Epigenetic therapy in cancer

A project submitted

By

Md. Shadman Ridwan Abid

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This work is dedicated to my parents to whom I owe my achievements

Table of Contents

Certification statement

This is to certify that, this project titled 'Epigenetic therapy in cancer'submitted in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University, constitutes my own work under the supervision of Dr M. Zulfiquer Hossain, Associate Professor, Department of Pharmacy, and Dr Eva Rahman Kabir, Professor and Chairperson, Department of Pharmacy, BRAC University. This project is the result of my original research and has not previously been submitted for a degree or diploma in the university. To the best of my knowledge and belief, the project contains no material previously published or written by another person except where due reference is made in the paper itself.

Signed,

_____

Countersigned by the Supervisor

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Acknowledgement

I would like to begin with my gratitude to Almighty Allah for the help in the completion of this research and preparation of this paper.

I would like to thank my supervisor Dr M. Zulfiquer Hossain, Associate Professor, Department of Pharmacy, and Dr Eva Rahman Kabir, Professor and Chairperson, Department of Pharmacy, BRAC University for their continuous provision, guidance and patience since the first day of the project work. They have continuously inspired and motivated me with their knowledge and expertise which made me more passionate about the project since it began.

List of Tables

List of Figures

Abbreviation

DNMT= DNA Methyltransferase

HDACi= Histone Deacetylase Inhibitor

CRC= Colorectal Cancer

CPT= Camptotheci

APL= Acute Promyelocytic Leukemia

AML= Acute Myeloid Leukemia

CML= Chronic Myeloid Leukemia

NSCLC= Non-Small Cell Lung Cancer

SCLC= Small Cell Lung Cancer

PKMT= Protein Lysine Methyltransferase

PCR2= Polycomb Repressive Complex 2

EZH2= Enhancer of Zeste 2

HMTases= Histone Methyltransferase

CDA= Cytidine Deaminase

MDS= Myelodysplastic syndromes

LCA= Laccaic acid A

HAT= Histone Acetyltransferase

PTCL= Peripheral T-cell lymphoma

CTCL= Cutaneous T-cell lymphoma

Abstract

Epigenetic therapy has received much attention in the field of oncology in recent years. A growing recognition of the influence of epigenetic modifications in tumorigenesis and the clinical success of several drugs that reverse the aberrant epigenetic alterations have positioned epigenetic therapy at the forefront of anti-cancer drug development. Several epigenetic enzymes such as DNA methyltransferase, histone deacetylase, topoisomerase, and EZH2 have been successfully targeted by small molecule inhibitors. Potential epigenetic modifiers are continuously being optimized for bioavailability, half-life, metabolism, and most importantly target specificity. Discovery of new compounds has also broadened the pipeline of latest epi-drugs. Considering the prospect of success of epigenetic therapy against lethal malignancies, in this review we aspire to describe how different abnormal epigenetic patterns such as excessive DNA methylation, histone deacetylation, and defective chromatin remodeling contribute to cancer and present an overview of the current implementation of epi-drugs along with combined therapy in cancer treatment.

1. Introduction

Cellular functionality and its regulation are determined by its genetic as well as epigenetic characteristics. DNA sequences are determinants of genetic characteristics while DNA methylation, covalent modification of histones and regulation of chromatin structure as well as non- coding RNA are major epigenetic modulators (Kelly, & Issa, 2017). Balancing of both genetic and epigenetic regulation is critically important for normal cellular development and function. Deregulation of these processes can lead to abnormal cell growth and survival and eventually cause cancer.

Presently, the instruments of transient and spatial regulation of gene activity that do not rely on the sequence of DNA bases are collectively referred to as epigenetic processes. Epigenetic alterations are complex and diverse, involving DNA methylation, histone modification, chromatin remodeling, and alteration in micro RNA expression. All of these mechanisms singly or together determine chromatin structure and lead to either activation or suppression of a gene. Epigenetic events can lead to suppression of tumor suppressor genes or over-expression of oncogenes, and thus play a pivotal role in tumorigenesis and metastasis. Epigenetic modifications are catalytic reversible reactions carried out by enzymes. Anticancer agents have, therefore, been designed to target these enzymes in a variety of malignancies. The goal of the current study is to describe the major classes of anti-cancer epi-drugs, their mechanism of action, efficacy, potency, advantages, disadvantages, and side effects. Recent advances and the future of anti-cancer epigenetic therapy will also be briefly discussed.

1.1 Epigenetics and Cancer

Epigenetic inactivation of tumor suppressor genes has been implicated in a variety of cancers.

1.1.1 Colorectal Cancer

Abnormal epigenetic modifications have been observed in colorectal cancer. Along with DNA hypermethylation on CpG island, epigenetic silencing of microRNAs significantly

contributes to colorectal cancer (CRC). For example, miR-34b/c and miR-148a have been found to be hypermethylated during CRC progression (Kalimutho et al., 2011).

1.1.2 Hematologic Malignancies

In several hematologic malignancies such as acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML), distinct epigenetic alterations have been shown to play a role in leukemogenesis. Several small molecule histone deacetylase (HDAC) inhibitors including Belinostat (PXD-101), Panobinostat (LBH589), AR-42, and 4SC-202 are presently in clinical use against AML and CML. Moreover, another group of drugs that includes GSK126, GSK343 and EI1 targets EZH2 to inhibit aberrant histone hypermethylation.

1.1.3 Ovarian Cancer

Epigenetic abnormalities have been found in ovarian as well as endometrial carcinomas. DNMT inhibitors and HDAC inhibitors are currently being tested to reverse the epigenetic changes including abnormal DNA methylation and unusual histone modifications in cancer cells.

1.1.4 Prostate Cancer

In prostate cancer, certain epigenetic modifications often occur prior to and more frequently than mutations. Suppression of certain tumor suppressor genes by epigenetic alterations such as histone acetylation, hypermethylation and hypomethylation have been implicated. Protein lysine methyltransferase (PKMTs) activity has been described as a major mediator of prostate cancer (Andreoli, Moura Barbosa, Parenti, & Rio, 2012).

1.2 Types of Epigenetic Abnormalities in Cancer

1.2.1 Abnormalities in DNA Methylation Pattern

Cancer cells show aberrant pattern of DNA methylation and post-translational histone alterations, accompanied by dysregulated gene expression. They have hypermethylation of particular CpG islands in the promoter regions of tumor suppressor genes. Promoter hypermethylation leads to silencing of these genes and plays a decisive role in carcinogenesis. Interestingly, cancer cells also experience global DNA hypomethylation in the rest of DNA, causing genome instability. DNA methylation is catalyzed by DNA methyl transferases (DNMTs).

Nucleoside analogs, 5-azacytidine and 5-aza-2-deoxycytidine have received approval from the USFDA for clinical use against myelodysplastic disorders. They incorporate into DNA and establish a covalent complex with DNMTs. This results in inhibition of DNMTs, followed by degradation, which leads to hypomethylation of DNA and re-expression of tumor suppressor genes. A wide range of potential DNMT inhibitors have been tested. DNMT inhibitors are classified into different groups depending upon their specificity and mode of actions. Hypomethylation and reactivation of tumor suppressor genes have been achieved by these inhibitors.Azacytidine, Decitabine, Zebularine are the most commonly used DNMT inhibitors (DNMTi). There is ongoing clinical investigation to improve DNMTi-based therapy. Hydralazine was initially endorsed for use as an antihypertensive, but it was recently discovered to repress DNMT activity. It is currently under assessment in various clinical trials.

A superior comprehension of the genes affected by DNA methylation in cancer and development of promoter-specific DNMT inhibitors will enable us to realize the full potential of this class of epigenetic drugs.

1.2.2 Abnormalities in Histone Acetylation

Abnormalities in Histone Acetylation

Histone acetylation releases DNA from histones and promotes gene expression. On the other hand, histone deacetylation represses gene expression. Histone deacetylation often collaborates with DNA hypermethylation to repress tumor suppressor genes in cancer cells. Chemical agents that inhibit histone deacetylases (HDAC) have been found to upregulate gene expression and exhibit anti-cancer effects in vitro and in vivo. For example, Vorinostat has been approved by FDA for clinical utilization in cutaneous T-cell lymphoma. Vorinostat and trichostatin-A (TSA) are broad-spectrum HDAC inhibitors (HDACi), have a structure containing hydroxamic acid, and non-specifically upregulate 2–10% of genes in the human genome. HDAC inhibitors are most effective in upregulating tumor suppressor genes when they are used in conjunction with DNMT inhibitors.

1.2.3 Abnormalities in EZH2 Activity

Modifications of epigenetic controllers, including the histone lysine methyltransferase EZH2, have been found in various cancer types. With the emergence of EZH2 inhibitors, we can now more fully assess the role of EZH2 in carcinogenesis. EZH2 has been shown to play a role in silencing of tumor suppressor genes in cancer cells.

EZH2 is the main catalytic subunit of the Polycomb Repressive Complex 2 (PRC2). It is responsible for repressing target genes through trimethylation of histone H3 on lysine 27(H3K27me3). However, the catalytic activity of PRC2 also requires EED and SUZ12 along with EZH2. PRC2 interacts with an elaborate network of cellular proteins. Moreover, there are many different histone methyltransferases (HTMases).To avoid affecting non-tumorigenic pathways; any approach ought to have a high specificity towards EZH2. Several research groups are looking for compounds that will inhibit specific HMTases. GSK126, GSK343, EI1, EPZ005687, UNC1999 are selective and potent EZH2 inhibitors which reduce H3K27me3 levels in cancer cells.

1.2.4 Abnormalities in Topoisomerase Enzymatic Activity

Topoisomerases are universal and evolutionarily conserved nuclear enzymes, which regulate the DNA topology by passing an intact DNA helix through a transient single (topo-I) or twofold (topo-II) strand break, enhancing key nuclear trans-activities, such as transcription, translation, chromosome isolation, replication, mitotic division and so forth. As the level of topoisomerases is upregulated in cancer and growing cells, anti-tumor drugs have been developed to target these enzymes. Contemporary topoisomerase inhibitors exert their cytotoxic effects by trapping topoisomerases in a complex with DNA and triggering apoptosis. Distinct inhibitors counteract aberrant activities of topoisomerase I and II individually or together. Irinotecan, Topotecan, CRLX101, Belotecan are common topoisomerase I inhibitors. Aclarubicin, Suramin, Novobiocin are topo II inhibitors.

1.2.5 Non-coding RNA Modulation

Non-coding RNA interacts with mRNA and other proteins to regulate the expression of tumor suppressive or oncogenes depending on the regulatory targets.(Lujambio, & Lowe, 2012). Tumor suppressive microRNA (miR-16) has been used for the treatment of NSCLC or mesothlioma as a supplementary anticancer agent.

2. Inhibition of Aberrant DNA Methylation

DNA methylation is the most characterized, functionally stable and reversible epigenetic alteration. DNA methylation is characterized by addition of (-CH3) group from the ubiquitous methyl donor SAM, to the carbon-5 position of cytosine residues in CpG dinucleotides.(Cheray, Pacaud, Hervouet, Vallette, and Cartron, 2015).These CpG dinucleotides are often found in CpG islands that are mainly situated close to or inside gene promoter regions (possessing almost 60% of mammalian gene promoters).CpG islands of tumor suppressor genes are usually free of methylation in typical human cells. Cancer cells show hypermethylation of particular CpG islands, especially in the promoter region of tumor suppressor genes. This is accompanied by global hypomethylation in the rest of genomic DNA. The end result is epigenetic activation of tumor suppressor genes and genomic instability.DNA methylation is a catalytic reaction which is catalyzed by DNA methyltransferases or DNMT enzymes. The DNMT family of enzymes consists of five members includingDNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Cheray, Pacaud, Hervouet, Vallette, and Cartron, 2015).

In mammalian cells, the transcription machinery binds to the promoter region of a gene to initiate transcription of DNA into RNA. Promoter regions are CpG-rich in almost half of the genes in our genome. Nearly 80% of the CpGdinucleotides outside CpG islands are mostly methylated (Herman and Baylin, 2003). On the contrary, in gene promoters dinucleotides of CpGislands are typically unmethylated regardless of the transcription status of the gene.

When the promoter region of a particular gene is methylated, the transcription factors do not recognize their binding sites on the DNA effectively, and this results in repression of the corresponding gene. In many types of tumors, the genomic DNA methylation pattern is changed either through hypermethylation (increased methylation) or hypomethylation (decreased methylation). In the pericentromeric chromosomal region of cancer cells, particularly CpG dinucleotides are hypomethylated which may induce genomic instability. More critically, aberrant silencing of tumor suppressor genes occurs due to hypermethylation of CpG dinucleotides in the promoter region. An elevated level of methylation in tumor cells most likely results from expanded action of DNMTs, usually as a result of over expression. Disruption in regulation of DNMT has been demonstrated in

several cancers including breast, lung, stomach, and colon cancer and in leukemia.(Gnyszka, Jastrzebski, and Flis, 2013). Fortunately, epigenetic changes are possibly reversible, unlike hereditary or environmentally induced genetic mutations. Hypermethylation is an attractive target for anti-cancer therapeutics because of its reversibility and flexibility.

The DNMTs of mammalian cells are comprised of two regions: a C-terminal portion that is mainly catalytic and a multi-domain N-terminal region which varies in size and encodes mostly regulatory functions. TheC-terminal portion consists of five hundred amino acid residues. The N-terminal potion of DNMT1 usually contains 621amino acid residues that are not absolutely essential for DNMT1 functions. (Subramaniam et al., 2014), though they are essential for distinguishing hemimethylated DNA from unmethylated DNA. The C-terminal catalytic domain that every DNMT shares, is associated with binding of co-factor (motifs I and X) and catalysis of substrate (motifs IV, VI, and VIII) (Subramaniam et al., 2014).

It has been demonstrated that DNMT3a and DNMT3b function as de novo methyltransferases establishing DNA methylation patterns during embryogenesis while DNMT1 acts to maintain the methylation patterns which were established during embryogenesis through consequent cycles of DNA replication. In several types of tumor, DNMT1 and DNMT3a as well as DNMT3b have been found to be over expressed (Pollock and Richon, 2009). For instance, significant level of DNMT3b appeared to be critical for the hypermethylation of CpG islands in breast cancer cells.

2.1 DNMT Inhibitors

Based on specificity, agents that inhibit DNA methylation are of two wide classes: global DNMT inhibitors and specific DNMT inhibitors. On the other hand, based on their mechanism of action DNMT inhibitors are classified into two categories: nucleoside inhibitors and non-nucleoside inhibitors

Table 2.1: List of DNMT inhibitors

GeneralDNMT inhibitor	DNMT1 specific inhibitor	DNMT3B specific inhibitor
Azacytidine	EGCG	NanaomycinA
Decitabine	Laccaic acid A	
Zebularine	MG98	
	Procainamide	

2.1.1 Azacytidine and Decitabine

Azacytidine as well as decitabine are known as cytidine analogs in which pyrimidine ring is modified at the 5- position. These compounds were at first utilized in leukemia treatment as chemotherapeutic agents. Subsequently, their hypomethylating functions were discovered Nucleoside transporter-1 transports this type of cytidine analogs into cells where they are modified to active triphosphate frames. For instance, azacytidine is converted into 5-azacytidine 5'- triphosphate by uridine cytidine kinase while decitabine is transformed into 5-aza-2'- deoxycytidine-5'- triphosphate by deoxycytidine kinase and afterward subjected to degradation by cytidine deaminase (CDA). Cancer cells replicate their DNA in the S phase of cell cycle like normal human cells and integration of these drugs into the human genome of cancer cells during the S phase is absolutely crucial. Azacitidine and decitabine have been approved by FDA to treat myelodysplastic syndrome (MDS) because they re-activate expression of silenced tumor suppressor genes (Gnyszka, Jastrzebski, and Flis, 2013).

As a ribonucleoside analog, azacytidine readily incorporates into RNA. It also incorporates into DNA but to a lesser degree. Decitabine which is a deoxyribose analog incorporates only into the DNA. 5-azanucleosides create a complex modified pyrimidine structure through incorporation of nitrogen moiety in carbon-5 position and initiates proteosomal degradation. Hence, these compounds remain bound to DNA covalently. Furthermore, DNMT activity is blocked. Additionally, invasion of trapped DNMT is facilitated by covalent protein adduction with DNA-damage signaling and loss of functionality (Gnyszka, Jastrzebski, and Flis, 2013).Accordingly, methylation of cytosine deposits is repressed even in the replicated daughter cells.

Azacytidine and decitabine have been clinically successful but their short half-life and rapid degradation at elevated temperature have limited their use. Moreover, irreversible decomposition occurs in alkaline solutions. In vitro experiments illustrate that azacytidine shows half-life of 7 hours and decitabine shows half-life of 21 hours in neutral aqueous solutions (Gnyszka, Jastrzebski, and Flis, 2013). Nevertheless, azacytidine shows chemical stability at 4 ˚C.

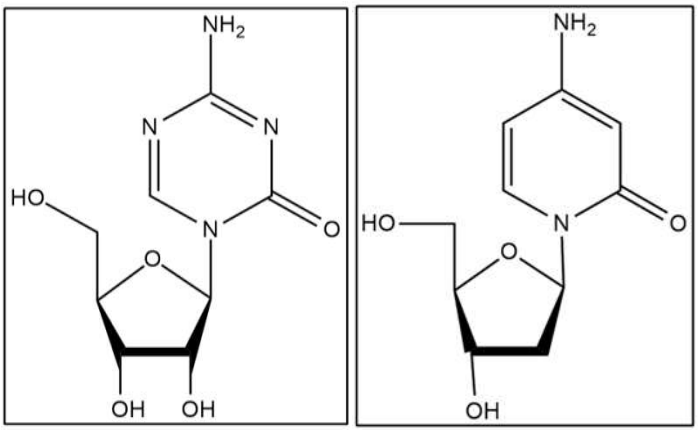

Figure 2.1.1 (A) Azacytidine Figure 2.1.1 (B) Decitabine

2.1.2 Zebularine

Zebularine falls under the nucleosideanalog family and is characterized by its chemical stability, low cytotoxicity and bioavailability. Zebularine specifically inhibits DNMT by forming a close complex of zebularine and the substituted DNA. (Cheray, Pacaud, Hervouet, Vallette, and Cartron, 2015).This mechanical pathway inhibits the activities of DNMT by modification of cytosine at carbon-5 position. Zebularine follows a similar pathway of activation and metabolism as azacytidine and gets incorporated into DNA. Uridine-cytidine kinase initiates the phosphorylation reaction followed by conversion of Zebularine into 2'-deoxyzebularine-5'-diphosphate.Ribonucleotide reductase acts as a catalyst in this conversion. The crucial step for incorporation of Zebularine into DNA is the conversion of 2'- deoxyzebularine-5'-diphosphate to 2'-deoxyzebularine-5' triphosphate. Thus Zebularine inhibits cytosine C5 from being activated for methylation.

Zebularine shows different half-life at different pH. At 37˚C, the half-life is ~44 hours in phosphate buffered saline (PBS) of pH1.0 and about 508 hours of half-life at pH 7.0 which

is permissive for oral administration (Yoo, Cheng, and Jones, 2004). Zebularine shows low toxicity. That is why frequent dosing of the drug via intravenous infusion can be applied to establish prolonged inhibition of DNMT.

Lack of target specificity of DNA demethylating agents is the greatest shortcoming of these drugs. Even after global hypomethylation by DNA demethylating agents it is not possible to reactivate certain genes completely (Esteller, 2005). On the flip side, global DNA demethylating agents have widespread side effects.

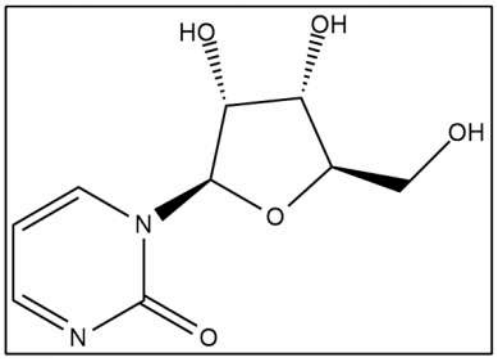

Figure 2.1.2 Zebularine

2.1.3 EGCG

Epigallocatechin-3-O-gallateis has been reported as a specific DNMT1 inhibitor. EGCG contains gallic acid that directly interacts with the catalytic site of DNMT1 and inhibits with high affinity.

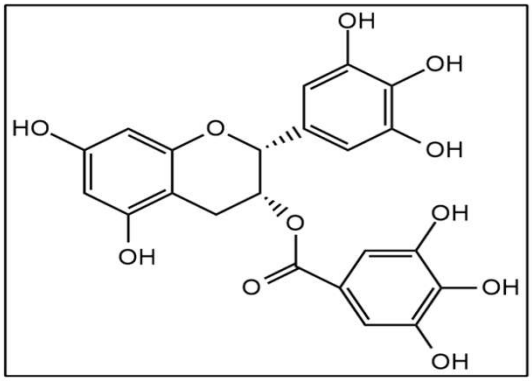

Figure 2.1.3 EGCG

2.1.4 Laccaic acid A

Laccaic acid A (or LCA) is natural product containing highly substituted anthraquinone (Cheray, Pacaud, Hervouet, Vallette, and Cartron, 2015). It also directly inhibits DNMT1 competitively and has been shown in breast cancer cells to re-express methylated and silenced genes.

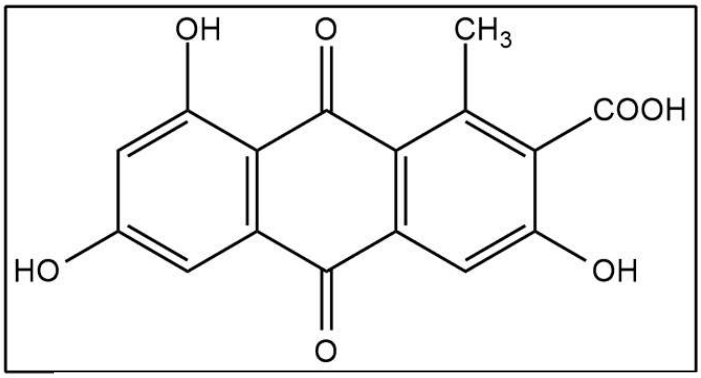

Figure 2.1.4 Laccaic acid A

2.1.5 MG98

MG98is an anti-sense oligonucleotide containing twenty base pairs that particularly binds to the 3'-untranslated regions (3'UTR) of DNMT1 and inhibits DNMT1 expression. Nevertheless, the FDA has not approved MG98 because of its toxicity.

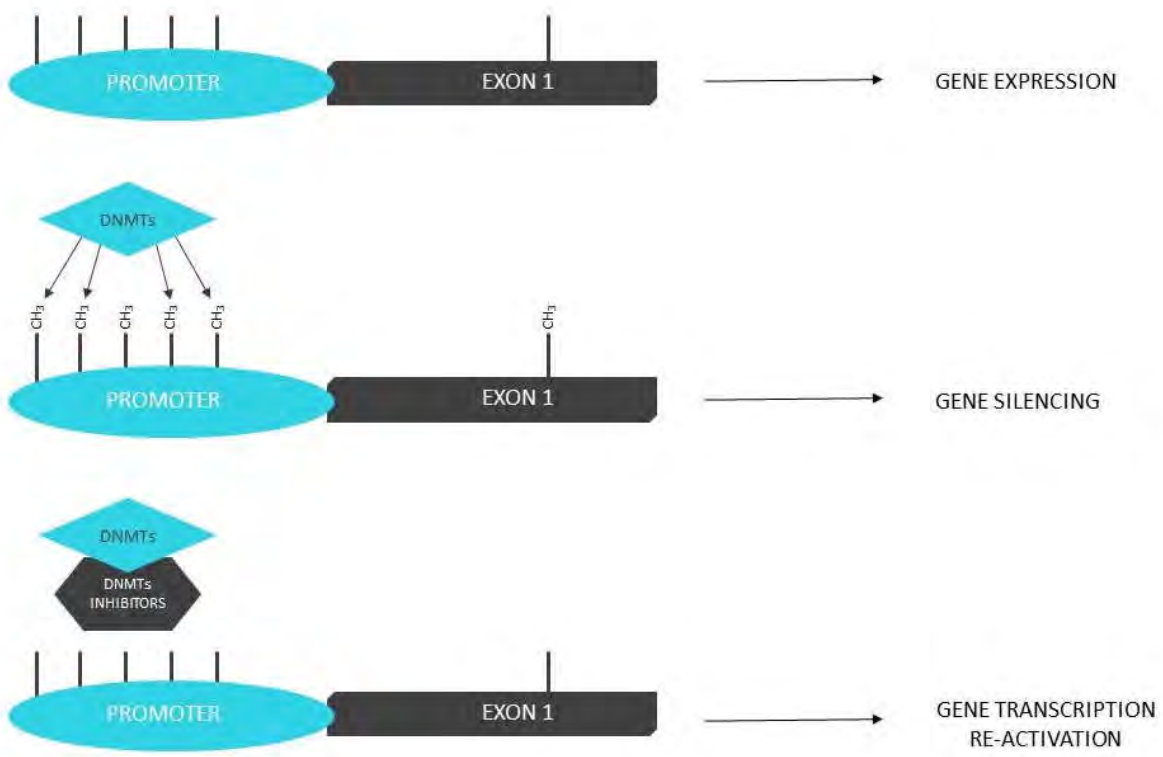

Figure 2.1.5 Gene reactivation by DNMT inhibitors

2.1.6 Procainamide

Procainamide is a non- nucleoside inhibitor and is responsible for global DNA hypomethylation. It induces re-expression of specific detoxifier gene GSTP1 which is silenced due to hypermethylation in prostate cancer (Lin et al., 2001). Procainamide binds with GC-rich sites of DNA sequences and prevents methylation. However, its effectiveness remains questionable.

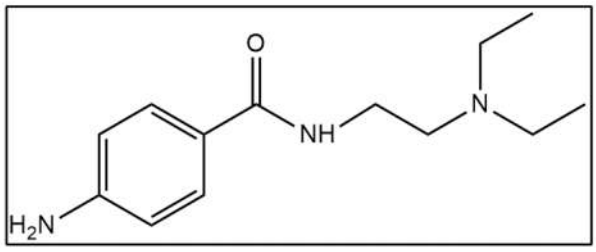

Figure 2.1.6 Procainamide

2.1.7 NanaomycinA

Nanaomycin A was isolated from a Streptomyces strain for and considered a quinine antibiotic. Though Nanomycin was proposed to be an effective DNMT1 inhibitor, later on the drug was found ineffective againstDNMT1 (Kuck et al., 2010). The particular inhibitory role of Nanaomycin A on DNMT3B was discovered in cancer cell lines subsequently. However, its precise mode of action remains unclear.

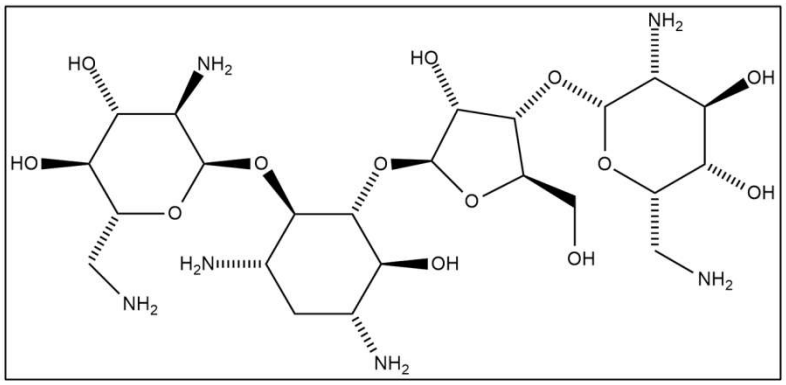

Figure 2.1.7 Nanaomycin A

3. Inhibition of Histone Deacetylation

Histone acetylation is one of the post translational histone modifications that can modify DNA accessibility, alter chromatin structures, and regulate gene expression without changing the DNA sequence. The chromatin contains nucleosomes with147 DNA base pairs on each nucleosome core particle. Five classes of histones have been discovered including H1, H2A, H2B, H3 and H4. Histone H1 is called the linker histone and rest are called core histones (Mottamal, Zheng, Huang, & Wang, 2015).

Reversible acetylation of histone and non-histone proteins occurs at a post-translational level in eukaryotic cells. Histone acetyltransferases (HATs) are responsible for protein acetylation while deacetylation is accomplished by histone deacetylases (HDACs) through antagonistic activities.Excessive acetylation results in apoptosis whereas aberrant histone deacetylationin association with DNA hypermethylation has been identified with malignancy through silencing of tumor suppressor genes. Disruption of HAT and HDAC functions leads to development of a wide range of human cancers. (Marks, Richon and Rifkind 1210-1216). Accordingly, HDACs have received much attention in anti-cancer therapeutics. HDAC inhibitors (HDACi's) have emerged as a rapidly developing area of clinical research. Histone deacetylases involve a group of 18 genes, which are assembled into classes I–IV in view of their homology. Classes I, II, along with IV consist of 11 genes, which are referred to as classical HDACs, while the rest 7 genes from class III are called sirtuins (Witt et al., 2009). These 11 family members from classes I, II and IV contain a Zn molecule positioned in their active site and pan- HDAC inhibitors mainly inhibit their functionality. Other classes of HDAC do not contain any Zn molecule and as a result, they cannot be inhibited by current inhibitors. Comprehensive studies suggest that HDAC inhibitors enhance apoptosis and cell cycle arrest in leukemia to different extents during G0~G1 phase or G2~M phase. (Bi &Jiang 2006). HDAC inhibitors function in both pathways by either directly inhibiting HDAC or indirectly activating HAT. HDAC is linked to tumor growth through promoting transcriptional repression of chromatin. On activation of HAT, hyperacetylation occurs that leads to transcriptional activation of tumor suppressor genes and growth arrest of tumor cells. HDAC inhibitors block tumor angiogenesis, affect survival of tumor cells and inhibit intracellular stress response pathways. HDAC inhibitors induce degradation of the pro-

angiogenic transcription factor HIF-1 by enhancing acetylation. HDAC inhibition initiates formation of reactive oxygen species in the cells (Lane, & Chabner, 2009).

3.1 HDAC Inhibitors

HDAC inhibitors (HDACi's) have emerged as a rapidly developing area of clinical research. Histone deacetylases involve a group of 18 genes, which are assembled into classes I–IV in view of their homology. Classes I, II, along with IV comprise of 11 genes, which are referred to as classical HDACs, while the rest 7 genes from class III are called sirtuins (Witt et al., 2009). These 11 family members from classes I, II and IV contains a Zn molecule positioned in their active site and pan- HDAC inhibitors mainly inhibit their functionality. Other classes of HDAC do not contain any Zn molecule so that they could not be inhibited by recent inhibitors. Comprehensive studies suggest that HDAC inhibitors enhance apoptosis and cell cycle arrest in leukemia to different extents during G0~G1 phase or G2~M phase. (Bi &Jiang 2006). HDAC inhibitors function in both pathways by either directly inhibiting HDAC or indirectly activating HAT. HDAC is linked to tumor growth through promoting transcriptional repression of chromatin. On activation of HAT, hyperacetylation occurs that leads to transcriptional activation of tumor suppressor genes and growth arrest of tumor cells. HDAC inhibitors block tumor angiogenesis, affect survival of tumor cells and inhibit intracellular stress response pathways. HDAC inhibitors induce degradation of the pro-angiogenic transcription factor HIF-1 by enhancing acetylation. HDAC inhibition initiates formation of reactive oxygen species in the cells(Lane, & Chabner, 2009).

Table 3.1: List of HDAC inhibitors

Hydroxamic acids	Trichostatin A, Vorinostat
Carboxylic acids	Valproate, Butyrate
Aminobenzamides	Entinostat, Mocetinostat
Cyclic peptides	Aapicidin, Romidepsin
Epoxyketones	Trapoxins

3.1.1 Vorinostat

Vorinostat, a hydroxamic acid, received FDA approval in 2006 for treating cutaneous manifestation in CTCL patients. FDA approval was based on two phase II clinical trials with a 30% response rate in patients with CTCL 2. The usual dose of Vorinostat is 400 mg once a day or 200 mg twice a day. In the case of solid tumors, the dose can be increased to 600 mg. Compared to previous agents, vorinostat has shown noticeable improvement in reducing pruritus. Vorinostat has been found to be an effective radiosensitizer in preclinical research for the treatment of glioblastoma (Shi et al., 2014). Vorinostat is used for the treatment of both type I and II endometrial cancer because of its ability to modify the signaling pathway of insulin-like growth factor-I (IGF-I) by altering the expression of related genes and produce anti-proliferative response in cancer cells. In murine and human lung cancer cell lines and genetically engineered mouse lung cancer models, vorinostat induces histone acetylation, death of cancer cells and expression of p27 whereas it reduces expression of cyclin D1 and cyclin E(Ma et al., 2013). Vorinostat emerged as a well-tolerated agent against squamous cell carcinoma. Moreover, vorinostat is recognized as a potent agent against gastrointestinal cancer, brain metastasis, advanced solid tumors, melanoma, and multiple myeloma. Pancreatic, refractory colorectal and lung cancers are the rest the diseases on which Vorinostatexhibits its inhibitory action. Patients experience Vorinostat as a well-tolerated drug though some adverse side effects have been documented including nausea, fatigue, diarrhea, thrombocytopenia and pulmonary embolism.

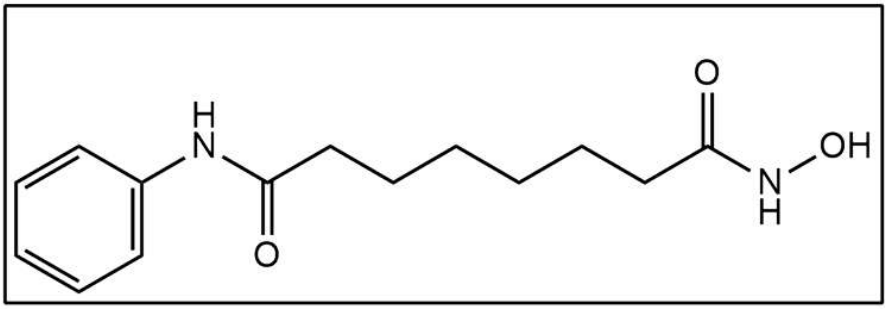

Figure 3.1.1 Vorinostat

3.1.2 Depsipeptide

Depsipeptide (Romidepsin) is another FDA-approved HDAC inhibitor containing bicyclic peptide that shows significant cytotoxic activity in in vitro as well as in vivo (Tumor xenograft model) investigations. Depsipeptide has passed phase II trial showing overall response rate of 34% in 96 patients according to an international study. It is considered safe for treatment of CTCL. Depsipetide has been identified as a potent agent against lung cancer and thyroid cancer. Depsipetide in undergoing clinical trials for possible use in treating renal, colorectal, breast, and hematological malignancies. Depsipetide has low toxicity, but showed limited clinical utility.

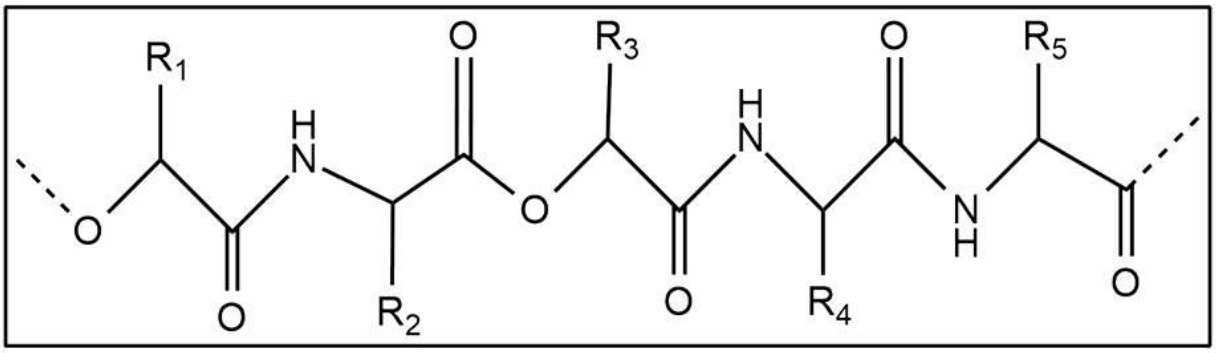

Figure 3.1.2 Depsipeptide

3.1.3 Belinostat

Belinostat is the third FDA-approved HDAC inhibitor after showing 25% response rate similar to other FDA-approved inhibitors. The response rate was found after phase II trial on 120 PTCL patients. Belinostat has also been evaluated through phase I and II clinical trials for solid as well as hematological malignancies. For establishing a second line treatment of refractory malignant pleural mesothelioma, belinostat has been put through clinical tests in 13 patients to evaluate response rate. Belinostat shows efficacy against micro papillary ovarian tumors and resistant epithelial cancer. Myelodysplastic syndrome (MDS) is one class of cancer which abrogates the formation of new blood cell by bone marrow. Belinostat was experimented in phase II clinical trial to measure its efficacy against MDS. (Cashen et al., 2011).

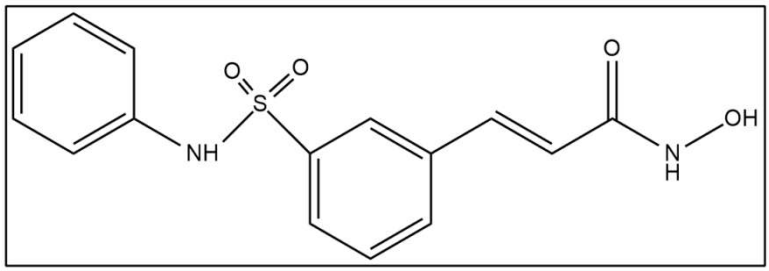

Figure 3.1.3 Belinostat

3.1.4 Pracinostat

After several clinical trials, pracinosta thas been established as an HDAC inhibitor that exerts its inhibitory actions against myelofibrosis. It also has shown clinical effect against solid tumors. It is fairly well tolerated, but more in-depth investigation of its mechanism of action is currently underway.

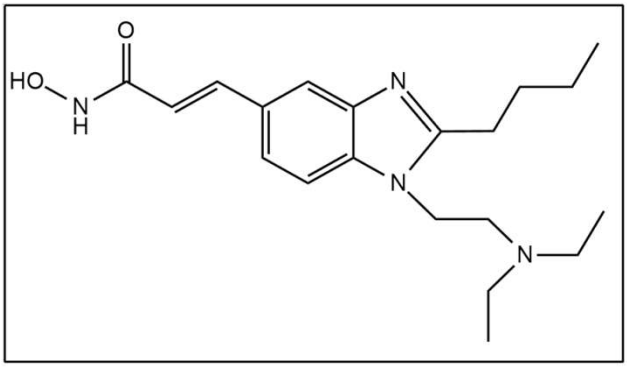

Figure 3.1.4 Pracinostat

3.1.5 Valproic acid

VPA has been used for more than thirty years for the treatment of epilepsy. It is a short chain of fatty acid.(Shi et al., 2014). Valproic acid has been introduced in the clinical trials as a single agent or as part of combination therapy. VPA was studied in phase I clinical trial and found to be effective against CNS tumors in pediatric patients. VPA treatment has been associated with histone acetylation in 50% of patients. Patients suffering from myelodysplastic syndrome have been treated with VPA in phase I clinical trial. 24% patients of patients responded to the treatment.

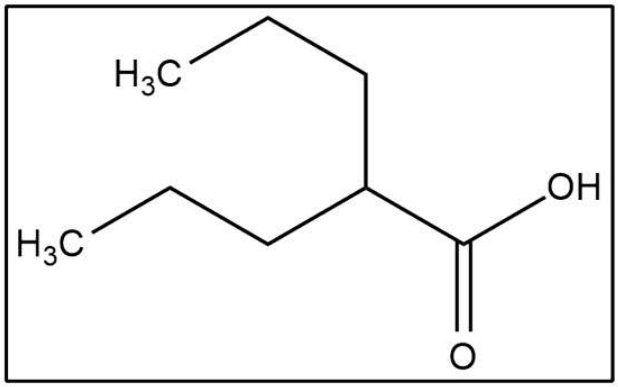

Figure 3.1.5 Valproic acid

3.1.6 Mocetinostat (MGCD0103)

Mocetinostat (MGCD0103) is a selective HDAC inhibitor that specifically acts against class I and IV histone deacetylases. Mocetinostat has been tested in both phase I and II clinical trials. In one trial, a 110 mg dose was given daily to bone marrow cancer patients. Unfortunately, only 3 patients out of a population of 29 individuals (10%) responded to the treatment (Boumber, Younes, & Garcia-Manero, 2011). Excellent biological activity was found when mocetinostat had been examined under phase II trial in lymphoma cells. The efficacy of 5-azacitidine has been increased with simultaneous addition of mocetinostat. Mocetinostat also induces cell cycle arrest and apoptosis in pancreatic cancer cells. During phase II studies it has been documented that, at a fixed dose 45 mg in 24 hours mocetinostat induces H3 histone acetylation , inhibits HDAC activity in peripheral WBC and acts against advanced-stage solid tumors. The half-life of Mocetinostat has been documented ~ 7 – 11 h.MGCD0103 is excreted via biliary as well as fecal routes, identical to other HDAC inhibitors.

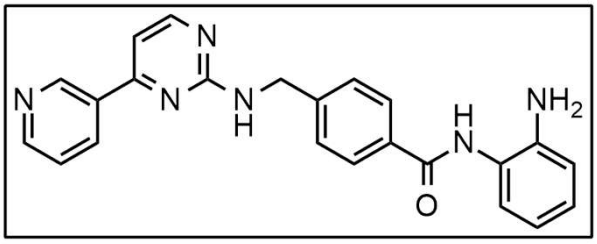

Figure 3.1.6 Mocetinostat (MGCD0103)

3.2 Doublet Therapy Theory

As a single agent HDAC inhibitors may show inadequate cytotoxic activity due to resistance mechanisms that reduce the therapeutic efficacy. Nevertheless, co-treatment of HDAC inhibitor with bortezomib or other drugs show more promising therapeutic effects at low doses.(Bastian et al. 2013). Combination therapy with HDAC inhibitors shows much promise. Vorinostat is being evaluated under clinical trials with temozolomide as a combined therapy along with radiotherapy for the patients who are diagnosed with glioblastoma multiforme. Vorinostat, which showed poor performance previously in the treatment of PTCL patients, showed much better outcomes when it was recently combined with cyclophosphamide, prednisone, vincristine, and doxorubicin. Combined therapy of depsipeptide and gemcitabine was experimented inpatients for the treatment of pancreatic, breast, and ovarian cancers in phase II clinical trials(Jones et al., 2012). The efficacy of belinostat has been significantly increased with the combination of cisplatin, cyclophosphamide, and doxorubicin against thymic epithelial malignancies in phase I trial. Pracinostat, when combined with 5-FU, pacritinib, and tozasertib, was effective against colorectalcancer, AMLand CML. It is suggested that HDAC inhibitors in combination therapy induce synergistic effects against aberrant histone acetylation as well as malignancy.

4. Aberrant Topoisomerase Enzyme Activity and Inhibition

DNA topoisomerases are essential enzymes that maintain super-helical tension during compaction of the extremely long DNA molecule of one cell into a very small nucleus. They are functionally efficient enzymes that lead to separation of DNA strands for transcription and replication. The double helix structure of DNA does not show any free rotation that produces supercoiling during DNA strand separation. Supercoiling can be two types either positive or negative, both generated in flanking regions where actually DNA strands get separated. Positive supercoiling occurs in the front site of DNA transcription or replication while negative supercoiling is generated at the back. In the absence of DNA topoisomerase function, positive supercoiling inhibits replication and transcription. Moreover, negative supercoiling accelerates the interference of normal DNA metabolism by forming D loops (invasion of a DNA duplex by a complementary single-stranded DNA segment), R loops (persistent annealing of RNA with its DNA template behind RNA polymerase) Z-DNA and guanosine quartets with abnormal DNA structure (Pommier, Leo, Zhang, & Marchand, 2010). Topoisomerases are important enzymes that cause temporary breakage in single (Topoisomerase I) or double (Topoisomerase II) DNA strands and regulate supercoiling, entanglements. Thus these enzymes are essential to maintain DNA transcription as well as replication process. Though Topoisomerase I and II enzymes show distinct cleaving characteristics, they both exert nucleophilic attack on phosphodiester bonds in DNA.

DNA topoisomerase enzymes facilitate DNA breakage as well as relegation and play a important role in DNA metabolism. Because of their role in DNA replication, transcription, recombination, chromatid segregation, topoisomerases have been comprehensive targets for anticancer treatment.

Topoisomerase I Inhibitors are usually used in treatment of several cancers such as lung, ovarian, breast, cervical and colon cancer. On the other hand, topoisomerase II inhibition is applied as anticancer therapy against lymphoma, lung cancer, testicular cancer, and leukemia.

4.1 Topoisomerase I Inhibitor

Topoisomerase I inhibitor specifically targets topoisomerase I that is highly expressed in tumor cells. Cytotoxic effects of topoisomerase I inhibitors are mediated by duration of exposure rather than dosage.

Table 4.1: List of Topoisomerase inhibitors

Topoisomerase I inhibitors	Topoisomerase II inhibitors	Topoisomerase I&II inhibitor
Irinotecan	Aclarubicin	DACA
Topotecan	Suramin	
CRLX101	Novobiocin	
Belotecan	Merbarone	
Gimatecan	Doxorubicin	

4.1.1 Camptothecin Derivatives

Camptotheca acuminata is the primary source of antiproliferative agents from which camptothecin was isolated as a topoisomerase I inhibitor. Eventually many derivatives of camptothecin have been developed with better water-solubility and pharmacokinetic characteristics. Topotecan and Irinotecan are the optimized CPT derivatives applied in ovarian and colorectal cancer treatment. Belotecan has also been approved in South Korea for use against SCLC. Nevertheless, these inhibitors are becoming less popular as they have significant side effects and display unsatisfactory curative action as a single agent, chemical instability, and spontaneous inactivation of drug by opening of structural E ring at neutral pH, conversion of lactone form to carboxylate form. Specifically, CPT derivatives share a typical auxiliary element of potent electrophilic α-hydroxylactone in the E ring that can be quickly hydrolyzed to inert carboxylate frame under physiological conditions (Dong et al., 2017). However, the invention of non-Camptothecin derivatives of Top I inhibitors has overcome the drawbacks of previous inhibitors. Most promising among them are Indenoisoquinolines and Dibenzonaphthyridinones that address the structural instability and multi drug resistance (Khadka& Cho, 2011).

4.1.1.1 Irinotecan

Irinotecan is a core five-ring chemical structure that links with bulky and long side chains. CPT-11 is another name of Irinotecan. CPT-11 shows effective therapeutic effects against cancer cells which are resistant to vincristine, colchicines, doxorubicin and vinblastine. (Tsuruo, Matsuzaki, Matsushita, Saito, & Yokokura, 1988). The drug becomes pharmacologically active when it turns into SN-38 as an active metabolite afterthe cleaving of the side chain. The formation of SN-38 is catalyzed by liver carboxylesterases (CE). Carboxylesterase -2 enzyme in tumor cells initiate the activation of SN-38 and exerts specific antiproliferative effects in tumor cell other than normal cells. SN-38 is formed due to conversion into active lactone ring from carboxylate form but it lasts for a very short period of time. The half-life of lactone ring is only 9.5 minutes after that it rapidly converts back into carboxylate form(Rivory, Canal, Mathieu-Boue, & Robert, 1995). With the increase of dose, the plasma concentration of Irinotecan remains constant because of largedistribution of drug in tissues at steady state. SN-38 is further metabolized into inactive SN-38 G by the enzymatic reaction of uridine diphosphate glycoronosyltranferase enzymes in liver(RB & MJ, 1997). It shows promising anti malignant function against colorectal cancer which is resistant to 5-fluorouracil (5-FU) (Van Cutsem E, 1999).

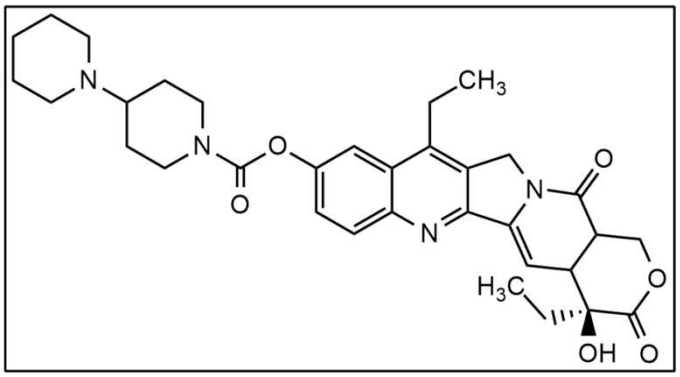

Figure 4.1.1.1 Irinotecan

4.1.1.2 Topotecan

Topotecan is a semisynthetic water-soluble CPT derivative. It shows impressive antitumor function against various solid tumors such as breast, colon, ovarian, and esophageal cancers, osteosarcoma, myelodysplastic syndrome, and leukemia. Topotecan exhibits strong anti-cancer activity and stability due to its reduced albumin binding property (Mi, Malak, & Burke, 1995). It shows wide distribution with significant penetration in CSF. Topotecan can be used for the treatment of brain metastases because of its permeability across blood-brain barrier.

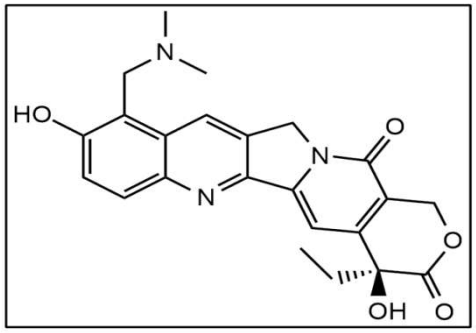

Figure 4.1.1.2 Topotecan

Topotecan has been tested in clinical trials and found to be effective against advanced level ovarian cancer that was resistant to other chemotherapeutic regimens. Topotecan demonstrated significant antitumor potency in children in a pediatric phase I clinical trial (Stewart et al., 1996). Its anti-malignancy property against CNS tumor in both children as well as adults is quite remarkable. The drug is mainly eliminated via the renal route. Topotecan shows oral bioavailability of about 40%.

4.1.1.3 CRLX101

To overcome the drawbacks of topotecan and to enhance antiproliferative activity, several conjugated drugs have been tested in clinical trials. CRLX101 is a copolymer of cyclodextrin–polyethylene glycol and CPT conjugate. It is connected to a hydrophilic cyclodextrin-based linear polymer via ester bond (Moukharskaya, &Verschraegen, 2012). This drug shows anti malignancy against colon carcinoma and tumors that show resistance to irinotecan.

4.1.1.4 Belotecan

Belotecan is a newly discovered CPT analog with better solubility and clinical tolerability. It is possible to administer it orally. In Belotecan (Camptobell or CKD602) a water-solubilizing group is introduced at position 7 of the ring B of CPT. All derivatives contain at least a pentacyclic ring system of CPT and an intact a-hydroxylactone group in the E ring which are required for the in vitro and in vivo activity of CPT's.

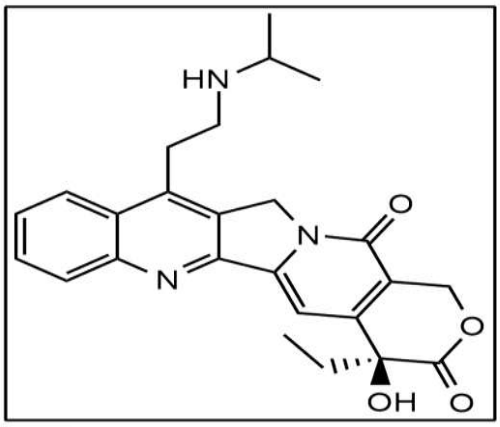

Figure 4.1.1.4 Belotecan

4.1.1.5 Gimatecan (7-t-butoxyiminomethyl-campthotecin)

Gimatecan is more efficient in stabilizing DNA cleavable complex than Topotecan or SN-38 and is present in the lactone form in plasma. It can be administered orally and has a relatively long half-life.

4.1.2 Topoisomerase I inhibitor and NSCLC

New generation topoisomerase I inhibitors including Topotecan and Irinotecan offer significant overall survival against non-small cell lung cancer (NSCLC). These topoisomerase I inhibitors act through a common mechanism: stabilization of DNA topoisomerase I cleavable complex. This complex inhibits DNA replication and causes cell death. Topoisomerase I inhibitors are effective radio-sensitizers. This was demonstrated in in vivo experiments. Phase II clinical trial was conducted by Saka and colleagues in 24 advanced NSCLC patients with concurrent administration of intravenous irinotecan and

thoracic radiation therapy and showed promising results (Choy, Kim, Pyo, &MacRae, 2001). The adverse effects included pheumonitis, esophagitis, neutropenia and fever. Topoisomerase I inhibitors are considered effective against NSCLC.

4.2 Topoisomerase II Inhibitor

Agents that target topoisomerases II enzymes can be divided into two major groups. One group is DNA Topo II poison that inhibits the topoisomerase activity by covalently binding to DNA and stabilizing DNA cleavable complex to inhibit further DNA replication. Another group includes several agents that interfere with at least one step of the catalytic cycle of topoisomerase and are known as catalytic inhibitors. Topo II poisons show significant antitumor function while catalytic inhibitors are widely used as antineoplastic agents or cardio receptors, and enhance the efficacy of other agents.

Topo II poisons not only increase the level of DNA cleavable complexes but also increase their life-time by inhibiting re-ligation of cleaved DNA strands. Daunorubicin and Doxorubicin are used as Topo II poison under the class of Anthracyclines. Daunorubicin is used against acute leukemia while Doxorubicin is highly recommended for treatment of bone cancer, breast cancer, bladder cancer, Hodgkin's lymphoma, and multiple myeloma. At low doses, DOX causes poisoning of topo II by increasing complex level while it also inhibits the formation of the complex at high concentration(Rocha, Busatto, Guecheva, & Saffi, 2016).

Topo II poisons are further subdivided into two groups: intercalating poisons and non intercalating poisons. The intercalating poisons include Doxorubicin, Mitoxantrone and other anthracyclines. Anthracyclines independently show large cytotoxic effects. Doxorubicin accelerates membrane damage by producing free radicals and traps DNA through cross linkage. Topo II poisons under non-intercalating category such as teniposide, epipodophyllotoxins, fluoroquinolones and etoposide do not tightly bind to DNA. Instead they interact with the protein to form a covalent complex.

4.2.1 Aclarubicin

Aclarubicin is a strong intercalating agent. As it intercalates into DNA, it stalls the interaction of topoisomerase II with DNA (Sorensen et al., 1992). Aclarubicin falls under the Anthracycline class of anti-cancer agents that clinically acts against acute myelocytic leukemia. Aclarubicin also shows inhibitory action against topoisomerase I enzyme.

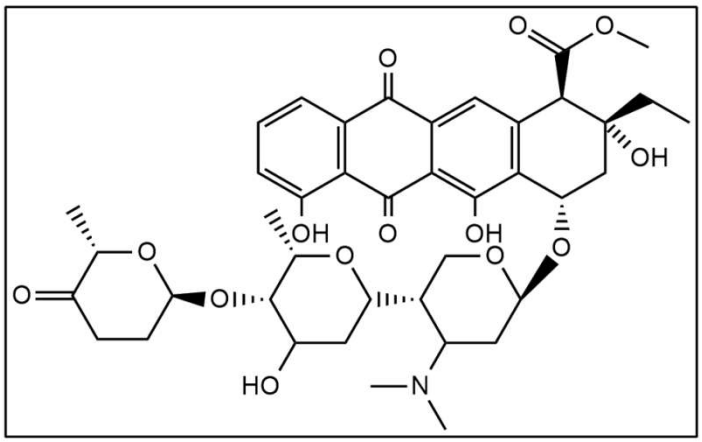

Figure 4.2.1 Aclarubicin

4.2.2 Suramin

Suramin is one kind of polyanionic drug which shows antitrypanosomal along with antifiliarial characteristics (Eisenberger & Reyno, 1994). At high concentrations Suramin regulates various cellular enzyme activities, but it affects topoisomerase II as well as receptors of some growth factors at lower doses. Suramin exhibits abnormally long half-life of more than 250 hours that suggests requirement for dose adjustment for individual patients. It has strong anti-malignant properties, but it also has many serious side effects including lymphopenia and neuropathy. The use of suramin has been successful in low doses and currently it is undergoing clinical development (Qu et al., 2002). Suramin at non-toxic low dose is combined with other anticancer agents such as 5- fluorouracil, vincristine, and doxorubicin.

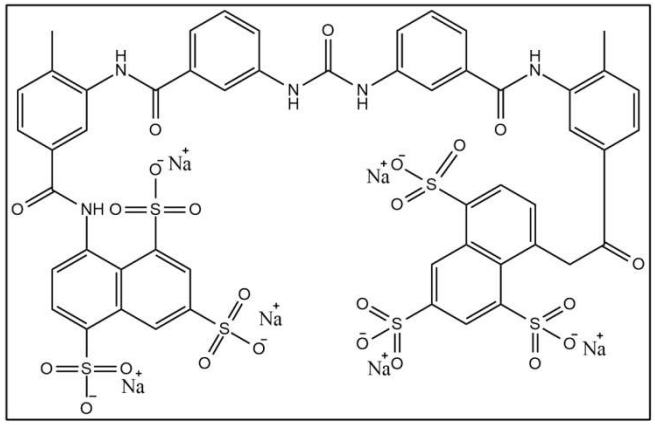

Figure 4.2.2 Suramin

4.2.3 Novobiocin

Novobiocin is classified as an aminocoumarin drug that primarily targets DNA gyrase B enzyme and then blocks the binding site of ATP to topoisomerase II. Novobiocin enhances the cytotoxic potentiality of Teniposide and Etoposide in several malignant cell lines by inhibiting the efflux of the drugs and increasing the intracellular concentration that leads to formation of more cleavable complexes. In addition, it acts as an effective inhibitor of arginine-specific mono- ADP- ribosyltransferases (Lodhi IJ et al. , 2001). Clinically 4g/day oral dose for one week is recommended for novobiocin. Presently, addition of antiemeticswith novobiocin limits the side-effects and increases the concentration of the drug in plasma to around 150 mM. Doublet therapy of novobiocin with high concentration of cyclophosphamide and thiotepa has been tested in phase II trials in patients suffering from advanced breast cancer (Larsen, Escargueil, &Skladanowski, 2003).

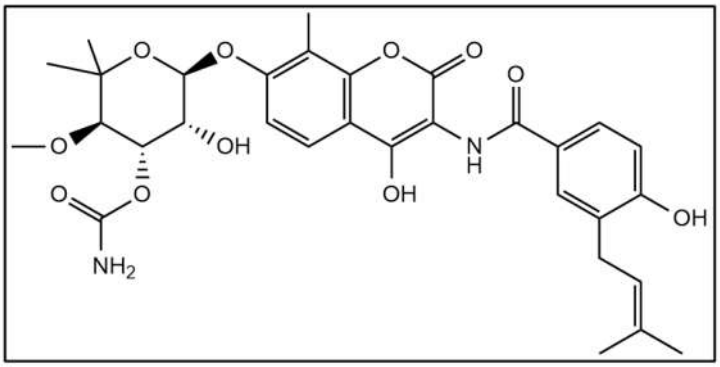

Figure 4.2.3 Novobiocin

4.2.4 Merbarone

Merbarone is derived from conjugation of aniline and thiobarbituric acid linked by amide linkage. Among seven hundred barbituric acids tested, merbarone was the only one with anti-malignant activity to cure L1210 leukemia and inhibit several murine tumors. It acts as a catalytic topo II inhibitor and interestingly does not affect DNA-protein binding. Nevertheless, it shows minimal effects against topoisomerase I. QAP1 is one kind of purine analogue that targets DNA-ATPase and inhibits the catalytic activity of topoisomerase II.

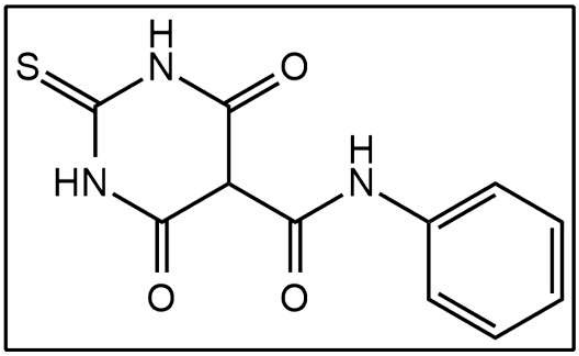

Figure 4.2.4 Merbarone

4.2.5 Doxorubicin

Doxorubicin is an anthracycline antibiotic that displays cytotoxic activity against a variety of tumor cells including small cell lung cancer. It is isolated from *Streptomyces peucetius* bacterial culture and has been an essential part of standard chemotherapy in cancer treatment.

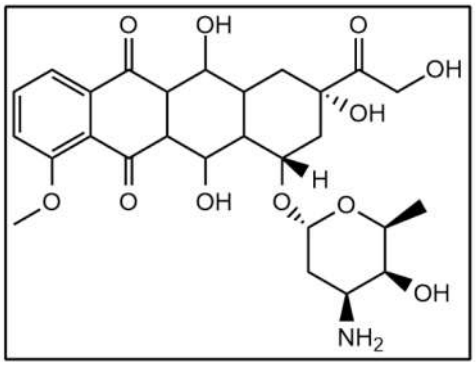

Figure 4.2.5 Doxorubicin

4.2.6 Epirubicin

Epirubicin, a semisynthetic derivative of DOX, shows some positive characteristics such as increased volume of distribution and enhanced total body clearance of drug. Similarly Idarubicin derived from Daunorubicin shows broad spectrum of action, better lipophilicity, and increased cellular uptake.

4.2.7 Bisdioxopiperazine Derivatives

Many bisdioxopiperazine derivatives such as ICRF-154, ICRF-159, MST-16 and ICRF-187 are promising topo II inhibitors that stall catalytic activity of topoisomerase II. They are intense chelating agents and they restrict Topo II in the reactant cycle after the strand passage before the second ATP is hydrolyzed (Nitiss, 2009). ICRF-193 inhibits the function of topoisomerase II by forming a noncovalent structure where DNA is surrounded by the topoisomerase. Nevertheless no activity has been found against topoisomerase I.

4.2.8 Quinolone Derivatives

A-62176 and A-74932 are quinolone derivatives, classified under the family of Quinobenoxazines that target specifically DNA gyrase enzyme and inhibits topoisomerase II enzyme activity. Colon carcinoma and breast carcinoma cells can be 50% inhibited by the impressive cytotoxicity of these drugs at 0.01-0.06 μg/ml concentration.

4.3 Topoisomerase I &II Inhibitor

Development of combined Topo I and Topo II inhibitor has gained much interest for implementation in anticancer therapy. Targeting both topoisomerase enzymes not only enhances anti-tumor function but also reduces the drug resistance as showed with camtothecins. Previously etoposide was combined with topotecan that lead to complexity in dosing schedule and toxicities. Bortezomib is a suitable agent for combined therapy with other topo II targeting agents (Nitiss, 2009).

4.3.1 DACA

[2-dimethylamino]ethyl]acridine-4-carboxamide (DACA) is a cytotoxic drug which binds to DNA throughintercalation and enhances DNA cleavage by inhibiting both topoisomerase I and II(Seo, 2015). The carboxamide chain at 4 position of the ring enables it to interact with DNA, casing DNA damage and cytotoxicity.

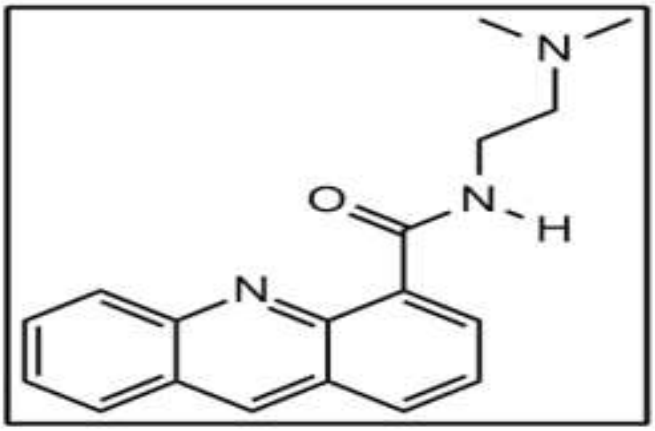

Figure 4.3.1 DACA

5. Targeting EZH2 as Epigenetic Therapy

Aberrant alteration of DNA and its histone proteins through histone deacetylation, DNA methylation, or trimethylation of lysine 27 on Histone 3 (H3K27M3) result in a deregulated epigenome and the silencing of tumor suppressor genes like p16.

Tumor suppressor genes can be epigenetically silenced when polycomb group of proteins interact with the regulatory elements of the genes. Polycomb group of proteins are mainly multiprotein complexes containing several catalytic subunits that induce silencing of targeted genes.The polycomb repressive complex 2 (PRC2) is capable of regulating H3K27M3 in association with Enhancer of Zeste homolog 2 (EZH2) and other EED, YY1, SUZ12 catalytic subunit.

The characteristics and functions of all the components of PRC2 are not yet understood. EZH2 is a major regulator of the epigenome. EZH2 requires other subunits of PCR2 like EED, SUZ12 for catalytic activity. Moreover, EZH2 interacts with chromatin stimulated by RbAp46/48 and AEBP2 (Cao, & Zhang, 2004). In humans, functions of PCR2 are essential for maintaining self-renewing capacity of stem cells (Lee TI, Jenner RG, Boyer LA *et al.2006)*

EZH2 has been found in hematopoietic stem cells and is believed to conserve their potential.(McCabe, & Creasy, 2014). EZH2 resides mainly in the nucleus but also can be found in cytoplasm.(Su et al., 2005). All members of EZH family consist of four domains which are homologous in nature: homologue domain I, homologue domain II, cysteine rich domain, and C-terminal SET domain.

EZH2 and EZH1 are both homologs of EZ in vertebrates. Though both of them interact with catalytic subunits of PCR2 complex, they differ in their expression patterns and functional roles. EZH2 is considered to be responsible for methyl transferase activities. In the hematopoietic system, EZH2 regulates cell proliferation and cell differentiation of T cells (Su et al., 2005).Aberrant alteration of EZH2 functions due to somatic mutation causes myelodysplastic syndrome, nonHodgkin lymphoma, B-cell lymphoma and T-cell acute lymphoblastic leukemia.

Earlier research demonstrated that E2F1 transcription factor regulatesEZH2 gene promoter activity and EZH2 mRNA is expressed via RB-E2F1 pathway (Bracken, 2003). Several studies show that EZH2 is responsible for progression of a varieties of cancers. When EZH2 is over-expressed, it transfers methyl group onto lysine 27 of histone 3 and represses targeted genes including p16. Silencing of p16, a major tumor suppressor gene, plays a decisive role in a variety of malignancies such as breast, cancer, prostate, skin, and bladder cancer.

EZH2 plays a significant role in normal development of B-cells, lymphomagnesis and formation of germinal center (Su et al., 2002). Mutant EZH2 causes aberrant proliferation of B cells accompanied by increased plasma cells that produces antibody. Overexpression of EZH2 in B cells results in multiple myeloma, and EZH2 is considered one of the oncogenic targets in MM. Several EZH2 inhibitors including GSK126, EPZ-6438, and EPZ005687 show anti-lymphoma activities.

5.1 Down-Regulating EZH2 in NSCLC

Non-small cell lung cancer displays abnormal epigenetic modifications, resulting from over-expression of EZH2. EZH2 interacts directly with histone deacetylase enzyme 1 and 2 from class I HDACs. HDAC requires the supportive EZH2 function that mediates the transcriptional repression of targeted genes. HDAC inhibitors down regulate varieties of PCR2 proteins (Fiskus et al., 2006). To treat NSCLC with HDAC inhibitors, EZH2 downregulation is required. To accomplish that DZNep is combined with an HDAC inhibitor. DZNep and the Vorinostat co-treatment provide synergistic effects to suppress the proliferation of NSCLC cell lines. Like in other class of cancer cell lines, the synergistic effect is associated with inhibition of EZH2 and PCR2 activity, preventing trimethylation of Lysine (K27) residue on histone H3 (H3K27me3). In addition to EZH2 depletion, the co-treatment also accumulates p27Kip1, reduces cyclin A and induces apoptosis of cancer cells. Suppression of wild type as well as mutant EGFR cell has also been observed (Takashina et al., 2016).

5.2 Therapeutic Programs of Targeting EZH2

5.2.1 Inhibition of EZH2 Transcription

SKI-606 (bosutinib) inhibits EZH2 transcription in a variety of cancer cells and recently it has been approved for chronic myelogenous leukemia in adult patients. SKI-606 (bosutinib) reduces the expression of EZH2 in breast cancer by limiting the phosphorylation of Src (Non receptor tyrosine kinase) responsible for mammary tumors. SKI-606 decreases mRNA of EZH2 to 50% and induces re-expression of E-cadherin to form organized epithelium of normal cells. Further experimentation is currently improving efficacy of SKI-606.

Methotrexate which is a dyhydrofolate reductase inhibitor exhibits anticancer effects in NSCLC by down-regulating expression of EZH2 at the transcriptional level.

5.2.2 Inhibition of EZH2 Translation

siRNAs targeting EZH2 mRNA have been used to down-regulate EZH2 expression in prostate cancer cells (Varambally et al., 2002). shRNA (short heparin RNA), targeting EZH2 mRNA, has been used to inhibit EZH2 protein translation and inhibit tumor formation in hepatocellular cells and delay cell cycle in G2/M phase in breast cancer cells (Gonzalez et al., 2008).Earlier it has been demonstrated that defects in micro RNA causes over expression of EZH2. Micro RNA-101 is supposed to have tumor suppressing functions and increasing the level of miRNA has been a successful strategy to treat various cancers including gastric, lung and bladder cancer. miR-26a arrests the cell cycle at the G1 phase and suppresses the growth of nasopharyngeal carcinoma cells. miR-214 reduces invasion of breast cancer cells and suppress the tumorigenicity of HCC cells (Han Li, & Chen, 2015) .

5.2.3 Inhibition of EZH2 by Depletion

3-Deazaneplanocin A

The most common EZH2 inhibitor is 3- deazaneplanocin A. Known as DZNep, it depletes the EZH2 level in cancer cells, inhibits hydrolysis of S-adenosyl homocysteine and demethylates H3K27. DZNep also depletes other PCR2 proteins EED and SUZ12 along

with EZH2. DZNep shows selectivity in its effect against cancer cells without affecting normal cells. DZNep strongly hinders CSC, inhibits tumor formation activity of HCC and causes apoptosis of AML.

DZNep is derived from 3-deazaadenosine as a cyclopentanyl analog. It increases the intracellular concentration of AdoHcy (S-adenosyl-Lhomocysteine) by preventing the action of AdoHcy hydrolase enzyme that converts the AdoHcy into adenosine as well as homocysteine through hydrolysis reaction. As a result, KMTase activity, which is dependent on S-adenosyl-L-methionine, is inhibited. Undermethylation of mRNA also results from stalling methyltransferase activity by DZNep(Jiang et al., 2008). Epigenetic therapy is often more effective when combination therapy is implemented. DZNep,along with HDAC inhibitors such as Trichostatin A, de-represses the FBXO32 gene (FBXO32 is part of the E3 ligase complex, an essential component of stem cells) and other genes, silenced by PCR2.

DZNep depletes the expression of EZH2 as well as SUZ12 in colon and breast cancer cells. To demonstrate anti-malignant effects, DZNep was used on OCI-AML3 and HL-60 cancer cells with promising results. Despite the promising potential of DZNep some drawbacks still limit its use such as short half-life of the drug in plasma, non-specificity of histone methylation inhibition, and its toxicity.

3-deazaadenosine is another compound that acts in a manner similar to DZNep. It depletes EZH2 level from cancer cells, inhibits growth of cancer cell and induces cell cycle arrest.

Panobinostat (LBH589) initially known as a pan HDAC inhibitor has recently been found to degrade EZH2 protein as well. The drug blocks interactions of EZH2 with DNMT1 and indirectly inhibits DNA methylation along with EZH2 depletion. Panobinostat combined with DZNep gives synergistic effect to deplete more EZH2. Significant accumulation of drug and decrease of EZH2 expression have been reported when DZNepwas combined with Probinostat. The experiment on primary AML cells has demonstrated that combined treatment of DZNep(500nM) along with PS (20nM) produces more lethality of cancer cells by inhibiting over-expression of EZH2 and SUZ12 as well as EED than any single agent (Fiskus et al., 2009). Combined therapy of DNZepand HDAC/DNMT inhibitors re-express certain genes which are targeted by EZH2 and silenced. (Sun et al., 2009).

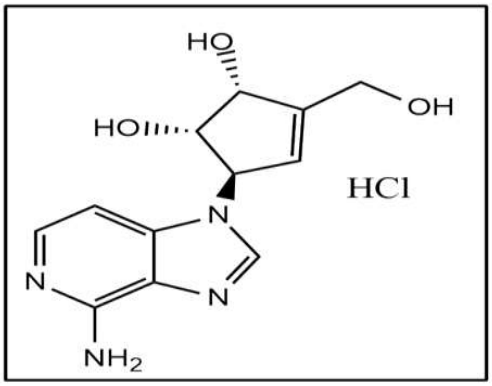

Figure 5.2.3 3-Deazaneplanocin A

5.2.4 Modification in EZH2 Phosphorylation

EZH2 differs from EHZ1 in having a critical threonine residue which is vulnerable to phosphorylation. Phosphoryltation of Tyr 345 enhances EZH2 activity, while phosphorylation of another residue including Tyr487 impairs the EZH2 binding with other proteins of PCR2. Additionally, phosphorylation of Ser21 attenuates EZH2 by reducing methyltransferase activity.

5.3 Selective EZH2 Inhibitors

GSK343, GSK126, GSK503, EPZ-6438, EI-1 and EPZ005687 are the highly selective EZH2 inhibitor containing dimethyl-pyridone and very effective against mutant and wild type EZH2. (McCabe, & Creasy, 2014).

5.3.1 GSK126

GSK126 competitively binds with EZH2 and inhibits SAM from interacting with EZH2. GSK126 shows effective potency in vivo experiments after intraperitoneal administration.The high relevance of EZH2 has been found in CRPC and primary PCa. EZH2 protein can be down regulated by using chemotherapeutic agents such as campthothecin. However, CRPC cells are subjected to apoptosis more efficiently with EZH2 inhibitor co-treatment.

GSK126 is one class of selective EZH2 inhibitors that inhibits both Polycomb-dependent (Pcd) as well as Polycombindependent(PcI) functions and stalls methyltranferase activity.

GSK126 de-represses the BRACHYURY, DAB2IP, FOXJ1 andHOXA9 genes which have been repressed by EZH2 activity while it inhibits the expression of TMEM48, KIAA0101, and CSK2 genes (Wu et al., 2016).

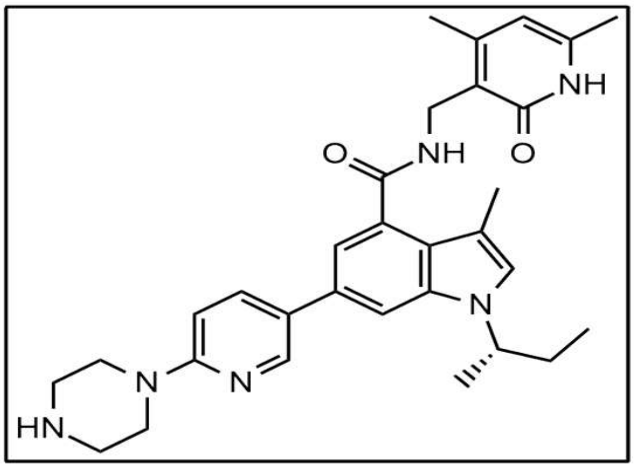

Figure 5.3.1 GSK126

5.3.2 GSK343

GSK343 has been discovered after the discovery of GSK126. It resembles GSK126 in activity against EZH2 but differs in structural components. It contains indazole ring with several substituted compounds like piperazine-substituted pyridine (Xu, Konze, Jin, & Wang, 2015).

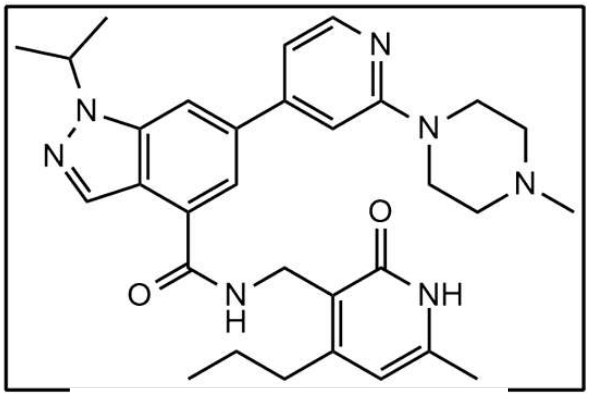

Figure 5.3.2 GSK343

5.3.3 UNC1999

UNC1999 is structurally different from GSK143 in pyridine substitution. This small modification in the structure provides better pharmacokinetic properties for UNC1999. UNC1999 shows more beneficial effects of oral bioavailability along with high selectivity of both wild type and Y641 mutant form of EZH2 while other compounds mostly require injection. It provides ten-fold less activity on EZH1 than EZH2 protein (Konze et al., 2013).

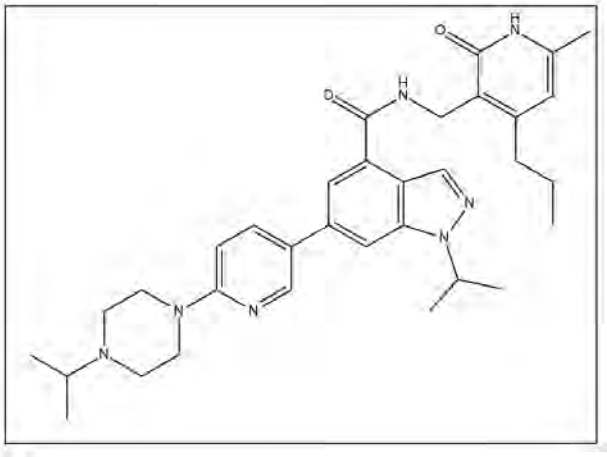

Figure 5.3.3 UNC1999

5.3.4 EPZ005687 and EPZ-6438

Several competitive EZH2 inhibitors have been identified such as EPZ005687 and EPZ-6438. EPZ005687 acts against both wild type and mutant EZH2 such as Y641-mutant. It exhibits 500 times more selectivity for EZH2 than other methyltranferase enzymes, specifically 50 times over EZH1(Kim, & Roberts, 2016). EPZ005687 is also selective against mutant lymphoma cell in a dose-dependent manner. EPZ-6438 is better than EPZ005687 with respect to pharmacokinetic characteristics including better oral bioavailability.

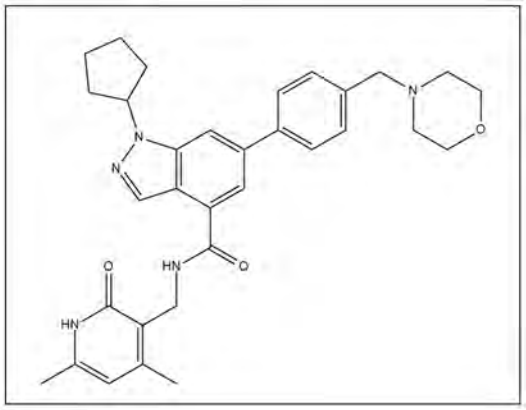

Figure 5.3.4 EPZ005687

5.3.5EI1

EI1 is another S-adenosylmethionine competitive inhibitor that selectively inhibits wild type or mutant EZH2 more than 10000 times over other groups while 90 times more selective over EZH1(Qi et al., 2012). EI1 reduces trimethylation on lysine 27 of histone 3 without leaving any impact on EZH2 levels and causes cyclic arrest, apoptosis of EZH2-mutated cancer cells like *EZH2*-mutant DLBCL cells or *SMARCB1*-mutant rhabdoid tumor cell.

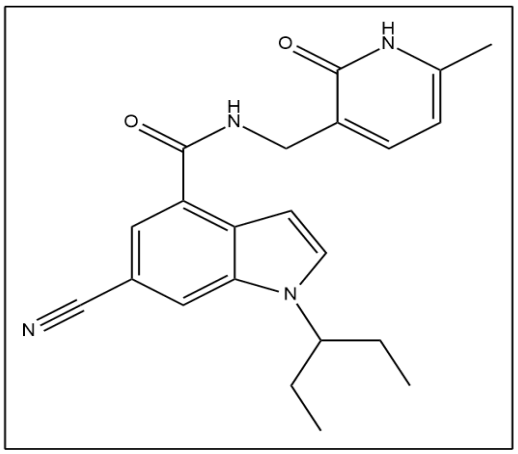

Figure 5.3.5 EI1

5.4 Therapy Resistance and Combination Therapies

There are two EZH2 mutants (Y111L and Y661D) that show resistance to inhibitors. Combined therapy has proven to be effective in countering drug resistance. EPZ-6438, the EZH2 inhibitor, shows synergistic effects with conventional chemotherapy against EZH2-mutated NHL cells (Knutson et al., 2014). EPZ-6438 is also co-administered with glucocorticoid receptor agonist that prevents germinal center proliferation in Non-Hodgkin lymphoma. Consequently GSK126, another potent EZH2 inhibitor enhances apoptosis of cell lines of prostate cancer along with etoposide chemotherapy.

It is suggested that simultaneous inhibition of EZH2 and DNMT1 would be more effective treatment against malignancy because EZH2 interacts with and directly regulates DNMT1 along with DNMT3a and DNMT3b methyltransferase enzymes.

6. Conclusion

Epigenetic contributors to the development of cancer are currently broadly acknowledged. Notwithstanding continued efforts, our capacity to successfully focus on the epigenome is still limited. Undoubtedly combining traditional anticancer therapy with epigenetic modifiers to reverse the abnormal epigenetic patterns holds tremendous potential for effective treatment of hematologic as well as solid malignancies. Epigenetic drugs have been a promising new class of anticancer therapeutics that have demonstrated positive responses in both preclinical and clinical experiments. A few FDA-approved drugs target different components of the epigenetic apparatus, most prominently DNA methylation, histone deacetylation, aberrant activity of EZH2 and topoisomerases. Regardless of recent advances in the utilization of epigenetic medication, specificity has remained a major challenge. Epi-drugs often induce global changes in epigenetic patterns. Efforts are currently underway to target epigenetic enzymes to specific genomic regions, using the CRISPR-Cas9 technology. This will go a long way in reducing side-effects of epi-drugs. It is essential to discover more specific epigenetic drugs with minimal toxicity which will be compelling particularly in patients with intractable malignancies. As was found years ago with chemotherapy, an emerging insight is that epigenetic medications often function best when they are administered in conjunction with other therapeutic programs. The most intriguing and therapeutically encouraging avenue right now is the combination of epigenetic drugs with immunotherapy, which is now being tested in numerous clinical trials. The field of epigenetic therapy is set to grow at an exponential rate with fresh ideas and insights gained from basic as well as translational research.

7. Appendix: Glossary

- **Genetics:** Genetics is the study of heredity. Heredity is a biological process where a parent passes certain genes onto their children or offspring. Every child inherits genes from both of their biological parents and these genes in turn express specific traits. Some of these traits may be physical for example hair and eye color and skin color etc. On the other hand some genes may also carry the risk of certain diseases and disorders that may pass on from parents to their offspring.
- **Epigenetics:** Epigenetics is the study of potentially heritable changes in gene expression (active versus inactive genes) that does not involve changes to the underlying DNA sequence, a change in phenotype without a change in genotype which in turn affects how cells read the genes
- **Epigenetic change**: Epigenetic change is a regular and natural occurrence but can also be influenced by several factors including age, the environment/lifestyle, and disease state. Epigenetic modifications can manifest as commonly as the manner in which cells terminally differentiate to end up as skin cells, liver cells, brain cells, etc. Or, epigenetic change can have more damaging effects that can result in diseases like cancer. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are currently considered to initiate and sustain epigenetic change.

- **DNA:** DNA, or deoxyribonucleic acid, is the hereditary material in humans and almost all other organisms. Nearly every cell in a person's body has the same DNA. Most DNA is located in the cell nucleus (where it is called nuclear DNA), but a small amount of DNA can also be found in the mitochondria (where it is called mitochondrial DNA or mtDNA).The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T)

- **RNA:** Ribonucleic acid (RNA) is a linear molecule composed of four types of smaller molecules called ribonucleotide bases: adenine (A), cytosine (C), guanine (G), and uracil (U). RNA is synthesized from DNA by an enzyme known as RNA polymerase during a process called transcription. RNA is then translated into proteins by structures called ribosomes. There are three types of RNA involved in

the translation process: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA).

- **Histone:** Histones are a family of basic proteins that associate with DNA in the nucleus and help condense it into chromatin. Nuclear DNA does not appear in free linear strands; it is highly condensed and wrapped around histones in order to fit inside of the nucleus and take part in the formation of chromosomes. Histones are basic proteins, and their positive charges allow them to associate with DNA, which is negatively charged. Some histones function as spools for the thread-like DNA to wrap around.

- **DNA methylation:** DNA methylation is an epigenetic mechanism that occurs by the addition of a methyl (CH_3) group to DNA, thereby often modifying the function of the genes. The most widely characterized DNA methylation process is the covalent addition of the methyl group at the 5-carbon of the cytosine ring by methyltransferase group resulting in 5-methylcytosine (5-mC), also informally known as the “fifth base” of DNA. These methyl groups project into the major groove of DNA and inhibit transcription
- **EZH2:** The EZH2 gene encodes a histone methyltransferase that constitute the catalytic component of the polycomb repressive complex-2 (PRC2), which functions to initiate epigenetic silencing of genes involved in cell fate decisions. EZH2 specifically methylates nucleosomal histone H3 at lysine-27 (H3-K27)

- **PRC2:** PRC2 (Polycomb Repressive Complex 2) is one of the two classes of polycomb-group proteins or (PcG).The other component of this group of proteins is PRC1 (Polycomb Repressive Complex 1).This complex has histone methyl-transferase activity and primarily tri-methylates histone H3 on lysine 27 (i.e. H3K27me3), a mark of transcriptionally silent chromatin. PRC2 is required for initial targeting of genomic region (PRC Response Elements or PRE) to be silenced,
- **Topoisomerase:** Topoisomerases are enzymes that participate in the over-winding or underwinding of DNA. The winding problem of DNA arises due to the intertwined nature of its double-helical structure. During DNA replication and transcription,

DNA becomes overwound ahead of a replication fork. If left unabated, this torsion would eventually stop the ability of DNA or RNA polymerases involved in these processes to continue down the DNA strand

- **Epigenetic drug:** Epigenetic drugs are small-molecule drugs named for their ability to alter gene expression by targeting epigenetic regulators, could enable powerful new strategies to combat diseases marked by aberrant gene expression, like cancer.
- **CpG island:** The CpG island is a short stretch of DNA in which the frequency of the CG sequence is higher than other regions. It is called the CpG islands, where "P" simply indicates that cytosine and guanine are connected by a phosphodiester bond.
- **Transcription:** Transcription is the first step of gene expression, in which a particular segment of DNA is copied into RNA (especially mRNA) by the enzyme, RNA polymerase.
- **Translation:** Translation is the process of translating the sequence of a messenger RNA (mRNA) molecule to a sequence of amino acids during protein synthesis.
- **Post translational modification:** Post-translational modification (PTM) refers to the covalent and generally enzymatic modification of proteins during or after protein biosynthesis. Proteins are synthesized by ribosomes translating mRNA into polypeptide chains, which may then undergo PTM to form the mature protein product
- .**Non-coding RNA: A** non-coding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into proteins. Epigenetic related ncRNAs include miRNA, siRNA, piRNA and lncRNA. In general, ncRNAs function to regulate gene expression at the transcriptional and post-transcriptional level.
- **Promoter:** In genetics, a promoter is a region of DNA that initiates transcription of a particular gene.
- **Mutation:** Mutation is the changing of the structure of a gene, resulting in a variant form that may be transmitted to subsequent generations, caused by the alteration of single base units in DNA, or the deletion, insertion, or rearrangement of larger sections of genes or chromosomes.
- **Cell cycle:** The cell cycle or cell-division cycle is the series of events that take place in a cell leading to its division and duplication of its DNA (DNA replication) to

produce two daughter cells. In cells with a nucleus, as in eukaryotes, the cell cycle is also divided into three periods: interphase, the mitotic (M) phase, and cytokinesis.

- **Nucleoside:** Nucleosides are the basic building blocks of nucleic acids: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).
- **Nucleotides:** Nucleotides are the phosphate esters of nucleosides: ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and a phosphate group.
- **Cytotoxic drug:***Cytotoxic*drugsdescribe a group of medicines that contain chemicals which are toxic to cells, preventing their replication or growth, and so are used to treat cancer.
- **Intercalation:** Intercalation is the insertion of molecules between the planar bases of DNA. This process is used as a method for analyzing DNA and it is also the basis of certain kinds of poisoning.
- **Acute myelocytic leukemia:** Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal white blood cells that build up in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its incidence increases with age
- **Histone acetylation & deacetylation:** Histone acetylation and histone deacetylation involve the addition or removal of an acetyl group on lysine residues in the N-terminal tail and on the surface of the nucleosome core of histone proteins. Acetylated and deacetylated histones are considered epigenetic tags within chromatin by relaxing (euchromatin) or tightening (heterochromatin) chromatin structure, subsequently increasing or decreasing gene transcription levels.

- **Lymphopenia:** Lymphocytopenia, or lymphopenia, is the condition of having an abnormally low level of lymphocytes in the blood. Lymphocytes are a white blood cell with important functions in the immune system. The opposite is lymphocytosis, which refers to an excessive level of lymphocytes.

- **Neuropathy:** Neuropathy is a term that refers to general diseases or malfunctions of the nerves. Nerves at any location in the body can be damaged from injury or disease. Neuropathy is often classified according to the types or location of nerves

that are affected. Neuropathy can also be classified according to the disease causing it. (For example, neuropathy from the effects of diabetes is called diabetic neuropathy

- **Myelodysplastic syndrome:** Myelodysplastic Syndromes (MDS) are a group of diverse bone marrow disorders in which the bone marrow does not produce enough healthy blood cells. MDS is often referred to as a "bone marrow failure disorder". MDS is primarily a disease of the elderly (most patients are older than age 65), but MDS can affect younger patients as well.
- **Aminocoumarin drug:** Aminocoumarin is a class of antibiotics that act by an inhibition of the DNA Gyrase enzyme involved in the cell division.
- **Anthracycline antibiotic:** Anthracycline antibiotics are an important group of antitumor drugs widely used in cancer chemotherapy. However, despite the increasing interest in these chemotherapeutic agents, their mechanism of action is not yet completely understood.
- **Chemotherapy:** Chemotherapy is the use of medication (chemicals) to treat disease. More specifically, chemotherapy typically refers to the destruction of cancer cells. However, chemotherapy may also include the use of antibiotics or other medications to treat any illness or infection
- **DNA ligase enzyme:** DNA ligase is a specific type of enzyme, a ligase, that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond between 5-phosphate of one DNA strand and 3-hydroxyl of another.
- **DNA gyrase enzyme:** DNA gyrase is an essential bacterial enzyme that catalyzes the ATP-dependent negative super-coiling of double-stranded closed-circular DNA. Gyrase belongs to a class of enzymes known as topoisomerases that are involved in the control of topological transitions of DNA
- **Bioavailability:** In pharmacology, bioavailability (BA) is a subcategory ofabsorption and the fraction of an administered dose of unchanged drug that reaches the systemic circulation, one of the principal pharmacokinetic properties of drugs. By definition, when a medication is administered intravenously, its bioavailability is 100%

- **Pharmacokinetics:** Pharmacokinetics is the study of drug absorption, distribution, metabolism, and excretion.
- **Pharmacodynamics:** Pharmacodynamics is the study of the biochemical and physiologic effects of drugs (especially pharmaceutical drugs).Pharmacodynamics is the study of how a drug affects an organism, whereas pharmacokinetics is the study of how the organism affects the drug.

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