A NOVEL INHIBITOR OF SIRTUIN: ELUCIDATION OF MODE OF ACTION AND MOLECULAR PATHWAY IN COLORECTAL CANCER CELL LINE

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A NOVEL INHIBITOR OF SIRTUIN: ELUCIDATION OF MODE OF ACTION AND MOLECULAR PATHWAY IN COLORECTAL CANCER CELL LINE

by

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LIST OF ABBREVIATION

%	Percent
µL	Microlitre
µM	Micromolar
5-FU	5-Fluorouracil
BAX	BCL2 associated X protein
BCL2	B-cell lymphoma 2
BH3	BCL2 homology 3
CO_2	Carbon dioxide
CRC	Colorectal cancer
ddH_2O	Deionised distilled water
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EGF	Endothelial growth factor
ERK	Extracellular-signal-regulated kinase
FBS	Foetal bovine serum
FOXO	Forkhead box protein O
G1	Gap 1 phase
G2	Gap 2 phase
GADD45A	Growth arrest and DNA-damage-inducible,alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOF	Gain of function
GOI	Gene of interest
HCl	Hydrogen chloride
HIF1	Hypoxia-inducible factor-1
hr	Hour
IC_{50}	Half maximal (50%) inhibitory concentration
JNK	Jun N-terminal kinase
KRAS	Kirsten rat sarcoma
M	Mitotic phase

MC	Mitotic catastrophe
MDM2	Murine double minute 2
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MN	Micronucleus
MYC	Myellocytomatosis
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium Hydroxide
NF-kB	Nuclear factor kappa B
^oC	Degree Celsius
PARP	Poly-(ADP-ribosyl)-polymerase
PBS	Phosphate buffered saline
PE	Plating Efficiency
PUMA	P53 upregulated modulator of apoptosis
QPCR	Quantitative polymerase chain reaction
RPMI	Roswell Park Memorial Institute
S	Synthesis phase
SA-β-Gal	Senescence- associated β-galactoxidase
SDS	Sodium dodecyl sulfate
sec	Second
SF	Survival fraction
SIRT1	Sirtuin 1
SIRT2	Sirtuin 2
SIRT3	Sirtuin 3
SIRT4	Sirtuin 4
SIRT5	Sirtuin 5
SIRT6	Sirtuin 6
SIRT7	Sirtuin 7
TBST	Tris-buffered saline with Tween 20
TGF-β	Transforming growth factor beta
TRAF2	Tumour necrosis factor receptor-associated factor 2

VEGF	Vascular endothelial growth factor
WHO	Word Health Organization
α	Alpha
β	Beta

PERENCAT SIRTUIN NOVEL

ELUSIDASI CARA TINDAKAN DAN LALUAN MOLEKUL DALAM TITISAN SEL KANSER KOLOREKTAL

ABSTRAK

Kanser kolon merupakan salah satu jenis kanser yang paling banyak didapati di seluruh dunia. Walaupun kebanyakan pesakit menunjukkan respon yang baik terhadap rawatan kimoterapi pada peringkat awal, akan tetapi terdapat banyak kes kanser berulang selepas rawatan yang disebabkan oleh kewujudan rintangan terhadap rawatan, maka wujudnya keperluan untuk membangunkan agen terapeutik baru untuk kanser kolorektal. Sirtuin 1 (SIRT1) dan sirtuin 2 (SIRT2) adalah sasaran penting kerana molekul-molekul ini terlibat dalam perkembangan kanser. Pengekspresan SIRT1 yang terlampau dilaporkan dalam kanser kolorektal, manakala sirtuin 2 (SIRT2) dilaporkan berupaya untuk menindas gen perencat kanser dan memainkan peranan penting dalam kelangsungan hidup sel-sel kanser. BZD9L1 adalah sejenis perencat terhadap molekul-molekul SIRT1 and SIRT2 yang telah dilaporkan mempamerkan aktiviti-aktiviti anti-kanser. Projek ini bertujuan untuk mengkaji molekul-molekul yang terlibat dalam peranan BZD9L1 merencatkan pertumbuhan sel kanser serta regulasi dengan laluan transduksi isyarat yang lain. Kesan BZD9L1 terhadap kelangsungan hidup sel, perkembangan, penghijrahan, kitaran sel, penuaan selular, apoptosis dan molekul-molekul yang terlibat dalam aktiviti-aktiviti ini telah dikaji dalam HCT116 dan HT-29 sel kanser kolorektal. BZD9L1 didapati mengurangkan kelangsungan hidup sel-sel HCT116 dan HT-29, tetapi tidak menunjukkan kesan signifikan terhadap kelangsungan sel kolon epitelium biasa CCD 841 CoN. Peningkatan dos BZD9L1 didapati mengurangkan

percambahan sel dan menghalang penghijrahan dalam kedua-dua jenis sel. Walau bagaimanapun, kompaun ini tidak mempunyai kesan ke atas pengagihan kitaran sel dan penuaan selular. Di samping itu, BZD9L1 mengakibatkan pengaktifan PARP dan kondensasi kromatin, serta menunjukkan apoptosis dalam kedua-dua sel HCT116 dan HT-29. BZD9L1 juga menunjukkan peningkatan pengekspresan faktor transkripsi yang terlibat dalam laluan molekular Notch, NF-kB, Myc/Max, ERK dan JNK dalam HCT116 tetapi menunjukkan pengurangan pengekspresan faktor transkripsi yang berkaitan dengan laluan Wnt, Notch, p53, TGF, NF-kB, Myc/Max, HIF dan ERK dalam sel HT-29. Ini menunjukkan peranan dwifungsi laluan kanser dalam mencapai hasil rawatan yang sama. Kesimpulannya, BZD9L1 mempamerkan aktiviti anti-kanser sitotoksik yang menyebabkan kematian sel secara apoptosis dalam sel kanser kolorektal. Oleh itu, penindasan aktiviti SIRT1 dan SIRT2 dengan menggunakan BZD9L1 merupakan strategi terapeutik yang berpotensi dalam rawatan kanser kolorektal.

A NOVEL INHIBITOR OF SIRTUIN:

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ABSTRACT

Colorectal cancer (CRC) is one of the most common types of cancer worldwide. Although most patients are responsive to standard chemotherapy treatments at the initial stage of diagnosis, many succumb to relapse due to acquired chemoresistance, hence driving the need for the development of novel therapeutic agents for colorectal cancer. Sirtuin 1 (SIRT1) and sirtuin 2 (SIRT2) are important targets as they have been implicated in tumour progression. SIRT1 is overexpressed in colorectal cancer, while SIRT2 represses tumour suppressor genes and play key roles in cancer cell survival. BZD9L1 is a novel SIRT1 and SIRT2 inhibitor with reported anti-cancer activities. This project aimed to investigate the molecular players involved in BZD9L1-mediated colorectal cancer cell inhibition as well as its regulation of cancer signaling pathways. The effect of BZD9L1 on cell survival, proliferation, migration, cell cycle, senescence, apoptosis and the molecular players mediating these outcomes were studied in HCT116 and HT-29 colorectal cancer cell lines. BZD9L1 reduced the viability of HCT116 and HT-29 cells but displayed non-significant effect on the cell viability of normal human colon epithelial CCD841 CoN cells. BZD9L1 significantly reduced cell proliferation and inhibited cell migration in increasing doses in both cell lines. However the compound had no effect on cell cycle distribution and cellular senescence. In addition, BZD9L1 induced cleavage of PARP and chromatin condensation, indicating apoptosis in both cell lines. BZD9L1 also upregulated the transcription factors associated with Notch, NF-

kB, Myc/Max, ERK and JNK pathways in HCT116 but downregulated the transcription factors related to Wnt, Notch, p53, TGF, NF-kB, Myc/Max, HIF and ERK pathways in HT-29 cells, highlighting bifunctional roles of these cancer pathways which result in the same treatment outcome. In conclusion, BZD9L1 exhibited anti-cancer activities as a cytotoxic drug that mediates apoptotic cell death in colorectal cancer. Hence, inhibiting SIRT1 and SIRT2 activities using BZD9L1 could be a promising therapeutic strategy in the treatment of colorectal cancer.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to cancer

Cancer is a disease caused by uncontrolled cell division and proliferation of abnormal cells. These mutated cells gain the ability to divide indefinitely with enhanced growth rate which often results in the formation of solid cell masses known as tumours. Tumours can be categorized as benign (non-cancerous) or malignant (cancerous), with the source of tumour origin termed as the primary site. Benign tumours are usually less life threatening due to the ease of complete removal through surgery. The case however worsens if tumours start to gain the ability to invade into neighboring tissues, which often results in the injury of vital organs, hemorrhage, and disruption of metabolic activities. The tumour becomes metastatic when cancerous cells detach from the primary tumour and migrate to distant organs such as lungs, colon, brain etc. through blood vessels and the lymphatic system.

The incidence of cancer has risen significantly in parallel with the constant expansion of world population and increase in aging community. The adoption of cancer-causing behaviors such as smoking, physical inactivity, and occupational as well as lifestyle exposure to carcinogenic compounds have been shown to encourage the risk of developing this deadly disease, particularly in poor and developing countries (Jemal *et al*., 2011, Torre *et al*., 2015). In addition, International Agency for Research on Cancer (IARC) predicts an expected growth of global cancer burden to 21.7 million new cancer cases by year 2030 with 13 million mortalities simply caused by the aging world population (Thun *et al*., 2010). This rise in global cancer burden is

not excluded from developing countries like Malaysia. According to the Malaysia Ministry of Health, about 100,000 Malaysians suffer from cancer each year (National Cancer Institute, 2016).

1.2 Colorectal Cancer

Colorectal cancer is the cancer of the colon or the rectum. Colorectal cancer arises from abnormal outgrowth of the inner lining of the large intestinal wall known as polyps, which if not removed may be malignant (American Cancer Society, 2017). Colorectal cancer is ranked as the third most common malignancy in the world (GLOBOCAN, 2012; Siegel *et al*., 2014) and is among the most common cancers in developing countries (GLOBOCAN, 2008; Ferlay *et al*., 2010). According to World Health Organization (WHO), colorectal cancer ranks the third most common in men (746,000 cases) and second most common in women (614,000 cases) worldwide (GLOBOCAN, 2012). During the past 20 years, many Asian countries had witnessed the rise in colorectal cancer especially in males (Center *et al.*, 2009). GLOBOCAN 2012 reported colorectal cancer as the second most common cancer in Malaysia with approximately 3000 newly reported cases every year (Veettil *et al.*, 2016). In a country with a population of more than 28 million, colorectal cancer is second most common among male and third most common among female Malaysians, with a growing number of cases every year (Chye and Yahaga, 2004; Omar *et al.*, 2006).

1.2.1 Development and staging of colorectal cancer

Staging refers to a scale of 0 to IV to determine the progression and the event of how much the cancer had spread from the primary site. Stage 0 tumours are benign and invasion of surrounding tissues were not present, whereas Stage IV tumours are malignant and the cancer has metastasized from the primary tumour to distant sites. Colorectal tumours are staged based on the evidence of invasion to colon wall and spreading to blood vessels and lymphatic systems. Although the tumour size in colorectal cancer are less important in terms of treatment outcomes, the cancer mortality and prognosis effectiveness have a close correlation to the stage advancement (John Hopkins Colon Cancer Center, 2017).

Colorectal cancer can be staged based on two staging systems, namely the TNM staging system and the Duke's classification. The TNM staging system was developed by the American Joint Committee on Cancer (AJCC) and is the most precise and widely used system in cancer diagnostic. Table 1.0 shows the comparison between the TNM staging system and Duke's classification, as adapted from John Hopkins Colon Cancer Center (2017) and Figure 1.1 shows the stages of colorectal cancer development classified in accordance of American Joint Cancer Committee (Dukes, 1932; Zatzkin, 2016).

Table 1.1 Comparison of TNM staging and Duke's classification.

Stages	TNM Classification			Duke's classification
	T	N	M	
Stage 0	Tis	N0	M0	-
Stage I	T1	N0	M0	A
	T2	N0	M0	B1
Stage II	T3	N0	M0	B2
	T4	N0	M0	B2
Stage III	T1, T2	N1 or N2	M0	C1
	T3, T4	N1 or N2	M0	C2
Stage IV	Any T	Any N	M1	D

(Table was adapted from John Hopkins Colon Cancer Center, 2017)

Key for TNM Staging:

Primary Tumor (T)

Tis – carcinoma in situ: intraepithelial or invasion of lamina propria

T1 – tumor invades submucosa

T2 – tumor invades muscularis propria

T3 – tumor invades through muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissues

T4 – tumor directly invades other organs or structures and/or perforates visceral peritoneum

Regional Lymph Nodes (N)

N0 – no regional lymph node metastasis

N1 – metastasis in one to three regional lymph nodes

N2 – metastasis in four or more regional lymph nodes

Distant Metastases (M)

M0 – no distant metastasis

M1 – distant metastasis

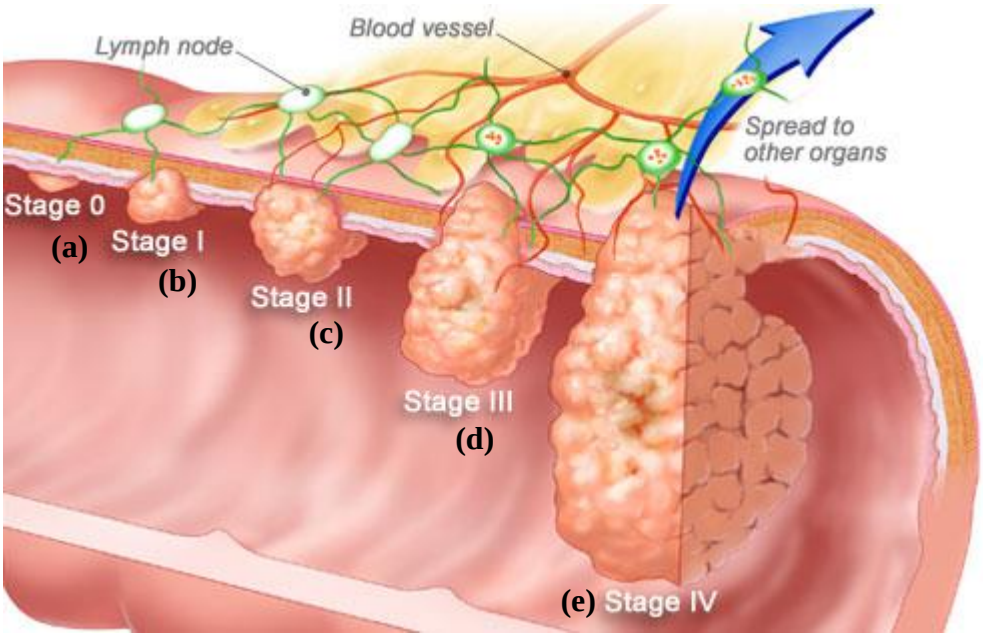

Figure 1.1 The stages of colorectal cancer development classified in accordance to American Joint Cancer Committee.

(a) Stage 0 (carcinoma *in situ*): Formation of tumour at the external lining of intestinal wall.
(b) Stage I: Tumours invade through the muscularis mucosae into the submucosa but do not reach the muscularis propria.
(c) Stage II: Divided into Stage II (B1) when tumours invade into the muscularis propria and Stage II (B2) when tumuors completely penetrate the smooth muscle layer into the serosa.
(d) Stage III: Tumour penetrates the smooth muscles and invade into the serosa following penetration into the lymph nodes.
(e) Stage IV: Metastasis occurs and tumour cells have travelled to distant organs such as lungs, liver and kidneys.

(Figure is reproduced from Zatzkin, 2016)

1.3 Colorectal cancer therapy

In cancer therapy, treatment options include physical removal of tumors via surgery which is common for early stages. The treatment options for colorectal cancer depends largely on the location of the tumour outgrowth, stages of development and patient condition. Patients diagnosed with Stage III and Stage IV cancer usually opted for surgical removal of tumours, along with radiotherapy and chemotherapy to reduce symptoms and remove as much cancerous tissue as possible. The use of chemotherapy in early stages of cancer also aims to reduce and curb tumor growth as well as to induce tumor shrinkage before surgery, and ultimately reduce the likelihood of developing metastasis if the disease progresses (Zheng *et al.*, 2015).

1.3.1 Chemotherapy

The clinical chemotherapy for colorectal cancer includes the individual use of fluorouracil and capecitabine (Xeloda) (Hirsch and Zafar, 2011), where both are anti-metabolites to cease DNA synthesis and repair, irinotecan (Campto) which is an inhibitor to topoisomerase I to interfere with cell proliferation (Saltz, 1998; Eyol *et al.*, 2016), a combination of folinic acid, fluorouracil and oxaliplatin (FOLFOX) (Yu *et al.*, 2009), and a combination of oxaliplatin and capecitabine (XELOX) (Lv *et al.*, 2012) .

Although most patients are responsive to standard chemotherapy treatments at the initial stage of cancer diagnosis, some exhibit *de novo* resistance while many succumb to relapse due to acquired chemo-resistance; rendering the standard chemotherapy regime ineffective. Despite medical advances in colorectal cancer treatment, cancer patients undergoing systemic chemotherapy only secure a mere 12.5 %

survival chances in the 5-year survival rate, whereas more than 90 % of patients experience failure in metastatic cancer treatment due to acquired resistance (Longley and Johnston, 2005; Siegel *et al.*, 2014). Moreover, conventional chemotherapy eliminates cancer cells by unselective induction of cell death on actively dividing cells, including some actively dividing normal cells which causes severe short-term and long-term side effects on the patients (National Cancer Institute, 2015). Chemotheraphies may cause nerve damage, fatigue, infertility, drop in platelet count and reduction of white blood cells in cancer patients. Patients may also experience hair fall, dry and sensitive skin, as well as brittle nails (Cancer Research UK, 2015). In addition, standard chemotherapy may increase the risk of developing some other cancers such as leukemia as a side effect (Aidan *et al*., 2013).

1.3.2 Targeted therapy

Targeted therapies are drugs or antibodies designed to inhibit cancer growth by disrupting the molecular pathway essential for cancer growth. Targeted therapies are gaining increased recognition, as they have been shown to increase patient overall survival rates and keeping cancer at bay (Munagala *et al*., 2011; Gish *et al.*, 2013; Dizon *et al*., 2016) . As targeted therapies utilize the availability of specific biomarkers to target cancer cells, only patients with cancer expressing these particular biomarkers are able to opt for this treatment (Petrelli and Giordano, 2008).

Several targeted therapy drugs have been developed for treatment of colorectal cancer to target main pathways of tumorigenesis either via inhibiting molecules responsible for promoting angiogenesis or blocking the tyrosine kinase receptors to halt downstream oncogenic signaling pathways. Colorectal cancer targeted therapy drugs such as bevacizumab (Fernando and Hurwitz, 2004), cetuximab (Baselga, 2001)

and panitumumab (Addeo *et al.*, 2010) are able to target specific cancer cell biomarkers thereby killing only cancerous cells but have minimal effects on normal non-cancerous cells. Novel treatments targeting molecules or biomolecules specifically expressed in cancer may therefore be a better option as compared to standard chemotherapy.

The main pathways involved in the colorectal cancer tumourigenesis potency and metastatic potential of colorectal cancer include the vascular endothelial growth factor (VEGF) and endothelial growth factor (EGF) pathways (Chee and Sinicrope, 2010). The VEGF family consists of the potent pro-angiogenic VEGFs (VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E), placental growth factors (PIGFs), and vascular endothelial growth factor receptors (VEGFRs) (VEGFR-1, VEGFR-2 and VEGFR-3) (Holmes and Zachary, 2005) that play key roles in inducing mitosis and regulating permeability of endothelial cells, as well as assisting the dissemination of tumour by increasing oxygen and nutrient delivery into the tumour via angiogenesis (Moreira *et al.*, 2007; Ranieri *et al*., 2006). VEGFs (VEGF-A, VEGF-C and VEGF-D) and VEGFRs (VEGFR-2, VEGFR-2/3 and VEGFR-3) are also found to induce lymphangiogenesis of tumours, which is the promotion of lymphatic vessel growth around metastatic tumours to enhance metastasis and lymph flow (Nagahashi *et al*., 2010).

For colorectal cancer treatment, U.S. Food and Drug Administration (FDA) approved clinical targeted therapy drugs including bevacizumab, ziv-aflibercept, cetuximab, panitumumab and regorafenib. Therapies targeting vessel formation through inhibition of VEGF includes bevacizumab (Avastin), ramucirumab (Cyramza), and ziv-aflibercept (Zaltrap) (Chee and Sinicrope, 2010; Verdaguer *et a*l., 2016). Bevacizumab is a monoclonal antibody developed against the ligand VEGF-A by

sequestering the ligand and preventing it from binding to VEGF receptors (VEGFR-2) which induces blood vessel formation (Kim *et al.*, 1993; Gordon *et al.*, 2001) . The use of this drug is able to increase chemosensitivity and radiosensitivity of colorectal tumours (Jain, 2005). Ramucirumab is a monoclonal antibody that binds to the extracellular domain of VEGFR-2 receptor, quenching available receptors for VEGF ligands. Ziv-aflibercept is a fusion protein comprising the extracellular domains of VEGFR-1 and VEGFR-2 fused to human IgG1-Fc which binds to free VEGF ligands, hence reducing freely available VEGF ligands (Stewart, 2011). These drugs have been approved for use in metastatic colorectal cancer in combination with standard chemotherapy drugs. Patients undergone this treatment have been reported to have a better overall survival rate, progression-free survival and slower tumour progression (Ranieri *et al*., 2006; Stewart, 2011).

The EGFR is a tyrosine kinase that is highly expressed in many cancer types including colorectal cancer. This growth-factor-receptor is involved in the alteration of cell phenotypes and increases the growth and survival ability of cancer cells (Huang and Harari, 1999). Targeted therapy drugs used to target changes to this pathway in colorectal cancer are Cetuximab (Erbitux) and Panitumumab (Vectibix). Cetuximab is a monoclonal antibody that binds to the extracellular domain of EGFR as a competitive inhibitor, and mediates anti-tumour properties by reducing cell proliferation, metastasis, angiogenesis, and promotes apoptosis (Goldstein *et al.*, 1995; Baselga, 2001). Panitumumab is an lgG2 monoclonal antibody that is highly specific to EGFR. Similar to Cetuximab, this drug will reduce cell proliferation and induce apoptosis. A major drawback of Panitumumab is that the drug is rendered ineffective against colorectal cancer with *V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog* (KRAS)

or *Neuroblastoma RAS viral oncogene homolog* (NRAS) mutation (Weber and McCormack, 2008).

Regorafenib (Stivarga) is another targeted therapy drug used as the last line of treatment for metastatic colorectal cancer patients that are chemo-resistant to standard chemotherapy (Majithia and Grothey, 2016). Regorafenib functions by inhibiting receptor tyrosine kinase (RTK), which in turn blocks tyrosine protein-kinase receptor tie2 (VEGRFR-TIE2) and inhibits angiogenesis (Clarke and Hurwitz, 2013; Grothey *et al*., 2013; Lim *et al.*, 2015) .

1.3.3 Limitations in current state of targeted therapy

Although the discovery of targeted therapy is a major breakthrough in cancer therapy, this method is not limitation free. As targeted therapy works by inhibiting specific biomarkers essential for cancer progression, the treatment is only effective against patients with tumours that contain that particular biomarker. RAS mutations are found in 30 % of all cancers, and in 35-45 % of colorectal cancer patients. Colorectal cancer patients with mutated KRAS gene were found to have low overall survival rate, and poorer or no response toward therapies including combination of targeted therapy drugs with standard chemotherapy drugs (Vincenzi *et al*., 2015). Many targeted therapy drugs target the EFGR pathway, and the absence of wild-type KRAS and NRAS genes rendered the therapy ineffective. KRAS and NRAS are genes from the RAS family that control fate of cells through the cell cycle by relaying signals from extracellular to the nucleus. Activation of these mutated genes causes constant signaling and promotion of survival genes regardless of blocked EFGR. Thus the discovery and development of novel targeted therapy agent is essential.

Another limitation is the emergence of circumvention of drug resistance to each administered drug. Although most patients showed major effects at the early stage of treatment, some patients do not show encouraging responses despite the presence of the biomarker target. Many patients failed to achieve complete response or only showed partial response, where complete resistance will eventually show after approximately a year. This could be contributed by the complexity of oncogenic pathway crosstalks in the presence of several mutations, or the failure of drugs to detect mutations due to low specificity (Saijo, 2012). Taking targeted therapy agent cetuximab as example, mutation at the S492R of EFGR gene in colorectal cancer results in resistance to cetuximab, and panitumumab have to be used as an alternative to overcome the resistance instead. In multi-cascaded signaling pathways such as the VEGF pathway in colorectal cancer, resistance can be achieved when inhibitors (e.g. bevacizumab) was used due to the variety of alternative signaling molecules (PIGF and fibroblast growth factors, FGF) which can bypass the inhibition to achieve proliferation. Furthermore, inhibition of some targets might cause further mutations in the tumours. The inhibition of angiogenesis may cause hypoxia in the tumours due to activation of hypoxia genes thus leading to promotion of cancer stem cells and aggressive proliferation of the tumour cells (Chen and Zhang, 2015).

In the perspective of research, although more than 800 drugs are under clinical development, only a handful amount of 8 % successfully penetrate the market (Schilsky *et al*., 2010). The complexity of cancer regulatory mechanism, different drug sensitivity and resistance, lack of biomarkers, conflicting and burdensome regulatory requirements are factors contributing to the low success rate (Schilsky *et al*., 2010).

Despite the use of effective strategies in combating cancer by utilizing the high specificity of targeted therapies, unpredicted side effects are unavoidable. The

common side effects from targeted therapies usually arisen as a result of dysregulation of targets of the inhibited molecule in normal tissues (Widakowich *et al.*, 2007). For example, EFGR is involved in the proliferation, survival and differentiation particularly of the skin (Chen *et al*., 1987) and gastrointestinal tract; and play an important part in the maturation cycle of keratinocyte (Jost *et al.*, 2000, Lynch *et al.*, 2007). When EFGR is inhibited by anti-EFGR agents such as panitumumab or cetuximab, the patient may experience papulopustular eruption (Lynch *et al.*, 2007), acne-like rash or folliculitis of the skin (Yano *et al*., 2003), or even post-chemotherapy diarrhea due to the extensive crypt damage in the colon (Playford *et al.*, 2004).

1.4 Epigenetic modification in colorectal cancer

Cancer is a genetic as well as an epigenetic disease (Sadikovic *et al.*, 2008). In the classical view of cancer, the disease has long been hypothesized to be a result of accumulated genetic mutations on the oncogenes and tumor-suppressor genes; where the activation of cell survival genes and suppression of tumor-suppressor genes would cause an uncontrolled cell division and proliferation (Virani *et al.*, 2012). It is only until the recent 20 years that the regulation of DNA on epigenetic diseases, including cancers are intensively studied (Dawson and Kouzarides, 2012). Epigenetic mechanisms are essential in the maintenance of regular function and development of genes expressed specifically in different tissue types, and alteration of global epigenetic landscape is one of the hallmarks of cancer (Sharma *et al.*, 2010). A body of growing evidence has shown that the intrinsic genome stability is influenced by multiple interlocking feedback mechanisms, including the DNA methyltransferases (DMNTs) and histone-modifying enzymes (such as acetylation, methylation and phosphorylation) where perturbations may lead to alterations in gene expression which

ultimately results in cellular transformation and malignant outgrowth (Lund and van Lohuizen, 2004; Ropero and Esteller, 2007). Acetylation and deacetylation is the addition or removal of an acetyl group to the histone nucleaosome core, which corresponds to the activation or deactivation of genes as part of gene regulation.

This study will focus on the histone acetylation/deacetylation process which are highly regulated by two groups of enzymes known as histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively. Among the earliest described mechanism for chromatin modification, HDACs are involved in epigenetic regulation via the removal of the acetyl- group from ε-lysine residues. HDACs are divided into four classes (class I, II, III and IV), with high structural and mechanism similarity among class I, II and IV (Bosch-Presegue and Vaquero, 2015). Class III HDACs comprise of the sirtuin family which was first discovered in yeast *Saccharomyces cerevisiae*. This originally discovered yeast protein silent information regulator 2 (Sir2p) homolog is a factor involved in the rescue of mating deficiency (Rine *et al.*, 1979), and the sirtuin family is highly conserved from yeast to human (Landry *et al.*, 2000) Sir2p was described as a factor in the epigenetic silencing of the mating-type loci and nucleolar ribosomal DNA (rDNA) as well as the telomeres via the promotion of a heterochromatin-like structure with hypoacetylated histones H3 and H4 N-terminal tails (Bosch-Presegue and Vaquero, 2011, Bosch-Presegue and Vaquero, 2015).

1.4.1 Mammalian sirtuins

The mammalian sirtuins are class III HDACs, which are NAD^+-dependent protein deacetylases and mono-[ADP-ribosyl] transferases. The mammalian sirtuin family are comprised of 7 members (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7)

each varies on catalytic activity, enzyme function and cellular localization (Figure 1.2). Each member of the sirtuin family possesses a conserved catalytic domain of approximately 250 residues flanked by variable lengths of C-terminal and N-terminal sequences which enhance their enzymatic activities and contribute to the different specificities and catalytic activities (Pan *et al*., 2012; Bosch-Presegue and Vaquero, 2015; Yeong and Oon, 2016).

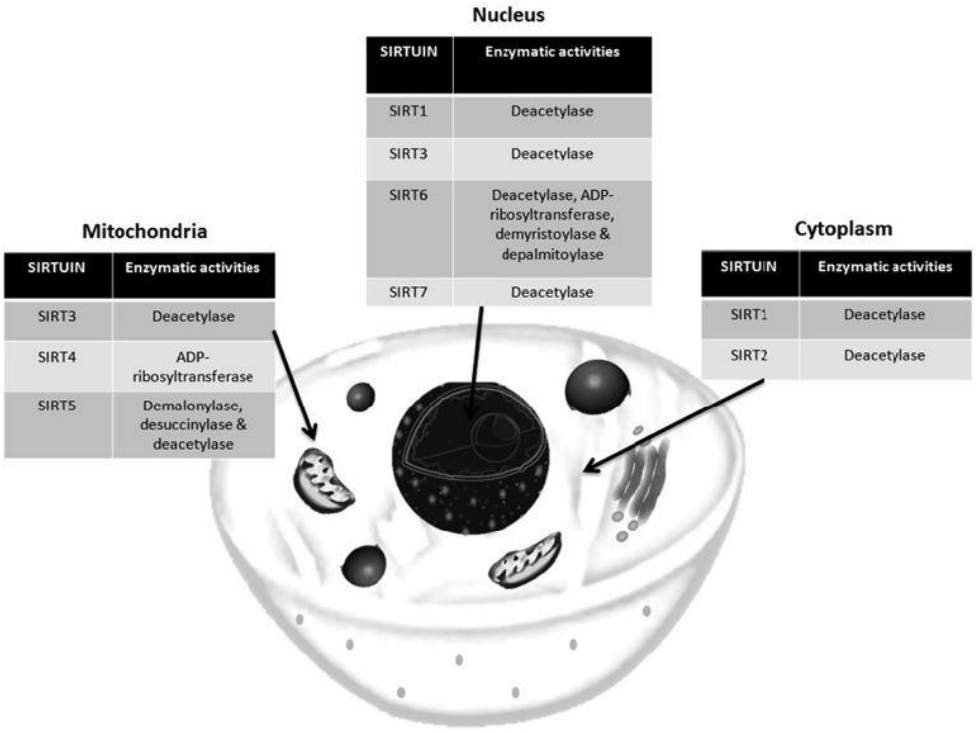

Figure 1.2 Localization and enzymatic activities of mammalian sirtuins.

SIRT1 (deacetylase) is found in the nucleus but is constantly shuttled in and out of the nucleus through the nuclear membrane. SIRT2 (deacetylase) is found mainly in the cytoplasm and only transported to the nucleus during G2/M phase of the cell cycle. SIRT3 (deacetylase) is mostly present in the mitochondria, and a small amount is also found in the nucleolus. SIRT4 (ADP-ribosyltransferase) and SIRT5 (deacetylase, demalonylase, desuccinylase) are only present in mitochondria. SIRT6 (deacetylase, ADP-ribosyltransferase, demyristoylase, depalmitoylase) is found only in the nucleus and SIRT7 (deacetylase) is found only in the nucleolus. (Figure is adapted from Yeong and Oon, 2016)

The most important and distinct function of mammalian sirtuins is the mediation of cellular homeostasis via the detection of changes in the cellular redox state caused by stress; which can be either oxidative stress, metabolic stress, or genotoxic stress (Rodriguez *et al*., 2013). By using NAD^+ as the enzyme cofactor, mammalian sirtuins are able to detect energy fluctuations in the cells due to changes in the intracellular redox potential and hence coordinate an appropriate response (Bosch-Presegue and Vaquero, 2011). Among many cellular processes modulated by sirtuins, the main function of sirtuins can be categorized into 4 processes, such as the involvement in chromatin regulation, determination of cellular fate by modulating cell cycle arrest or apoptosis in the present of cellular stress, regulation of cellular homeostasis, and contribution to developmental and cellular differentiation (Bosch-Presegue and Vaquero, 2011).

1.4.2 Role of sirtuins in cancer

As sirtuins are main key players in homeostasis maintenance and longevity, deregulation of the family is often linked to the emergence of homeostatic diseases such as cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer. SIRT1 and SIRT2 possess high potential as a target for cancer therapy and of research interest. However, recent studies have reported a contradicting interplay of sirtuins in cancer progression. Findings on SIRT1 and SIRT2 in cancer demonstrate interesting role as both sirtuins demonstrated as tumor suppressor gene and oncogene. On one hand, evidence showed that SIRT1 and SIRT2 are able to protect organisms against cancer via curbing oxidative stresses; while other studies suggested the longetivity effect of SIRT1 and SIRT2 contributes to cancer progression. The bifunctional roles of SIRT1 and SIRT2 are summarized in Figure 1.3.

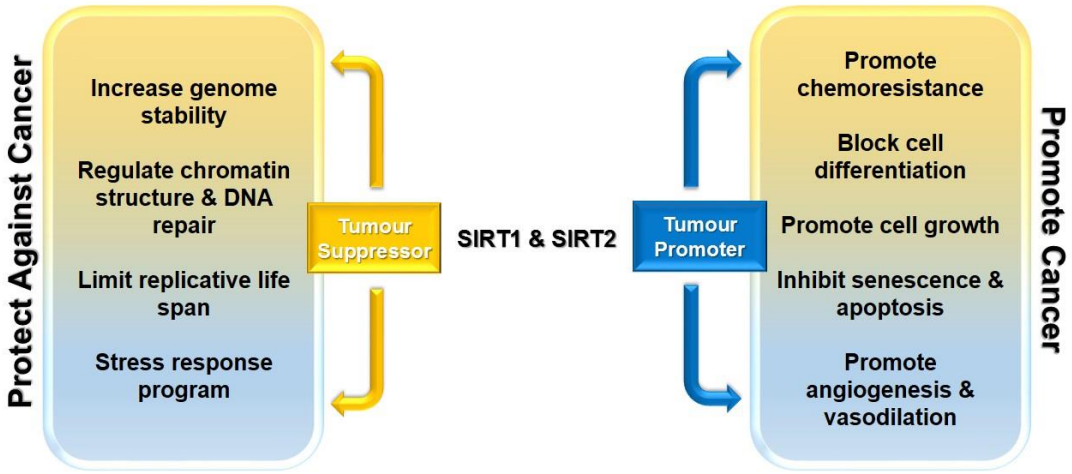

Figure 1.3 The bifunctional role of SIRT1 and SIRT2 in cancer.

SIRT1/SIRT2 can behave as both tumour suppressor and tumour promoter. SIRT1/SIRT2 act as tumour suppressor by increasing genome stability, regulating chromatin structure and promoting DNA repair, limiting cellular replicative life span and mediates the stress response program. Both SIRT1 and SIRT2 act as a tumour promoter by promoting chemoresistance, blocking cell differentiation, promoting cell growth, inhibiting senescence and p53-/FOXO3- mediated apoptosis, and promoting angiogenesis and vasodilation (Bosch-Presegue and Vaquero, 2011).

1.4.2 (a) SIRT1 and cancer

SIRT1 has become an interesting target for the development of anti-cancer drugs since the last decade due to its overexpression in a variety of cancers (Bradbury *et al.,* 2005; Hida *et al*., 2007; Huffman *et al*., 2007, Zhao *et al*., 2011) including colorectal cancer (Stunkel *et al.,* 2007). Research over the years have found SIRT1 to possess bifunctional roles as both tumour promoter and tumour suppressor.

The role of SIRT1 as tumour suppressor is demonstrated in a study done by Firestein *et al* (2008) where SIRT1 overexpression in mice model with a mutated APC (*APC$^{min/+}$)* gene showed reduced colon cancer formation by deacetylating β-catenin and promoting cytoplasmic localization of β-catenin. Another study also found that SIRT1 sensitizes cells to tumour necrosis factor alpha (TNF-α)-induced apoptosis through the deacetylation of RelA/p65 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) protein (Yeung *et al.*, 2004). Apart from mediating pathways stated above, SIRT1 was found to mediate *BRCA1* signaling by negatively regulating and inhibiting Survivin by deacetylating H3K9 of the protein's promoter region. The absence of Survivin will curb the translation of anti-apoptotic proteins (Wang *et al*., 2008b). SIRT1 in maintaining genome integrity and DNA damage repair is another important role as a tumour suppressor. SIRT1 mutant (SIRT1$^{-/-}$) cells were found to form aneuploidy and structural aberrations due to abnormal mitosis, and more susceptible to DNA double strand breaks due to impaired DNA repair system (Wang *et al*., 2008a). Apart from the molecular perspectives, *in vivo* studies performed on mice also showed promising results. For instance, transgenic mice with overexpressed SIRT1 has been found to undergo a more healthy aging process, having a lower probability of carcinomas and sarcomas development and being less susceptible to liver cancer (Fan and Luo, 2010).

SIRT1 has been demonstrated as a hallmark of cancer when it is found to play critical role in mediating multiple aspects of drug resistance through the reduction of drug penetration, suppression of anti-apoptotic mechanisms and promotion of cancer stem cells survival (Wang and Chen, 2013). The ability of SIRT1 to deacetylate the tumour suppressor gene p53 confirms its role as a tumour promoter through the repression of p53-dependent cell cycle arrest and p53-dependent apoptosis in response to cellular stress (Vaziri *et al*., 2001). SIRT1 was also found to cause epigenetic silencing of tumour suppressor FOXO family (a subgroup of Forkhead box group of transcription factors), resulting in the suppression of DNA damage repair, impaired cell cycle control mechanism and loss of FOXO3-mediated apoptosis (Brunet *et al*., 2004). Apart from that, SIRT1 was found to deacetylate c-Myc and promotes Myc/Max association which in turn stimulates cell proliferation (Mao *et al*., 2011) and drug resistance (Li *et al.*, 2014). Lastly, SIRT1 was found to induce replicative immortality of cancer cells by positively regulating the epithelial mesenchymal transition (EMT) and hypoxia inducible factors (HIF), hence inducing migration and enhanced uptake of nutrients into tumour cells (Dioum *et al*., 2009; Byles *et al*., 2012; , Chen *et al*., 2014).

1.4.2 (b) SIRT2 and cancer

The tumour suppressor role of SIRT2 can be seen in its regulation of the cell cycle. SIRT2 is involved in mediating the mitotic checkpoint, where cells are prevented to undergo mitosis in the presence of DNA damage (Dryden *et al*., 2003). It is postulated that SIRT2 mediates cell cycle through the regulation of chromatin condensation (Inoue *et al.*, 2007). SIRT2 was also shown to play an important role in chromosomal segregation as SIRT2-deficient mice were shown to be more prone in developing

tumours due to impaired chromosomal segregation (Kim *et al*., 2011). In addition, SIRT2 regulates migration and invasion through downregulation of the Wnt-signalling pathway. SIRT2 was also reported to interact with β-catenin, and absence of SIRT2 was found to enhance cellular migration and invasion through the upregulation of matrix metalloproteinase 9 (MMP9) and downregulation of chromodomain-helicase-DNA-binding protein 1 (CHD1) gene (Nguyen *et al*., 2014).

Although SIRT2 is less studied as compared to SIRT1, recent studies showed SIRT2 to repress tumour suppressor gene by deacetylating p53 and *KRAS* gene, thereby promoting tumourigenesis and cancer cell survival (Yang *et al*., 2013). SIRT2 also represses the FOXO family, and the suppression of FOXO3 by both SIRT1 and SIRT2 contributes to the formation of cancer via FOXO3 degradation (Li *et al*., 2011, Wang *et al.*, 2012).

1.5 Commercial sirtuin modulators

As sirtuin is gaining increasing recognition with its significant role in cancer, drug discovery efforts targeting sirtuins is emerging. A number of sirtuin inhibitors targeting SIRT1 and/or SIRT2 have been discovered and are currently being developed, including Nicotinamide derivatives, Suramin and its analogs, Splitomicin and its analogs, Hydroxynaphthaldehyde derivatives, Thiobarbiturates derivatives, Indole derivatives, Indole-isoxazolone derivatives, Tenovin derivatives, Peptidomimetics and other compounds with diverse structural cores (Yeong and Oon, 2016). The currently available sirtuin modulators, specific targets and type of inhibitors are depicted in Table 1.2.

Although many sirtuin modulators have been discovered and researched on, further development of these modulators are unable to reach clinical trials due to either unfavorable physiochemical properties or poor side effects. For instance, compounds such as isonicotinamide (Jackson *et al*., 2003, Schmidt *et al.*, 2004) and suramin derivatives (Figg *et al*., 1994, Gill *et al.*, 1995) were found to exhibit too much toxicity and thus proved to be less useful. Other compounds like nicotinamide derivatives, splitomicin derivatives (Freitag *et al.*, 2011) and some tenovin derivatives (Lain *et al.*, 2008) have either poor solubility in water or too lipophilic in nature. Thus, the essentiality of discovery of novel sirtuin inhibitor is imminent.

1.6 BZD9L1: A novel SIRT1 and SIRT2 inhibitor

BZD9L1 is a highly-fluorescent benzimidazole derivative and potent sirtuin inhibitor discovered recently in our laboratory. This synthetic compound is known to inhibit SIRT1 and SIRT2, with stronger inhibition towards SIRT2. Data from previous work has reported BZD9L1 to exhibit a linear progress curve over time which suggests the compound is a reversible inhibitor towards SIRT1 and SIRT2. In an assay performed with increasing concentrations of NAD^+, BZD9L1 was found to demonstrate competitive inhibition, through competition with substrate NAD^+ to bind at the same active site of SIRT1 and SIRT2 enzyme (Yoon *et al.*, 2015). BZD9L1 was found to demonstrate strong anti-proliferative effects across a panel of different tested cancer cell lines including leukemia (CCRF-CEM), breast cancer (MDA-MB-468) and colorectal cancer (HCT-116) (Yoon *et al*., 2015), with the strongest anti-proliferative activity observed in colorectal cancer.