model xenograf tumor tikus bogel digunakan untuk kajian *in vivo*, di mana pewarnaan hematoksilin dan eosin dilakukan untuk mengkaji peratusan nekrosis dan ujian imunohistologi terhadap seksyen tisu telah dijalankan untuk menyiasat ekspresi protein Ki67 dan CD34. BZD9L1 ditemui mengurangkan daya kehidupan sel, penghijrahan sel, pembentukan tiub, dan pembiakan saluran sferoid sel. BZD9L1 pada dos 10μM didapati menghalang ekspresi protein SIRT2 dan SIRT3 dalam sel

EA.hy926. Ujian sitokin angiogenesi membuktikan bahawa BZD9L1 boleh mengurangkan ekspresi sitokin Angiogenin, bFGF, PDGF-BB, dan PIGF berbanding kumpulan kawalan. Keputusan analisis sitometri aliran menunjukkan bahawa rawatan BZD9L1 menyekat kitaran sel pada fasa G1 dan apoptosis awal dalam sel endotelial. Rawatan BZD9L1 ke atas ko-kultur sferoid HCT116 menghalang invasi dalam sferoid yang dirawat dengan medium berkondisi secara signifikan (P * <0.05) dalam semua kumpulan rawatan. Di samping itu, pengurangan ketara (P * < 0.05) dalam pertumbuhan tumor telah diperolehi daripada eksperimen model xenograf tumor tikus bogel. Kajian histologi membuktikan bahawa rawatan BZD9L1 boleh mengurangkan nekrosis dan ekspresi protein Ki-67 dengan ketara (P * < 0.05) dalam kumpulan yang dirawat dengan dos tinggi (250mg/kg). Bagaimanapun, analisis imunohistokimia CD34 tidak dapat memberikan kesimpulan yang pasti disebabkan oleh nekrosis yang bertindih dengan ekspresi jelas protein CD34. Kajian ekspresi gen CD34 melalui tindak balas berantai polymerase masa nyata mewakili alternatif untuk mengesahkan potensi anti-angiogenesis BZD9L1. Kesimpulannya, kajian ini telah membuktikan keupayaan BZD9L1 untuk menghalang pertumbuhan sel endotelial EA.hy926 *in vitro* dan menghalang perkembangan tumor CRC *in vivo*.

BZD9L1: ELUCIDATION OF ITS ANTI-ANGIOGENIC POTENTIAL IN *IN-VITRO* AND *IN-VIVO* COLORECTAL CANCER MODELS

ABSTRACT

Colorectal cancer (CRC) is the third most common cancer globally. CRC depends largely on angiogenesis for growth and metastasis. Much effort has been made to selectively target the angiogenic pathways to restrain tumour growth. However, some CRC patients become resilient to these anti-angiogenic drugs and standard therapies. The class III histone deacetylase family of sirtuins (SIRTs) has been closely linked to cancer progression but less is known about its activity in regulating tumour angiogenesis. BZD9L1 is a novel sirtuin inhibitor with demonstrated anti-cancer activities. This study aimed to investigate the anti-angiogenic potential of BZD9L1 on EA.hy926 endothelial cells (EC) *in vitro* and HCT116 tumour xenograft nude mice. The *in vitro* experiments comprised of cell viability assay, scratch wound assay, tube formation assay, spheroid sprouting assay, western blotting, angiogenesis array, cell cycle and apoptosis analysis via flow cytometry and finally, indirect co-culture model. Nude mice tumour xenograft model was used for the *in vivo* study, where hematoxylin and eosin staining was done to study the percentage of necrosis in the tumour section and immunohistochemistry was conducted to investigate the protein expression of Ki67 and CD34. BZD9L1 was shown to reduce cell viability, cell migration, tube formation, and spheroid sprouting of EC. BZD9L1 at 10μM was also shown to inhibit SIRT2 and SIRT3 protein in EA.hy926 cells. Angiogenesis array results revealed that the compound reduces the cytokine concentration of Angiogenin, bFGF, PDGF-BB, and PIGF significantly ($P^* < 0.05$) compared to the control group. The compound caused cell cycle arrest at

the G1 phase and early apoptosis in EC from flow cytometry analysis. BZD9L1 treatment on the HCT116 spheroid indirect co-culture significantly ($P * < 0.05$) impeded invasion in spheroid treated with conditioned medium from all treatment groups. In addition, a significant ($P * < 0.05$) reduction in tumour growth was obtained in the *in vivo* nude mice xenograft models. Histology studies revealed that the compound reduced necrosis and Ki67 protein expression significantly ($P *< 0.05$) in high dose (250mg/kg) treated group. However, immunohistochemical analysis CD34 was inconclusive due to the presence of necrosis that overlaps the clear expression of the CD34 protein. CD34 gene expression study via quantitative real-time polymerase chain reaction represents the alternative to confirm the anti-angiogenic potential of BZD9L1. In conclusion, this study has demonstrated the ability of BZD9L1 to inhibit the growth of EA.hy926 endothelial cells *in vitro* and impede the development of CRC tumours *in vivo*.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer is a disease characterized by the unchecked division and survival of abnormal cells. Colorectal cancer occurs when abnormal development appears in the colon or rectum (CRC). A prominent cause of death, particularly in Western nations, colorectal cancer (CRC) has risen to the third-most prevalent cancer globally (Hassan et al., 2016). The Malaysian National Cancer Patient Registry's second annual report on colorectal cancer states that between 2008 and 2013, there were 21.3 instances of the disease per 100,000 people in Malaysia. Chinese people had the highest risk of colorectal cancer per 100,000 people overall (27.4 cases per 100,00 population). The colorectal cancer mortality rate was 9.8 incidences per 100,000 people from 2008 to 2013. Similarly, males had 1.42 times higher age-adjusted death rate for colorectal cancer than females. (Hassan et al., 2014).

According to the World Health Organization's (WHO) GLOBOCAN 2020 report, CRC was the second most prevalent cancer in women (9.4%) after breast cancer and the third most prevalent disease overall among males (10.6). With age, both the incidence and mortality rates for CRC significantly rise. CRC is second in terms of mortality but third in terms of incidence. The incidence rates have been growing progressively in South Eastern and South Central Asia and South America (Xi and Xu, 2021). The malady risk variables include diet, family ancestry, advancing age, smoking and obesity. In addition, low-fibre, red meat and high-fat diet, genetics and old age may contribute to the increased risk for CRC (Lewandowska et al., 2022).

The growth and spread of colorectal cancer are highly dependent on angiogenesis. Clinical trials for the treatment of CRC are now being conducted using

a variety of angiogenesis inhibitors that target tyrosine kinases and vascular endothelial growth factor (VEGF) signalling, thanks to advancements in therapeutic care for CRC patients. Epigenetics is a wide range of heritable changes that do not require primary DNA sequence modification. The regulation of pro- or anti-angiogenic molecules could affect tumour growth. Epigenetic regulation of the genes in endothelial cells in the vicinity of tumour cells plays a vital role in tumour angiogenesis. Methylation of DNA, modification of histone and silencing of micro-RNA (miRNA)-associated genes are currently considered to initiate and sustain epigenetic changes (V.Subramaniam et al., 2019).

SIRT1-7 are deacetylase and ADP ribosylase active proteins that play a variety of roles in cellular functions such as metabolism, stress response, and ageing. Sirtuins are class III histone deacetylase enzymes that require NAD+ as a cosubstrate for their enzymatic activity. Twenty years ago, sirtuins gained notoriety for their alleged potential in the treatment of ageing. Sirtuins have been extensively researched since then. Sirtuins have also been discovered to play important roles in neurodegenerative and cancerous illnesses. This has encouraged researchers to look for effective and selective SIRT inhibitors, which may result in a novel medicinal discovery (Lee et al., 2021).

Their potential roles in cancer, metabolic and neurodegenerative diseases have stimulated investigation to seek potent and selective SIRT inhibitors which could potentially lead to a new therapeutic breakthrough. The only highly fluorescent sirtuin inhibitor known thus far, BZD9L1, is a powerful sirtuin inhibitor that was very recently found. The findings showed that BZD9L1 can be viewed by fluorescence microscopy in living cells without any conjugation to external fluorophores. The substance is readily adsorbed by cells, where it is primarily found

in the cytoplasm. BZD9L1 has demonstrated potent antiproliferative effects in a variety of cancer cell lines, including HCT116, CCRF-CEM, and MDA-MB-468, making it an intriguing possibility for cancer treatment (Y. K. et al., 2015).

The prognosis and survival rate of a patient remains heterogeneous in which tumour attributes, dynamic host response factors, and quality of treatment may be accountable. Current chemotherapy regimens eliminate cancer cells by unselectively inducing cell death in neoplastic cells and normal cells, leading to severe short-term and long-term side effects. Some CRC patients become resilient to these anti-angiogenic drugs and standard therapies such as chemotherapy and radiation (Van der Jeught et al., 2018).

There is a need to look for alternative targeted therapy that could benefit patients. Targeted therapies are gaining increased recognition, as they have been shown to increase patient overall survival rates and keeping cancer at bay by disrupting molecular pathways essential for cancer growth. BZD9L1 targets SIRT1/2 that are highly expressed in colon cancer without destroying healthy surrounding cells (Tan et al., 2018). It can also act as an adjunct with chemo to improve therapeutic outcome and potentially improve survival rates (Tan et al., 2019).

Hence, we hypothesize that BZD9L1 is able to inhibit sirtuin and negatively regulate angiogenesis leading to tumour growth retardation and this work opens a new avenue for the establishment of a potential novel anti-angiogenic agent through sirtuin inhibition in tumour angiogenesis.

1.2 Objective

This project overall aims to study the potential of BZD9L1 in negatively regulating angiogenesis in colorectal cancer (CRC) *in vitro* and *in vivo* based on the following objectives:

1. To determine the effects of BZD9L1 in *in vitro* models
    a. To determine the mode of action of BZD9L1 on endothelial cells
    b. To elucidate the molecular mechanisms of BZD9L1 on endothelial cells
    c. To investigate the effect of BZD9L1 on CRC and endothelial cells using a co-culture model
2. To determine the effects of BZD9L1 in *in vivo* models
    a. To determine the effect of BZD9L1 on angiogenesis in CRC tumour xenograft model

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Tumour masses discovered in prehistoric human and reptile bones are the earliest reliable indications of malignancy in mammals (Faguet, 2015; Rothschild et al., 2003). Despite the absence of the word "cancer," the earliest description of the illness was discovered in Egypt in the 19th century. The George Ebers and Edwin Smith Papyrus is a recreation of a passage from a trauma surgery textbook written in ancient Egypt. It describes 8 instances of surgically excised breast tumours or ulcers with the fire drill, a cautery tool. These ancient Egyptian writings described cancer as an incurable disease and linked it to "the curse of the gods"(Di Lonardo et al., 2015). Cancer can develop practically anywhere in the millions of cells that make up the human body. Human cells frequently divide to produce new cells as needed by the body (a procedure known as cell proliferation and multiplication). In addition, as damaged or old cells die, new ones take their place. This step can occasionally go wrong, resulting in damaged or abnormal cells proliferating when they shouldn't. These cells can grow into tumours, which are tissue lumps. Tumours could be cancerous or benign.

The descriptions of the characteristics of cancer by Hanahan and Weinberg have been essential to our comprehension of these traits as well as to the rational development of efficient treatments. The hallmarks of cancer, an influential review written by Hanahan and Weinberg in 2000, sought to categorize the complicated biology of cancer into six main hallmarks (Hanahan and Weinberg, 2000). They included two developing hallmarks reprogramming energy metabolism and evading immune response as well as two enabling characteristics genome instability and

mutation and tumour-promoting inflammation in an updated evaluation published ten years later (Hanahan and Weinberg, 2011). Now ten years after, again Hanahan has added cellular plasticity as a new hallmark of cancer and non-mutational epigenetic reprogramming and polymorphic variations in organ/tissue microbiomes as 2 new enabling characteristics. Additionally, senescent cells of varying origins may be added to the roster of functionally important cell types in the tumour microenvironment (Hanahan, 2022).

Among the first six hallmarks, inducing or accessing vasculature has been a hot topic of interest among researchers due to the tumour's innate ability to commandeer blood vessels to promote tumour development and trigger metastasis. In the latest version of the hallmarks of cancer series, Hanahan added non-mutational epigenetic reprogramming which is the topic of interest for this study where we study epigenetic regulation and its effects on the tumour microenvironment (Hanahan, 2022). The addition of epigenetic regulation as part of the hallmarks of cancer has shone the spotlight on our research.

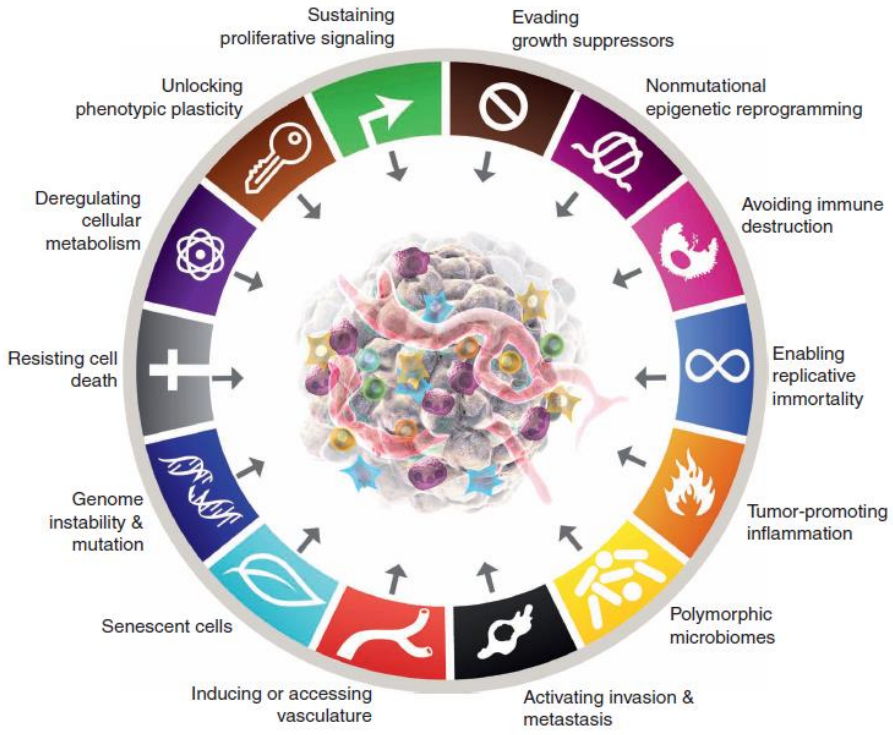

Figure 2.1 Hallmarks of Cancer. To date, a total of ten hallmarks and four enabling characteristics have been identified to drive the progression of cancer. Source: Hanahan, 2022

2.2 Colorectal Cancer

Colorectal cancer (CRC) describes the phenomenon of uncontrolled cell growth that starts in the colon or part of the large intestines. The third most common cancer in the world, colorectal cancer (CRC) is also the second most common cancer in females and the third most common cancer in males. CRC ranks third in incidence, but second in mortality, which increases with age (Xi and Xu, 2021). According to data from the Malaysia National Cancer Registry (MNCR) 2012-2016, colorectal cancer is the most common cancer in males and the second most common disease in women in Malaysia, after breast cancer (AM et al., 2019; Abdullah et al., 2021). The overall 5-year relative survival (RS) for colorectal cancer was 51.1%, as per the Malaysian Study on Cancer Survival (MySCan). Stage I has the highest RS when compared to stage III and stage IV. One of the malignancies that are susceptible to

early detection through screening and diagnosis is colorectal cancer. Nevertheless, more than 70% of CRC cancer patients in Malaysia had stage III or IV illness at the time of diagnosis. (Abdullah et al., 2021).

Recent progress in the treatment of CRC has resulted from targeting the angiogenesis and the tumour microenvironment. Metastatic CRC patients' survival is dramatically increased by anti-angiogenic therapy, such as bevacizumab, a monoclonal antibody against vascular endothelial growth factor. These therapies do not, however, entirely cure the disease, and a significant portion of patient tumours still exhibit chemoresistance or recurrence (Mathonnet et al., 2014).

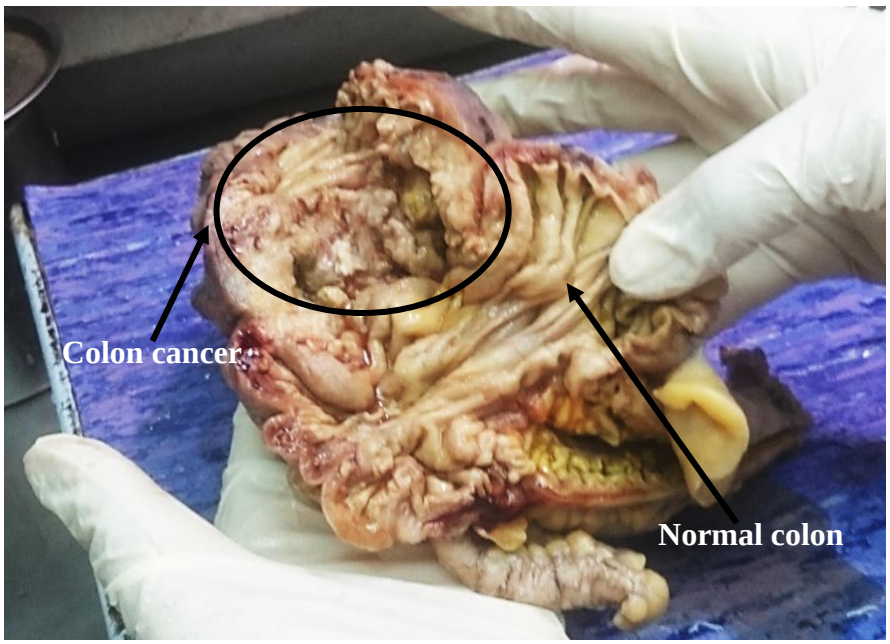

Figure 2.2 Image of colorectal cancer pathology sample. This image displays the phenotypic difference between the mucosa lining of normal and malignant parts of the colon. Source: Subramaniam and Oon, 2020

2.3 Tumour Angiogenesis

The development of new blood vessels from pre-existing vasculature is called angiogenesis. The primary mechanism behind tumour development and metastasis, angiogenesis, has been the focus of extensive research. Neovascularization is

fundamentally a four-step process, including tumour angiogenesis. The first step involves the enzymatic degradation of the vessel in the basement membrane and the stimulation of endothelial cells by pro-angiogenic factors. Secondly, the migration of endothelial cells towards environmental cues. Third, endothelial cell proliferation and tube formation and finally, the maturation of new blood vessels stabilized by mural cells. (Adair and Montani, 2010; Nishida et al., 2006; V.Subramaniam et al., 2019). The equivalence of pro- and anti-angiogenic factors governs the growth of new blood vessels, and the angiogenic switch is triggered when the pro-angiogenic stimulus is greater than the anti-angiogenic resistance. Growth factors are released by a hypoxic tumour microenvironment, which encourages vascular endothelial cells to proliferate and migrate (Ashwaq et al., 2018; Ribatti, 2013; Secord and Siamakpour-Reihani, 2017).

Variations in oxygen tension may activate several pathways, producing a variety of transcriptional factors in hypoxic conditions. Hypoxia is known to upregulate pro-angiogenic inducers leading to enhanced, rapid and chaotic blood vessel formation. Hypoxia-inducible factors (HIFs) are heterodimers made up of an oxygen-dependent (HIF-α) and an oxygen-independent (HIF-β) -subunit. HIFs have been known as master regulators of angiogenesis, as it upregulates pro-angiogenic genes including VEGF, FGF and PDGF(Abou Khouzam et al., 2021; Muz et al., 2015; Pinto et al., 2010). In addition to hypoxia, the canonical angiogenesis initiator VEGF has been discovered to express itself in most cancer types in response to various stimuli. PDGF, EGF, TGF-β, FGF, MMPs, chemokines, ephrins, apelin, VEGF and angiopoietins are additional pro-angiogenic factors that are increased for the activation of tumour angiogenesis (Lugano et al., 2020; Zuazo-Gaztelu and Casanovas, 2018).

Thus, targeting the proteins or mediators involved in increasing angiogenesis has created a fantastic foundation for future cancer treatment.

2.4 Cytokines involved in angiogenesis.

Cytokines play a crucial role in angiogenesis. Key cytokines include Angiogenin, Angiopoietin-2, EGF, bFGF, HB-EGF, HGF, Leptin, PDGF-BB, PLGF, and VEGF-A. These cytokines act as potent stimulators of endothelial cell proliferation, migration, and tube formation, facilitating the growth of new blood vessels.

2.4.1 Angiogenin

Angiogenin, a strong angiogenic factor, promotes the growth of new blood vessels from pre-existing ones. It is a member of the ribonuclease superfamily and has angiogenic and ribonucleolytic properties (Tello-Montoliu et al., 2006). Angiogenin induces endothelial cell proliferation, migration, and capillary tube formation, making it a key regulator of angiogenesis (Kishimoto et al., 2005).

Overexpression of ANG was found to boost the expression of important angiogenic factors, including MMP2, to enhance host angiogenesis and metastatic potential (Miyake et al., 2015). Genetic and pharmacological disruption of angiogenin angiogenesis in the gastrointestinal tract (Zhang et al., 2013).

2.4.2 Angiopoietin-2 (Ang-2)

Another significant cytokine implicated in angiogenesis is angiopoietin-2. It controls blood vessel maturation and stability in conjunction with angiopoietin-1. During angiogenesis, Ang-2 destabilises the blood vessels, allowing for sprouting and remodelling. (Scholz et al., 2015).

Several studies have shed light on the significance of Ang-2 in tumor angiogenesis and metastasis. One review paper examined the functions of VEGF and Angiopoietins in tumour angiogenesis and metastasis, highlighting the crucial part Ang-2 plays in tumour neovascularization (Saharinen et al., 2011). The function of Ang-2 in adaptive tumour resistance to VEGF signalling inhibition was the subject of a separate investigation. The study's findings revealed that Ang-2 was crucial in encouraging tumour resistance and reducing the effectiveness of VEGF-targeted treatments (Rigamonti et al., 2014).

2.4.3 Epidermal Growth Factor (EGF)

Various biological functions, including angiogenesis, depend on the cytokine known as epidermal growth factor (EGF). Strong growth factors, like EGF, encourage the division, migration, and proliferation of cells. EGF is important in angiogenesis because it encourages endothelial cell migration and proliferation, which helps the growth of new blood vessels (van Cruijsen et al., 2005).

One study investigated the mechanisms of EGF-induced apoptosis and cell migration in anaplastic thyroid cancer cells. The researchers found that EGF signaling contributed to both apoptosis and cell migration, suggesting its dual role in angiogenesis and cancer progression (Mincione et al., 2011). In a different study, the involvement of EGF-induced mechanotransduction in endothelial cell migration was investigated. This study shed light on the biological mechanisms underpinning EGF-mediated angiogenesis (Li et al., 2005).

2.4.4 Basic Fibroblast Growth Factor (bFGF)

Basic Fibroblast Growth Factor (bFGF) is a potent angiogenic cytokine that plays a critical role in promoting angiogenesis. It stimulates endothelial cell

proliferation, migration, and capillary tube formation, which are essential steps in the process of new blood vessel formation (Nakamichi et al., 2016).

bFGF has been reported to modulate EGF-induced signalling and cell motility in prostate cancer cells (Gan et al., 2010). Additionally, the role of bFGF in inducing angiogenesis was explored *in vivo* using an animal model. The study demonstrated that bFGF, in combination with endothelial progenitor cells, significantly promoted angiogenesis, offering therapeutic potential for vascular regeneration (Bastaki et al., 1997).

2.4.5 Heparin-Binding Epidermal Growth Factor (HB-EGF)

Heparin-Binding Epidermal Growth Factor (HB-EGF) is a member of the EGF family and is involved in various cellular processes, including angiogenesis. HB-EGF has been shown to stimulate endothelial cell proliferation, migration, and capillary tube formation (Mehta and Besner, 2007).

In one study, the expression and function of HB-EGF in human malignant gliomas were examined. HB-EGF was significantly expressed in gliomas and was involved in promoting mitogenic signalling suggests that it may be a promising therapeutic target for glioma angiogenesis (Shin et al., 2017).

2.4.6 Hepatocyte Growth Factor (HGF)

Hepatocyte Growth Factor (HGF) is a potent angiogenic cytokine that plays a crucial role in promoting angiogenesis. HGF stimulates endothelial cell proliferation, migration, and the formation of capillary-like tubes, which are essential steps in the process of new blood vessel formation (Rosen et al., 1997).

According to one study that looked at the role of HGF as a lymphangiogenic factor, HGF caused lymphangiogenesis via an indirect process involving the activation of VEGF-C (Cao et al., 2006).

2.4.7 Leptin

Leptin, primarily known for its role in appetite regulation and energy homeostasis, has also been identified as a cytokine that promotes angiogenesis. Leptin can induce endothelial cell proliferation and stimulate the production of other angiogenic factors, making it an important player in angiogenesis-associated processes.

In one study, researchers demonstrated that leptin may activate the Akt and Wnt signalling pathways, whereas siRNAs that inhibited the Akt and Wnt signalling pathways inhibited the leptin-induced angiogenesis of endothelial cell lines. EA.hy926 (Yu et al., 2019). Another study investigated the pro-angiogenic role of leptin in endothelial cells and its activation of the PI3K/Akt/eNOS signaling pathway (Goetze et al., 2002).

2.4.8 Platelet-Derived Growth Factor-BB (PDGF-BB)

Platelet-Derived Growth Factor-BB (PDGF-BB) is a potent angiogenic factor that plays a critical role in promoting angiogenesis. It induces endothelial cell migration and proliferation, essential processes in the formation of new blood vessels (Raica and Cimpean, 2010).

One study focused on the prognostic value of PDGF-BB expression in resected non-small cell lung cancer. The results showed that PDGF-BB expression was associated with prognosis, indicating its potential as a prognostic biomarker in lung cancer (Zhang et al., 2014).

2.4.9 Placental Growth Factor (PlGF)

Placental Growth Factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family and is known to play a critical role in promoting angiogenesis. PlGF binds to VEGF receptor-1 (VEGFR-1), stimulating endothelial

cell proliferation and migration. It works in synergy with other VEGF family members to regulate vascular development and angiogenesis in cancer (De Falco, 2012).

In vitro human umbilical vein endothelial cells (HUVECs) were used in a study to examine the pro-angiogenic effects of PlGF. PlGF and hypoxia inducible factor (HIF) increased angiogenesis in *in vivo* rat models (Zhou et al., 2019).

2.4.10 Vascular Endothelial Growth Factor-A (VEGF-A)

One of the most potent and well-researched angiogenic agents is vascular endothelial growth factor-A (VEGF-A). It is an essential regulator of angiogenesis and a member of the VEGF family. The proliferation, migration, and tube creation of endothelial cells are stimulated by VEGF-A, which aids in the development of new blood vessels from pre-existing ones (Shibuya, 2011).

The relationship between a certain VEGF-A gene polymorphism and vulnerability to esophageal cancer was the subject of a meta-analysis study. The researchers came to the conclusion that this polymorphism may increase the risk of esophageal cancer, emphasising the significance of VEGF-A in tumour angiogenesis (Bradbury et al., 2009; Wang et al., 2023). A different study shows that VEGFA stimulates Sox2, increasing EMT and tumour spread in breast cancer. Through the activation of Slug and the suppression of miR-452, a metastasis suppressor that targets SNAI2 and enhances cell invasion, VEGFA increases cell invasion. These results point to a unique method by which cancer stem cells acquire the ability to spread by upregulating Sox2 through VEGFA, miR-452 loss, and overexpression of Slug (Kim et al., 2017).

2.5 Angiogenesis models

Determining the mechanisms involved in angiogenesis, the formation of new blood vessels from pre-existing ones, is crucial for understanding its role in various physiological and pathological processes. In recent years, several *in vitro* and *in vivo* assays have been developed to investigate the complex mechanisms underlying angiogenesis.

2.5.1 *In vitro* assays

In vitro assays play a crucial role in studying the mechanisms involved in angiogenesis by utilizing isolated cells or tissues cultured under controlled conditions(Goodwin, 2007). These assays allow researchers to manipulate various factors and observe their effects on endothelial cells. The following are commonly used *in vitro* assays for studying angiogenesis:

1. Proliferation assays: These assays assess the ability of endothelial cells to divide and proliferate, which is the key in formation of blood vessels (Arnaoutova and Kleinman, 2010).
2. Migration assays: These assays measure the migratory capacity of endothelial cells towards specific stimuli, aiding in understanding the mechanisms underlying endothelial cell movement during angiogenesis (Pijuan et al., 2019)
3. Tube formation assays: These assays evaluate the ability of endothelial cells to organize into capillary-like structures, providing valuable information about vessel formation (Staton et al., 2009).
4. Spheroid assays: Spheroid assays involve the use of three-dimensional cultures of endothelial cells, mimicking the *in vivo* microenvironment.

These assays facilitate the study of angiogenic sprouting and complex cell-cell interactions (Shah et al., 2019)

2.5.2 *In vivo* assays

In vivo assays involve studying angiogenesis within living organisms or tissues and have provided significant insights into the complex interactions between cells and the extracellular environment (Rahman et al., 2020). The following are commonly used *in vivo* assays for capturing the process of angiogenesis:

1. Zebrafish assays: Zebrafish embryos, due to their transparency and rapid development, are a popular model for studying angiogenesis *in vivo*. These assays allow real-time visualization of blood vessel formation and growth (Chávez et al., 2016).
2. Chick chorioallantoic membrane(CAM) assays: Chick embryos, also transparent, provide an excellent model for studying angiogenesis *in vivo.* These assays enable the observation of blood vessel development and the manipulation of the environment to study angiogenic sprouting (Ribatti et al., 2020).
3. Mouse model assays: Mouse models offer a wide range of options for studying different aspects of angiogenesis, including vessel formation, remodeling, and maturation. These assays contribute to a comprehensive understanding of angiogenesis in a mammalian model (Eklund et al., 2013).

2.5.3 *Ex vivo* assays

Ex vivo assays involve the culture of tissues or organs outside the body to study angiogenesis. The following are commonly used ex vivo assays:

1. Aortic ring assays: Aortic rings, obtained from animals, are embedded in a gel matrix. These assays enable the observation of new blood vessel sprouting from the aortic rings, providing insights into angiogenesis (Kapoor et al., 2020).
2. Corneal assays: The cornea, a transparent and easily accessible tissue, is commonly used to study angiogenesis. These assays allow the observation of new blood vessel growth into the cornea following the induction of angiogenic factors (Nowak-Sliwinska et al., 2018).
3. Metatarsal assays: The development of new blood vessels (angiogenesis) in the growing metatarsal bones of embryos is studied using a laboratory technique called the metatarsal angiogenesis assay. It entails isolating and cultivating these bones to watch how blood vessels develop spontaneously. This assay is more accurate at simulating sprouting angiogenesis *in vivo* and aids researchers in their understanding of the intricate process of angiogenesis (Song et al., 2015).

2.5.4 Co-culture assays

Co-culture assays are valuable tools used to study angiogenesis by investigating the interactions between endothelial cells and other cell types within a controlled experimental setup (Li et al., 2018). These assays allow for the examination of paracrine signaling, cell-cell interactions, and the influence of various cell populations on angiogenic processes. The co-culture assays commonly employed in angiogenesis research include:

1. Endothelial Cell-Fibroblast Co-culture Assay:

Co-culturing endothelial cells with fibroblasts, a key stromal cell type, provides insights into the reciprocal paracrine communication between endothelial cells and the surrounding microenvironment during angiogenesis. Fibroblasts can secrete growth factors, extracellular matrix components, and other pro-angiogenic molecules that influence endothelial cell behavior (Truelsen et al., 2021).

2. Endothelial Cell-Pericyte Co-culture Assay:

The interaction between endothelial cells and pericytes is crucial for blood vessel stabilization and maturation. Co-culture of endothelial cells and pericytes allows for the examination of pericyte recruitment, vessel maturation, and the regulation of endothelial cell behavior (Tattersall et al., 2016).

3. Endothelial Cell-Tumor Cell Co-culture Assay:

Co-culture of endothelial cells with tumor cells provides a model to investigate angiogenesis in the context of cancer. Tumor cells can secrete pro-angiogenic factors and induce endothelial cell sprouting and tube formation (Oh et al., 2020).

4. Endothelial Cell-Macrophage Co-culture Assay:

Macrophages play a crucial role in modulating angiogenesis through their ability to secrete pro-angiogenic factors and remodel the extracellular matrix. Co-culture of endothelial cells with macrophages allows for the investigation of the crosstalk between these cell types and their influence on angiogenic processes (Tattersall et al., 2016).

5. Endothelial Cell-Stem Cell Co-culture Assay:

Co-culturing endothelial cells with stem cells, such as mesenchymal stem cells, offers a platform to study the paracrine effects of stem cells on angiogenesis. Stem cells can secrete angiogenic factors and promote endothelial cell proliferation, migration, and tube formation (Liang et al., 2017).

Co-culture assays have emerged as valuable tools in angiogenesis research, offering a more physiologically relevant model to investigate the complex interactions between different cell types involved in angiogenesis (Truelsen et al., 2021). These assays provide several benefits, including the recapitulation of cellular interactions, the ability to mimic the tumor microenvironment, and the examination of paracrine signaling (Jin et al., 2020). By co-culturing endothelial cells with various cell types such as fibroblasts, tumor cells, macrophages, and stem cells, researchers can gain insights into the reciprocal communication and influence of these cell populations on angiogenic processes (Doak et al., 2018; Pape et al., 2020; Kahrizi et al., 2023).

However, co-culture assays also have limitations that should be considered. They involve the simplification of the complex *in vivo* environment, potentially missing certain aspects of the native tissue architecture. The lack of spatial organization within co-culture systems may limit the accurate representation of angiogenesis processes that rely on specific cell positioning (Goers et al., 2014). Additionally, deciphering the specific contributions of individual cell types can be challenging due to the complex and dynamic nature of their interactions (Whiteley et al., 2022) . Furthermore, the use of artificial culture conditions in co-culture assays, including culture media and substrates, may introduce experimental biases, time-consuming and deviate from the natural *in vivo* conditions (Vis et al., 2020).

2.6 Existing anti-angiogenic regimen and therapy for colorectal cancer

Advancement in therapeutic care for CRC patients has led to the identification of various angiogenesis inhibitors targeting the tyrosine kinases and vascular endothelial growth factor (VEGF) signalling being explored in clinical trials for the treatment of CRC. However, the prognosis and survival rate of a patient remains

heterogeneous for which tumour attributes, dynamic host response factors, and quality of treatment may be accountable. Some CRC patients become resilient to these anti-angiogenic drugs and standard therapies. Bevacizumab, aflibercept, ramucirumab, and regorafenib are the four angiogenesis-blocking medications that have received approval from the US Food and Drug Administration for the treatment of metastatic colorectal cancer (Kanat and Ertas, 2019; Xie et al., 2020). Bevacizumab is a monoclonal antibody that is anti-VEGF that was discovered back in 1993 and approved for use against metastatic colon cancer in 2004, however it failed to show clinical significance when used as monotherapy, except in glioblastoma multiform (Boere et al., 2010; Ferrara et al., 2004; Haibe et al., 2020; Mésange et al., 2014). Aflibercept, a potent VEGF pathway targeting agent, as a single agent and when paired with cytotoxic chemotherapy, has been demonstrated to show no significant improvement and overall survival in clinical studies (Chung and Pherwani, 2013; Tannock et al., 2013). It also displayed increased toxicities, hypertension, proteinuria, fatigue, and headache (Patel and Sun, 2013; Syed and McKeage, 2015; Wang and Lockhart, 2012). Additionally, the anti-VEGFR 2 monoclonal antibody, Ramucirumab, was shown to increase VEGF-A protein expression which is essential in angiogenesis progression. Equally, in a clinical study, ramucirumab-treated patients were shown to experience more frequent proteinuria and a decline in life quality (Tiwari, 2016; Verdaguer et al., 2016). Regorafenib is a type II kinase inhibitor which in a previous study reported that inhibition of the VEGF/VEGF-R signalling system increases the aggressiveness of colorectal cancer cells (Tomida et al., 2017). Therefore, there is a need to look for an alternative targeted therapy that could benefit patients who develop resistance to the existing anti-angiogenic and standard regime.

2.7 CRC and Angiogenesis

In CRC, angiogenesis is crucial for tumour growth and is regulated by the proteins hypoxia-inducible factor-1 α (HIF-1α) and vascular endothelial growth factor (VEGF). Overexpression of HIF-1 is linked to tumour aggressivity, invasiveness, and resistance to radiation and chemotherapy in CRC. HIF-1α under hypoxia mediates the expression of VEGF, which is the main pro-angiogenic growth factor (Bałan et al., 2009; Mousa et al., 2015; Yin et al., 2020). According to a prior study, VEGF and IL-6 serum levels are much greater in CRC patients and are associated with the disease's advanced stages and metastatic spread (Mathonnet et al., 2014). The overexpression of VEGF has also been reported to have poorer prognostic among patients (Bałan et al., 2009; Giatromanolaki et al., 2006). Uttarawichien and colleagues previously reported that the suppression of VEGF successfully inhibited cell-induced angiogenesis in CRC (Uttarawichien et al., 2021). Additionally, anti-angiogenic therapies targeting specific pathways that promote angiogenesis have been shown to improve the prognosis of patients with metastatic CRC (Lopez et al., 2019).

The United States Food and Drug Administration (FDA) has approved the use of anti-VEGF medications for the treatment of CRC, including bevacizumab, cediranib, aflibercept, sunitinib malate, regorafenib, 5-fluorouracil, and ramucirumab. These medications have all demonstrated effectiveness in treating CRC (Mousa et al., 2015; Sun, 2012). Current chemotherapy regimens eliminate cancer cells by unselectively inducing cell death in neoplastic cells and even normal cells, leading to severe short-term and long-term side effects (Giatromanolaki et al., 2006; Hansen et al., 2021; Lopez et al., 2019; Pinto et al., 2010; Sun, 2012).

2.8 Epigenetics

Epigenetics is a wide range of mitotically or meiotically heritable changes that do not require primary DNA sequence modification. Chemical modifications imposed on the DNA molecule and histone proteins by environmental signals affect the gene's function and are collectively referred to as epigenetic phenomena. Alterations towards the pro- or anti-angiogenic genes could enhance tumour growth. The growth factors mentioned above have been linked to improving the angiogenic potential of the tumours through epigenetic modulation (V.Subramaniam et al., 2019). Methylation of DNA, modification of histone and silencing of micro-RNA (miRNA)-associated genes are currently considered to initiate and sustain epigenetic changes.

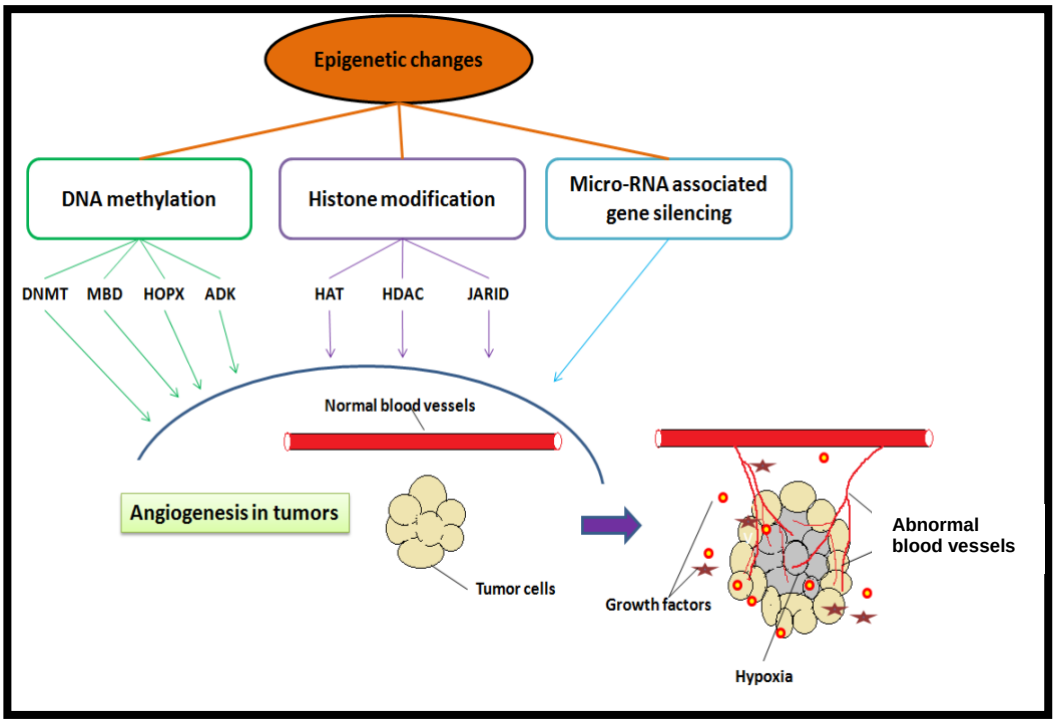

Figure 2.3 Schematic diagram proposing the effect of epigenetic changes on tumour angiogenesis. Changes that occur through DNA methylation, histone modifications and micro-RNA-associated gene silencing have been linked with the sprouting of new blood vessels that impact tumourigenesis and metastasis. Source: V.Subramaniam et al., 2019.

2.8.1 Histone modification

Histone modification involves post-translational modification to histone proteins. Histones package and order the DNA into specific structural units called nucleosomes. The histones, which are unique to eukaryotes, include histone(H) H1, H2A, H2B, H3 and H4. Histone acetylation occurs via HAT, which is essential for gene regulation. HDACs can also deacetylate residues of lysine. There are also two main types of HDACs: SIRT and classical HDAC(Tchio Mantho et al., 2017; V.Subramaniam et al., 2019). There are 18 enzymes known to belong to the HDAC superfamily. They are further divided into four classes: class I, which includes the enzymes HDAC1, HDAC2, HDAC3, and HDAC8; class IIa, which consists of the enzymes HDAC4, HDAC5, HDAC7, and HDAC9; class IIb, which includes the enzymes HDAC6 and HDAC10; class III, which includes sirtuins (SIRTs; SIRT 1–7;

enzymes that differ in evolution and mechanism from the other HDACs); and class IV (HDAC11) (Alaskhar Alhamwe et al., 2018; Zhao and Shilatifard, 2019).