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Characterization of BIX-01294 and its analogs E67 and E11 on growth and reactivation of epigenetically silenced tumor suppressor genes in human breast cancer cells.

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Characterization of BIX-01294, and its analogs E67 and E11 on growth and reactivation of epigenetically silenced tumor suppressor genes in human breast cancer cells.

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M.Sc , Bharathiar University, 2002

Advisor: Paula M. Vertino, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
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Abstract:

Epigenetic silencing of tumor suppressor genes in cancers are characterized by DNA hypermethylation and local alterations in histone modifications, including increased repressive histone modifications such as histone H3K9me2/3, H3K27me3 and a decrease in activating histone modifications such as histone H3K4me2/3. G9a and G9a-like protein (GLP) are euchromatin associated histone methyltransferases that repress transcription by methylating histone H3 at K9. BIX-01294 was originally identified as a G9a inhibitor during a chemical library screen for specific inhibitors against histone methyltransferases (HMTases); E67 and E11 are lysine mimics and analogs of BIX-01294 that have been reported to have better inhibitory activity towards GLP in comparison to BIX-01294 in *in vitro* enzyme assays. In this study, we have characterized the impact of three small molecule inhibitors- BIX-01294, E67 and E11 in breast cancer cells. We show that all three agents effectively inhibit the growth of breast cancer cells with IC_{50} values between 3.0 μM and 10.0 μM. BIX-01294 and E11 are the most potent; E67 is the least potent. As single agents, these inhibitors have minimal effects on the expression of epigenetically silenced tumor suppressor genes in breast cancer cells. We also show BIX-01294 and E67 are antagonistic to 5-Aza-2'-Deoxycytidine (5-azaCdR) induced demethylation at the *TMS1* locus. Our findings indicate that these inhibitors exert their effects *in vivo* without altering the protein levels of G9a and DNMT1. Finally, we show a significant decrease in global levels of histone H3K9me2 in cancer cells treated with these agents. Our results propose that these agents harbor different growth inhibitory activity, differential effects on DNA methylation of a tumor suppressor gene and similar effects on down-regulation of H3K9me2 modification, in breast cancer cells. Studying the mechanism of action of BIX-01294, E67 and E11 by themselves or in combination with 5'azaCdR will allow for the further development of epigenetic therapy of human breast cancer.

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Table of Contents

Chapter 1: Introduction…… 1

Figures ……26

Chapter 2: Material and Methods……... 35

Figures…….....35

Chapter 3: Results……42

Figures……...54

Chapter 4: Discussion …….. 81

Figures……..91

References ……....93

List of Figures and Tables

Chapter 1: Introduction

Figure 1: DNA methyltransferase reaction

Figure 2: The pictorial representation of various post translational histone modifications and their biological roles.

Figure 3: Domain organization of the core G9a components: G9a, GLP and Wiz

Figure 4: Schematic representation of the mouse G9a locus

Figure 5: DNA methylation and histone modification patterns are altered in cancers

Figure 6: Epigenetic drugs for cancer therapy

Figure 7: Chemical structure of the compound BIX-01294

Figure 8: Chemical structures of E67 and E11(BIX-01294 derivatives)

Figure 9: Diagram of the TMS1 gene

Chapter 2: Materials and Methods

Figure 10: Schematic of the experimental design

Chapter 3: Results

Figure 11: Effect of BIX-01294 and its analogs on the growth of breast cancer cells

Figure 12: Effect of BIX-01294 and its analogs on the growth of normal immortalized breast epithelial cells

Figure 13-16: Effect of BIX-01294, its analogs and/or 5-azaCdR on gene expression in MDA-MB231 cells

Figure 17: Effect of BIX-01294 and/or 5-azaCdR on DNA methylation at the TMS1 locus

Figure 18: Effect of E67 and/or 5-azaCdR on DNA methylation at the TMS1 locus

Figure 19: Effect of E11 and/or 5-azaCdR on DNA methylation at the TMS1 locus

Figure 20: Effect of BIX-01294, its analogs and/or 5-azaCdR on G9a and DNMT1 protein levels in breast cancer cells

Figure 21: Effect of BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K9me2 modification in breast cancer cells

Figure 22: Effect of BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K9me3 modification in breast cancer cells
Figure 23: Effect of BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K9me1 modification in breast cancer cells
Figure 24: Effect of BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K27me2 modification in breast cancer cells
Figure 25: Effect of BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K4me2 modification in breast cancer cells

Chapter 4: Discussion
Figure 26: Model for epigenetic regulation of BIX-01294, its analogs and 5'azaCdR at the promoters of epigenetically silenced tumor suppressor genes in MDA-MB231 cells

Tables: Results
Table 1: Approximate IC_{50} values of BIX-01294, E67 and E11
Table 2: Percent CpG demethylation at the *TMS1* locus in untreated and treated MDA-MB231 cells

CHAPTER 1

INTRODUCTION

I). BREAST CANCER AND BREAST CANCER GENETICS:

Cancer is a group of diseases that result from cells in the body that change and grow out of control. Most types of cancers eventually form a lump or mass called a tumor and are named after the part of the body from where the tumor originates. Breast cancer originates in the breast, which is made up of glands for milk production called lobules, and ducts that connect the lobules to the nipple. The remainder of the breast is made up of fatty, connective and lymphatic tissue. Breast cancers can be *in situ* that is confined within the lobules (lobular carcinoma) or ducts (ductal carcinoma); nearly all cancers detected at this stage are cured. Most diagnosed breast cancers are already invasive, meaning that they have started to grow beyond the confines of the normal duct and invade the neighboring tissues of the breast [1].

Breast cancer is the second leading cause of cancer deaths in women and is the most frequently diagnosed cancer. The American Cancer Society has estimated that in the US, 207, 090 women were diagnosed with invasive breast cancer and 39,840 women died of breast cancer in 2010. The five-year survival rate among women whose breast cancer has not spread beyond the breast at the time of diagnosis is 98.3%. Approximately 5-10% of breast cancer is inherited. Most of these familial breast cancers are associated with mutations in *BRCA1* or *BRCA2* (breast cancer associated genes) genes. Inherited mutations in ATM, p53, CHEK-2, genes also may increase the woman's chance of developing breast cancer. Individuals with mutations in these genes are prone to the development of other cancers as well. Given the prevalence and severity of this disease,

it becomes critically important to study the underlying molecular mechanisms that contribute to this disease, thereby opening up new avenues of therapy including genetic and epigenetic therapy in combination with conventional medical treatments.

II). EPIGENETICS AND TRANSCRIPTIONAL REGULATION:

The term ‘Epigenetics’ literally means ‘over’ or ‘on top of’ genetics, and was coined by Conrad Waddingdon in the early 1940s to explain why genetic variations sometimes did not lead to phenotypic variations and how genes might interact with their environment to yield a phenotype [2]. Epigenetic changes can be defined as stable molecular alterations of a cellular phenotype, such as the gene expression profile that are heritable across somatic cell divisions (sometimes germ line transmissions) but do not involve changes in the DNA sequence itself [3].

In the nuclei of all eukaryotic cells, DNA is packed into chromatin, a highly organized and dynamic DNA-protein complex, consisting of histone and nonhistone proteins, which is the prime regulatory platform for gene transcription. The nucleosome is the fundamental subunit of chromatin and is composed of an octamer of four core histones- two H2A/H2B dimers, one H3/H4 tetramer around which 146 bp of DNA are wrapped [4]. Histones are small basic proteins composed of a globular domain and a more flexible and charged amino- terminus (histone tail) that protrudes from the nucleosome surface [5].

Several epigenetic mechanisms regulate gene expression such as DNA methylation, post-translational histone modifications, and functional noncoding RNA [6]. Euchromatin (open chromatin) and heterochromatin (closed chromatin) are controlled by N-terminal modifications of histone tails, DNA methylation and by the binding of a

number of non-histone proteins such as G9a/GLP, LSD1, JARID1b, etc, which belong to a group of histone modifying enzymes.

Epigenetic modifying enzymes including DNA methyltransferases (DNMTs,) histone methyltransferases (HMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone ubiquitinases, etc, play critical roles in transcriptional regulation. There is no compelling evidence yet that histone modifications are subject to mitotic inheritance although genomic methylation patterns are faithfully inherited [7]. In general, genomic DNA methylation and patterns of histone modifications regulate the local transcriptional potential of genes.

III). GENOMIC DNA METHYLATION:

Genomic DNA methylation is a post-replication modification that occurs on the 5^{th} position of the pyrimidine ring of cytosines in the context of the CpG dinucleotide. DNA methylation is the covalent addition of a methyl group catalyzed by DNA methyl transferases using S-adenosyl-L-methionine (SAM) as the methyl donor (Figure 1).

CpG islands are sequences where CpGs are found at a high frequency in the genome[8]. Most of the 29,000 CpG islands in the human genome are present in the promoter region of almost half the genes and are usually unmethylated in normal cells [9, 10]. CpG islands are present in the 5'-regions of 60% of all genes; with the exception of some CpG islands located within the 3' ends of genes. The distribution of methylated and unmethylated CpG dinucleotides is non-random [11], certain genomic sites such as imprinted regions, genes on the inactive X chromosomes, endogenous retrotransposons, pericentromeric heterochromatin regions are hypermethylated whereas other sites such as CpG islands associated with gene promoter regions are usually unmethylated [12, 13].

DNA methylation is conserved in many eukaryotic organisms including many fungi, plants, and animals, although it has been lost in the budding yeast *Saccharomyces cerevisiae* and the nematode worm *Coenorhabditis elegans* [14]. DNA methylation is conserved in eukaryotes and it provides unique means for setting up heritable control of gene regulation which is important for genomic imprinting and X chromosome inactivation, control of cell differentiation and development, chromatin modifications and silencing of endogenous retroviruses [12, 14, 15]. DNA methyltransferases are evolutionarily conserved in eukaryotes and show considerable sequence conservation in their catalytic domains. Two mechanisms have been proposed that may contribute to gene repression by DNA methylation [16]. 1). Cytosine methylation prevents the binding of transcription factors at transcription factor binding sites thereby promoting gene repression. 2). Recognition of methylated cytosines by methyl CpG binding proteins such as MeCP2, MBD1, MBD2 and MBD3 followed by the recruitment of transcription co-repressor complexes by these proteins promotes gene repression.

Methylated cytosines are more prone to modifications by endogenous and exogenous mutational mechanisms than other bases of DNA. Mutation rates at CpG sites have been estimated to be higher by 40 times than other transition mutations [17]. DNA methylation plays an important role in maintaining transposons in an inert state in mammalian cells and inhibits the expression and transposition of endogenous retrotransposons, thereby decreasing the mutational load [18, 19]. Overall, the above information indicates that DNA methylation is a pivotal heritable epigenetic modification that is conserved in eukaryotes and plays important roles in embryonic development and gene regulation.

DNA Methyltransferases:

DNA methyltransferases can be classified into maintenance DNA methyltransferases and *de novo* DNA methyltransferases. DNA methylation is maintained by DNMT1 which functions during DNA replication to copy the methylation patterns onto newly synthesized DNA. Dnmt3A and Dnmt3B are *de novo* methyltransferases, which establish DNA methylation patterns during embryonic development. DNMT1 also does *de novo* methylation *in vitro* and *in vivo* [12, 15, 20], and maintenance of methylation in certain regions of the genome requires DNMT3A and DNMT3B [21]. Mouse embryos null for Dnmt1 or lacking Dnmt3A and Dnmt3B arrest early in gestation and die around day 9.5 due to genomic instability resulting from activation of endogenous retroviruses and demethylation of minor and major satellite repeats [18, 22].

DNMT1 has an inherent preference for hemimethylated DNA in order to function as a maintenance methyltransferase [23]. UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) and DNMT1 are chromatin-associated proteins, wherein UHRF1 confers the preference for hemimethylated DNA and helps to tether DNMT1 to the chromatin during replication to facilitate efficient CG methylation. UHRF1 and DNMT1 physically interact and co-localize in the chromatin [24]. Proliferating cell nuclear antigen also plays an important role in recruiting DNMT1 to replication forks during DNA replication [25]. HP1s are heterochromatic proteins (HP1α, β and γ) that bind to histone H3 methylated at K9 and are involved in silencing activities of both G9a and DNMTs. There is evidence that supports a model wherein HP1 mediates communication between G9a (H3K9 methyltransferase) and DNMT1 in the silencing of

euchromatic genes [26]. The above data solidifies the DNA methylation function of DNMT1 during DNA replication through its interactions with other chromatin-associated proteins.

Dnmt1 null-mice show growth retardation and embryonic lethality, therefore supporting a direct link between epigenetic gene regulation and mammalian development [18]. Similar to DNA methylation, post-translational histone modifications are pivotal in epigenetic regulation of genes.

IV). CHROMATIN STRUCTURE:

Post-translational modifications of histone proteins:

Local chromatin architecture is an important factor in the regulation of gene expression and is strongly influenced by post-translational modifications of the N-terminal tails of histones. Histones are subject to many post-translational modifications (PTMs) including acetylation, methylation of lysines (K) and arginines (R), phosphorylation of serine (S) and threonines (T), ubiquitination, sumoylation and biotinylation of lysines as well as ADP ribosylation. Post-translational modifications and their biological functions are pictorially represented in Figure 2.

The ‘histone code’ hypothesis as suggested by Strahl and Allis in 2000, proposes that combinations of histone modifications determines the activity state of the underlying gene [4]. These modifications regulate the accessibility for DNA binding and regulatory proteins (eg: transcription factors) either by altering the charge on the histones (eg: H4K16Ac) and thus changing the DNA-histone interactions or by recruiting structural proteins (eg: HP1). A brief description of several PTMs of histones is described below.

Histone lysine acetylation:

Histone acetylation is probably the best understood histone modification, where acetylation of histones occurs at ε-amino groups of evolutionarily conserved lysine residues located at the N-termini of histones. The negative charge of acetylated histones repels from the negatively charged DNA thereby leading to an open chromatin and easy access of DNA by transcription factors and RNA polymerase II. Histone lysine acetylation also serves as a recruitment platform because bromodomain containing proteins such as SWI/SNF and p300 encode histone acetyltransferase activity (HAT) and bind to acetylated lysines in nucleosomal promoters to promote transcriptional co-activation [27]. The steady state balance of this modification is achieved by the regulated activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [28, 29].

Histone lysine/arginine methylation:

Methylation can occur on lysine residues on N-terminal tails of histone H3 at positions 4, 9, 27, 36 and on lysine 20 on histone H4 and on one lysine residue 79 within the globular domain of histone H3. H1 is a linker histone that stabilizes higher-order chromatin structure and the H1 amino terminus possesses lysine methylation. Each of these lysine residues on the above mentioned histones could be mono-, di- or trimethylated. Together, all the above factors may make lysine methylation an ideal epigenetic mark for long-term maintenance of chromatin states [30]. Lysine methylation is regulated by the activities of histone methyltransferases and demethylases. Lysine methylation on histone H3 or H4 is involved in a number of biological processes including transcriptional regulation, genomic imprinting, X-chromosome inactivation,

DNA methylation and heterochromatin formation [31]. Methylation of arginine residues occurs in three distinct methylated states: monomethylation, asymmetric dimethylation and symmetric dimethylation catalyzed by protein arginine methyltransferases (PRMTs).

Several lysine demethylases exist that remove the mono/di/tri-methyl mark from modified lysines therefore leading either to gene activation or gene repression. Lysine Specific Demethylase I (LSD1) is a flavin-dependent amine oxidase, which demethylates H3K4me2/me1 and H3K9me2/me1 in an androgen receptor mediated pathway [31]. LSD2 also demethylates H3K4me2/me1 and has been linked to maternal imprinting. [32]

Jumonji domain containing Fe^{2+}- and α-ketoglutarate-dependent dioxygenases are a second class of lysine demethylases that demethylates mono-, di-, or tri-methyl lysines. For example, JMJD2A contains an N-terminal Jumonji domain and C-terminal PHD and Tudor domains. The Jumonji domain alone is capable of demethylating tri- and di-methylated H3K9 (H3K9me3/2) and H3K36 (H3K36me3/2) with a very low turnover rate [33]. PHF8 and KIAA1718 also belong to the Jumonji family of proteins, with a PHD domain that binds to H3K4me3 and a Jumonji domain that demethylates H3K9me2 or H3K27me2 or H3K36me2.

Methylation of histone tails can either lead to gene activation or gene repression depending upon the residue modified and the modification state. Regulatory potential is greatly increased because multiple methyl groups can be added to a single lysine residue leading to mono-, di-, or trimethylation of substrate proteins, these various levels of methylation are found in different chromosomal regions and correlate with distinct biological outcomes. For instance, H3K9me3 marks nucleosomes in heterochromatin,

whereas mono- and dimethylation of H3- H3K9me1 and H3K9me2 marks are found in euchromatin [34]. Trimethylation at H3K4, H3K36 or H3K79 are associated with active gene transcription while di/trimethylation of H3K9 and H3K27 are associated with gene repression. Genome-wide studies have shown that endogenous retroviral elements (ERVs) are marked by the repressive histone marks H3K9me2 and/or H3K9me3 in mouse embryonic stem cells [35, 36].

Histone phosphorylation occurs on histones H1 and H3 and is known to play important roles in both transcriptional regulation and mitosis. Histone ubiquitylation has been found to be critical to mitotic and meiotic growth. Biotinylation is an emerging field of research, where there is evidence that this modification is enriched in transcriptionally silent chromatin [30, 37]. Lysines 4, 9 and 18 of histone H3, and the K8 and K12 residues of histone H4 have been identified as the biotinylation sites [30].

Together the above information suggests that post-translational histone modifications perform functions based on its ability to modify chromatin either to an active form or to a repressed form. In the following section, a brief description of histone methyltransferases with specific focus on several H3K9 methyltransferases is given. We chose to describe H3K9 methyltransferases in more detail because it is the focus of this thesis work.

Histone lysine methyltransferases:

Mammalian histone lysine methyltransferases (HMTases) have been divided into two categories based on their *in vitro* substrate preferences. One category of enzymes including the H3K27 HMTase EZH2, H4K20 HMTases PR-SET7 [38, 39] and Suv4-20h1/h2 preferentially methylate histones in a nucleosomal context and the other

category of enzymes, which includes all of the characterized H3K9 HMTases (Suv39h, G9a/GLP, and SETDB1/ESET), preferentially methylate core histone octamers in a non-nucleosomal context and exhibit a low rate of catalysis for nucleosomal histones [40, 41]. All HMTases with the exception of the Dot1 family contain a characteristic SET domain. Dot1 family members, which methylate H3K79, contain conserved sequence motifs characteristic of class I methyltransferases such as DNMTs and PRMTs. A brief description of Suv39h and SETDB1/ESET H3K9 histone methyltransferases and detailed explanation of G9a/GLP is described below.

SUV39h:

Suv39h was the first discovered SET domain containing HMTase [42]. It specifically trimethylates H3K9 in heterochromatin and promotes gene silencing. This is the most studied H3K9 HMTase and contributes towards the establishment of pericentric heterochromatin. Suv39h appears to use monomethylated H3K9 residue as its *in vivo* substrate because monomethylated H3K9 levels were increased in Suv39h double knock out ES cells [35]. This data suggests cooperation of Suv39h with a monomethylase *in vivo*.

SUV39H dependent methylation of H3K9 transduces the signal to recruit HP1 to heterochromatic regions in mammalian chromatin [43]. As H3K9 can either be methylated or acetylated, competitive modification of this position could provide a 'molecular switch' for the induction of euchromatic or heterochromatic subdomains, if the abundance or the activity of the respective enzymes is altered [44]. For instance, forced over-expression of SUV39H1 induces ectopic heterochromatin formation by redistributing endogenous HP1β [45]. Suv39h1 is involved in *de novo* gene silencing and

it interacts with DNMT3B [46]. Disruption of both *Suv39h* genes in mice resulted in sub-Mendelian ratio births, mitotic defects and chromosome misaggregation [47]. This suggests that loss of Suv39h impairs mammalian genome stability.

SETDB1:

SETDB1 is a SET domain containing protein B1 (also called as ESET). It is a H3K9 methyltransferase that by itself dimethylates euchromatic H3K9 but trimethylates this residue in the presence of its cofactor hAM, human homolog of mAM, a murine ATFa-associated factor [48]. SETDB1 null mice embryos show peri-implantation lethality and are important for early development, as SETDB1 knockout is lethal between embryonic days 3.5 and 5.5 [49]. SETDB1 is the only euchromatic H3K9 HMTase that does H3K9 trimethylation in the presence of hAM, suggesting a dual role of this enzyme in connecting gene silencing to pericentric heterochromatin formation [50]. SETDB1 has been shown to interact with DNMT3A *in vitro* and *in vivo* through its N-terminal domain [50].

G9a/GLP:

G9a and GLP (G9a-like protein) are members of the Suv39h subgroup of SET domain-containing molecules and together they are the primary HMTases for mono- and di-methylation of H3K9 [41]. G9a and GLP preferentially methylate H3K9 and less efficiently H3K27 as shown by in vitro and in vivo studies [41, 51]. These enzymes exist as a heteromeric complex endogenously in various human and mouse cells and this heteromerization of G9a/GLP is mediated by its respective SET domains [41].

The G9a and GLP heteromeric complex seems to be the functional H3K9 histone methyltransferase *in vivo* [41, 52] as shown in mouse ES cells by sequential

immunodepletion analysis. The same studies also determined that the molecular stoichiometry of this heteromeric complex is nearly one to one suggesting that G9a and GLP function *in vivo* as either a heterodimer or as a multimer composed of equal amount of two enzymes. It is also reported that G9a forms a stoichiometric complex not only with GLP but also with Wiz, a multi-zinc finger containing molecule. Knockout or knockdown of GLP or Wiz concomitantly reduces G9a protein levels [53] (Figure 3).

G9a and GLP share 75% sequence identity in their respective SET domains. Human and mouse G9a exists as two splicing variants, the larger splice variant ~ 165 kDa and the smaller splice variant is ~140 kDa. These two splice variants differ in size because of the additional N-terminal peptides in the larger one [54] (Figure 4). G9a/GLP are histone methyltransferases that contain domains within the same polypeptide for synthesizing and binding a specific histone mark, such as SET domain for making a methyl mark and ankyrin repeats or chromodomain for recognizing the methyl mark (9). In summary, G9a/GLP are euchromatic H3K9 HMTases that exist as a heteromeric complex *in vivo* and share large sequence similarities in their respective SET domains.

***G9a* knockout and *G9a* mutation studies:**

G9a knockout mice are embryonic lethal around embryonic day 9.5 because of severe growth defects. $G9a^{-/-}$ mouse ES cells show significantly reduced DNA methylation [55, 56]. Another study [52] has shown that ES cells stably expressing catalytically inactive G9a maintains DNA methylation of its target promoter regions indicating that DNA methylation activity of G9a/GLP is independent of its SET domain mediated HMTase activity. In contrast, there is little impact on DNA methylation at constitutive heterochromatin in Suv39h1/h2 $^{-/-}$ mice and Suv39h1/h2 $^{-/-}$ mice are not

embryonic lethal. Together, these data suggest that G9a and Suv39h have non-overlapping functions *in vivo [54, 57]*.

Heterozygous mutations or deletions of the human *EHMT1 (GLP)* gene cause Kleefstra Syndrome, a neurodevelopmental disorder that is characterized by autistic-like features and severe intellectual disability [58]. Studies in mice have shown that G9a and GLP form a heterodimeric complex and that loss of either protein resulted in almost identical phenotypes such as early embryonic lethality, reduced H3K9 dimethylation and inappropriate gene transcription [41, 54]. All the above G9a knockout studies indicate that G9a is an essential gene during development.

Specificity determinant for G9a methylation of its histone targets:

Arginine in position 8 of histone H3 (H3R8) is an important specificity determinant for K9 methylation by G9a. Any other amino acid substituted at that position completely abolished the activity of G9a on the H3 peptide substrate [59] as demonstrated by *in vitro* peptide arrays. Data from human cells from this study shows that G9a automethylates it's N-terminus lysines. In addition to this, G9a/GLP is reported to methylate lysine 373 in the tumor suppressor p53 [60]. This indicates a potential role of G9a/GLP in human cancers and links p53 methylation to tumorigenesis.

Evolutionarily conserved relationship between H3K9 and DNA methylation:

Histone H3K9 methylation is important for global DNA methylation in *Neurospora crassa* as identified in a mutagenesis screen by the Selker group. They showed that DNA methylation was entirely eliminated by a mutation that disrupted a SET domain protein DIM-5, which catalyzes trimethylation of H3K9. H3K9 methylation is also important for some DNA methylation in plants, such as in *Arabidopsis thaliana*, where the loss of

function *Suvh4* alleles showed loss of cytosine methylation and reactivation of endogenous retrotransposon sequences. Suvh4 is similar to mammalian Suv39h [61].

G9a and DNMT3 (zebrafish *de novo* DNA methyltransferase protein, that is orthologous to mammalian DNMT3B) functionally cooperate in neurogenesis in zebrafish [62]. Knockdown of either protein by morpholinos exhibited a drastic reduction in brain size suggesting that G9a and DNMT3 cooperatively promote neurogenesis. It has been suggested that this occurs through silencing of *lef1*, a critical neural development gene [62]. G9a has been shown to recruit *DNMT3A/3B* through its ANK domain [55] independent of its SET domain and mediate *de novo* methylation of *Oct3/4* promoters in differentiated mouse ES cells and prevent reprogramming of embryonically silenced genes during development.

UHRF1 recognizes and binds to hemi-methylated DNA and mediates transcriptional silencing of p21 through the recruitment of G9a, DNMT1 and HDAC1 in a cell-cycle dependent manner. G9a and LSH, a chromatin-remodeling enzyme, act cooperatively to promote DNA methylation at a subset of gene promoters such as the *Rhox* loci, *EIf5* and *Wfdc15* in mouse embryonic fibroblasts. LSH is required for recruitment of G9a to the promoters of pluripotency genes during differentiation in mouse [63]. G9a has a vital biological role in the stable inactivation of pluripotency genes and could be a major influence in controlling the ability of somatic cells to undergo reprogramming [64, 65].

In vitro binding assays have demonstrated recruitment of HP1 (heterochromatin proteins) proteins to chromatin arrays with H3K9me2 modification, which is mediated by G9a. Increased HP1α, β and γ binding were found with increased G9a mediated

H3K9me2 modification. It is also established that HP1s, G9a and DNMT1 cooperatively repress *Survivin* gene expression (inhibitor of apoptosis protein) in human colon cancer cells [26].

Together all these data imply that G9a establishes euchromatic gene silencing by interacting with other repressive epigenetic factors including GLP, DNMTs, HP1s and UHRF1. Interactions between G9a/DNMT3, UHRF1/DNMT1/G9a, G9a/LSH implies a potential mechanism for the coordinate maintenance of DNA and H3K9 methylation in epigenetically silenced genes. Overall the above information suggests evolutionarily conserved interactions between proteins that promote gene silencing.

V). ABERRANT EPIGENETIC REGULATION IN CANCER:

In normal cells, approximately 60% of genes possess promoter associated CpG islands that are unmethylated, and CpG dinucleotides distributed in the intergenic regions are usually hypermethylated. Unmethylated CpG islands in the 5' regulatory regions of the genes exhibit high transcriptional potential and are associated with active histone modifications such as H3K4me/me3, H3K16 ac and H3/H4 ac [66, 67] (Figure 5). In addition to this, endogenous retrotransposons, endogenous retroviruses and developmentally programmed genes are marked by DNA methylation and histone methylation in normal cells thereby mediating its silencing.

In cancer cells, CpG islands that are normally unmethylated can become methylated (Figure 5) that leads to silencing of tumor suppressor genes; DNA repair genes; genes for hormone receptors and genes that inhibit angiogenesis. CpG dinucleotides in other regions (such as intergenic regions) become hypomethylated leading to poor

transcriptional repression of normally silenced genes such as oncogenes and retrotransposons [3].

Tumor Suppressor Genes:

Aberrant hypermethylation of CpG islands lead to silencing of tumor suppressor genes that in turn leads to cancer progression. Numerous studies have shown that CpG island promoter hypermethylation of tumor suppressor genes occurs early in tumorigenesis. This is a major event in the origin of many cancers. Hypermethylation of tumor suppressor genes such as *Retinoblastoma (Rb), VHL* (associated with von Hippel-Lindau disease), *p16, CDH1* (E-cadherin), *hMLH1* (a homolog of MutL *Escherichia coli)* and *BRCA1* (breast-cancer susceptibility gene 1) have been reported so far.

More than 100 genes have been reported to be hypermethylated in breast tumors or breast cancer cell lines[68]. H1N1 is an inhibitor of cell growth, migration and invasion that is frequently silenced in breast cancer by DNA methylation [69]. In studies from our own laboratory, *TMS1* (Target of Methylation-associated Silencing-1) also called as *ASC*, a tumor suppressor gene, is aberrantly methylated and silenced in human breast cancers[70]. Thus, DNA methylation patterns in cancer cells are distorted.

In cancer, the histone component of the epigenome also undergoes both widespread and gene-specific changes in addition to abnormal patterns of DNA methylation. Overall, cancer cells exhibit global decrease in the levels of H4K20me2/3, H3K9me2, and H4 acetylation, specifically H4K16 [71, 72] (Figure 5). Together, aberrant DNA methylation and global decrease in H3K9me2, and H4K20me3 indicates a global dysregulation of transcriptional repression in cancer cells, which may promote tumorigenesis through the de-repression of endogenous transposons (eg: Alu), loss of

checkpoint controls, an impaired DNA damage response and thereby leading to chromosomal instability [66].

Epigenetic therapy of cancer:

'Epimutations' has been used to describe disruption in epigenetic regulation that provoke aberrant gene expression patterns and give rise to cancer characteristics such as the epigenetic silencing of the tumor suppressor genes. In contrast to genetic mutations, epimutations are reversible. In recent years, a lot of attention is given to epigenetic drugs, which could restore the normal epigenetic landscape in cancer cells by inhibiting enzymes of the epigenetic machinery.

Till date the US Food and Drug Administration (FDA) have approved four drugs for cancer treatment: two DNMT inhibitors, vidaza and decitabine, in the treatment of myelodysplastic syndrome patients who develop acute leukemia; and two HDAC inhibitors, vorinostat and romidepsin for the treatment of rare cutaneous T cell lymphoma (CTCL) [2]. An overview of different compounds used in epigenetic therapy of cancer is shown in Figure 6.

DNMT inhibitors:

Presently, there are two commercially available FDA approved DNMT inhibitors-azacytidine (Vidaza, Phamion, Boulder, CO) and decitabine (Dacogen, Supergen Inc, CA). Decitabine is 5-aza-2'-deoxyazacytidine (5-azaCdR). Both these inhibitors are cytidine analogues; the 5-azacitidine can be metabolized to both ribose triphosphate and deoxy ribose triphosphate forms and be incorporated into DNA and RNA while 5-azaCdR is metabolized to the deoxyribose triphosphate form and incorporated into DNA. 5-azaCdR was originally synthesized over 40 years ago. Incorporation of these

cytidine analogs occurs during DNA synthesis that is followed by filling of the cytosine pockets of DNMTs. This inhibits the capacity of DNMTs to methylate DNA by a covalent bond formation between the modified base and DNMTs [73].

An important observation is that DNMT activity decreases much faster than incorporation of these analogs into DNA. A mechanism whereby the analogs induce selective degradation of DNMT1 by a proteasomal pathway has been proposed to explain this observation [74]. These cytidine analogs are considered to be unselectively incorporated into genomic DNA in proliferating cells including stem cells in normal tissues and likely to mediate its effects by cell cycle arrest, differentiation and apoptosis of cells.

The DNMT inhibitor Azacytidine (5-Aza) has also been used in combination with conventional chemotherapeutic drug cisplatin, where it reduced the nephrotoxicity of cisplatin in the murine model. 5-Aza treatment resulted in a decrease in the levels of metallothioneins, which induce oxidative stress either directly or indirectly. This was the first evidence showing that treatment with a DNMT inhibitor could reduce the chemotherapy-related toxicity in cancer [75].

HDAC inhibitors:

HDAC (histone deacetylase) inhibitors are a group of small molecules that can promote hyperacetylation of histones to alter gene expression and particularly to activate genes that are usually silenced in tumors. All HDAC inhibitors are marked by the presence of a metal-binding domain that can block substrate-Zn chelation at the HDAC active pocket. Major anticancer effects of HDAC inhibitors are cell cycle arrest in G1 or G2-M, induction of differentiation and apoptosis, inhibition of angiogenesis and

metastasis as well as enhance sensitivity to chemotherapy. Sodium n-butyrate, phenylacetate, trichostatin A, vorinostat (SAHA), romidepsin are some of the several HDAC inhibitors.

Small molecule histone lysine methyltransferase inhibitors:

Histone lysine methylation has important roles in the organization of chromatin domains and regulation of gene expression. H3K9 is one of the best-studied modifications in eukaryotic chromatin. It has major roles (H3K9me3 modification) in heterochromatin formation and silencing of euchromatin (H3K9me2 modification). H3K9me2 modification is particularly important because this mark is erased from the promoters of reactivated tumor suppressor genes in cancer following treatment with HDAC and DNMT inhibitors. Therefore, it becomes an attractive target for screening of small molecule inhibitors in the context of epigenetic therapy of cancer.

Chaetocin, a fungal compound, was the first HMTase inhibitor to be discovered. It is a competitive inhibitor of SAM, which is a cofactor of histone lysine methyltransferases. Chaetocin specifically inhibits the enzymatic activities of HMTases belonging to members of SUV39 family, including SUV39H1, G9a, GLP, DIM-5 and ESET [76, 77]. The complicated backbone structure of chaetocin and only partially characterized specificity obscure its biological potential as a research tool for epigenetic therapy.

BIX-01294 was first identified during the screening of specific inhibitors against histone lysine methyltransferases (HMTases) using recombinant G9a as the target enzyme [78]. From a chemical library of 125,000 preselected compounds, seven hits were identified, among those, BIX-01294 was a specific H3K9 HMTase inhibitor, which

does not compete with the cofactor SAM and it selectively impairs the G9a histone methyltransferase activity and generation of H3K9me2 *in vitro* and *in vivo[78]*. BIX-01294 consists of a central quinazoline ring linked to seven membered diazepane ring and benzylated six-membered piperidine ring [79] (Figure 7). BIX-01294 binds the SET domain of G9a/GLP; it resembles the bound conformation of histone H3 Lys4 to Arg8, residues N-terminal to the target lysine, but leaves the target lysine-binding channel unoccupied.

E67 and E11 are synthetic BIX-01294 analogs with moieties mimicking lysine and methyl-lysine [79]. These compounds were generated by structural modifications to BIX-01294 so that it mimics the side chain of a substrate lysine. Basically the length of the aliphatic chain and the presence of a terminal amino group for the 5-aminopentyloxy moiety were expected to extend into the active site of GLP and mimic the side chain of a substrate lysine. Chemical structures of E67 and E11 are shown in Figure 8.

The IC_{50} values (half maximal inhibitory concentrations) of BIX-01294 have been determined by mass spectrometry based *in vitro* assays. Initially, it was reported to be inhibitory against G9a but recent studies have shown that it is effective against GLP also (G9a IC_{50}= 1.9 μM; GLP IC_{50}= 0.7 μM, [80]); G9a IC_{50}= 250 nM vs GLP IC_{50}= 27 nM [81]). More potent G9a/GLP inhibitors have been developed by further structural modifications of BIX-01294 which has the following IC_{50} values *in vitro* (**E72**: GLP IC_{50}= 100 nM, **E67**: GLP IC_{50}= 273 nM, **UNC0321:** G9a IC_{50}= 9.0 nM and GLP IC_{50}= 15nM) [79].

Thus, BIX-01294 and its analogs act as competitive inhibitors of the N-terminal peptides of H3 (K4 to R8 or K9), [79] as shown by structural studies of GLP bound to a

substrate in comparison to GLP bound to BIX-01294 and its family of derivatives [79, 80]. BIX-01294 improves the reprogramming efficiency of neuronal precursor cells into induced pluripotent stem (iPS) cells when transduced with other transcription factors such as *Oct3/4, Klf4, Sox-2* and *c-myc.* This explains the important role of G9a in silencing of the above markers during differentiation by establishing the repressive H3K9 methylation [65].

In this study, we have focused on characterizing the *in vivo* effects of BIX-01294, E67 and E11 in breast cancer cells, which has not been studied earlier.

VI). OBJECTIVE

Epigenetic silencing of tumor suppressor genes in cancers are characterized by DNA hypermethylation and local alterations in histone modifications, such as histone H3K9me2/3, H3K27me3 and H3K4me2/3. G9a and G9a-like protein (GLP) are euchromatin- associated histone methyltransferases that repress transcription by methylating histone H3 at K9. BIX-01294 is a known G9a/GLP small molecular inhibitor; E67 and E11 are small molecular derivatives of BIX-01294.

At the beginning of this thesis, the only data available at the time were the original studies that identified and characterized BIX-01294 as a small molecule inhibitor of G9a [78]. Additional studies showed the effectiveness of BIX-01294 in reprogramming of mouse embryonic fibroblasts into induced pluripotent stem cells (iPSC) by G9a inhibition [65, 82]. E67 and E11 were characterized *in vitro* in terms of their effects on K_d and IC_{50} values against recombinant G9a and its growth inhibitory effects in mouse ES cells *in vivo [79]*. As E67 and Ell were reported to be G9a/GLP inhibitors and BIX-01294 was an already published G9a inhibitor, we were interested to characterize all three drugs in breast cancer cells, which had not yet been studied.

We hypothesized that these small molecule inhibitors would lead to expression of epigenetically silenced tumor suppressor genes in breast cancer cells through its effects on DNA methylation and chromatin modifications. The rationale was published data showing interactions between G9a and DNMTs in establishing a repressive euchromatin at its target genes [41, 83, 84]. Our main objectives were to determine the cytotoxic effects of these drugs on cancer and normal cells, to study the expression of epigenetically silenced genes, to determine the effects of HMTase inhibition on DNA

methylation, and to finally study the changes in global levels of histone modifications. All these studies were also concurrently done with 5-azaCdR, a DNMT inhibitor, to determine the effects of combined therapy and to see whether there is any effect when cells were treated with both these inhibitors. We specifically studied the expression of three epigentically silenced tumor suppressor genes and one transcription factor which is detailed below.

1. *TMS1* – (Target of methylation-induced silencing) is a novel CpG island associated gene that becomes aberrantly methylated and silenced in cells overexpressing *DNMT1*. *TMS1* was originally identified in a screen for downstream targets of aberrant DNA methylation [85]. *TMS1* is silenced in association with aberrant CpG island methylation in human breast cancer cells. *TMS1* encodes a novel CARD protein and promotes apoptosis in human cells. CARD is found within the prodomain of a number of caspases that are pivotal apoptotic proteins. *TMS1* is hypermethylated and silenced in MDA-MB231 cells [85] (Figure 9).

2. *ESR1*- (Estrogen Receptor) and estrogen plays important role in the development and function of the female reproductive organs and mammary glands. About one quarter of all breast cancers lack ER and are ER negative, which means that they are not responsive to hormonal therapies. These tumors are poorly differentiated and have the worst clinical outcomes. *ER* gene has CpG islands in its promoter and the first exon and these CpG islands are epigenetically silenced by hypermethylation caused by *DNMT1* over expression in human breast cancers including MDA-MB231 cells. Treatment of such cancer cells with 5-aza-2'CdR leads to transient re-activation of *ER* [86].

3. *CHD1*- (E-Cadherin): E-cadherin is the prototypic member of the classic cadherin family of transmembrane glycoproteins, functioning in cell-cell adhesion molecule at adherens junctions of epithelial cells via homotypic interactions with E-cadherin molecules on neighboring cells and cytoplasmic interactions with catenins and actin cytoskeleton. Disruption of the cadherin-catenin complex has been seen in cancers of many tissues including breast and is correlated with tumor dedifferentiation, lymph node metastasis and a worse patient prognosis. Proposed epigenetic mechanisms for E-cadherin inactivation include hypermethylation of the *CDH1* (gene that codes for E-cadherin) promoter and chromatin-mediated effects [87]. E-cadherin is epigenetically silenced by promoter hypermethylation in MDA-MB231 cells and is transiently reactivated by 5-aza-2'CdR treatment.

4. *EGR1*- (Early growth response gene 1) is a transcription factor that directly regulates a number of tumor suppressor genes such as *PTEN* (Phosphatase and tensin homolog deleted in chromosome 10), p53, p73, *FAS*, cyclin D2 and cyclin-dependent kinase inhibitors by binding to its promoter and activating its transcription [88]. *EGR1* is suggested to function as a tumor suppressor gene because it has been reported to be suppressed or absent in a variety of human tumor cell lines including breast cancer cells and its over expression can dramatically inhibit tumor cell growth *in vitro* and in xenografted mice [89]. Paradoxically, *EGR1* functions as an oncogene in prostate cancers. It is noteworthy that polycomb-group mediated histone H3K27me3; a key mark of genomic silencing is tightly correlated with *EGR1* repression. This is further supported that HDAC inhibitors efficiently remove H3K27me3 marks, recruit acetyl groups to histone H3 tails and thereby reactivate expression of *EGR1* in synovial sarcoma [90].

The ultimate goal of this study is to determine the therapeutic potential of these agents in cancer by studying the mechanism of epigenetic regulation of tumor suppressor genes in breast cancer cells treated with these agents.

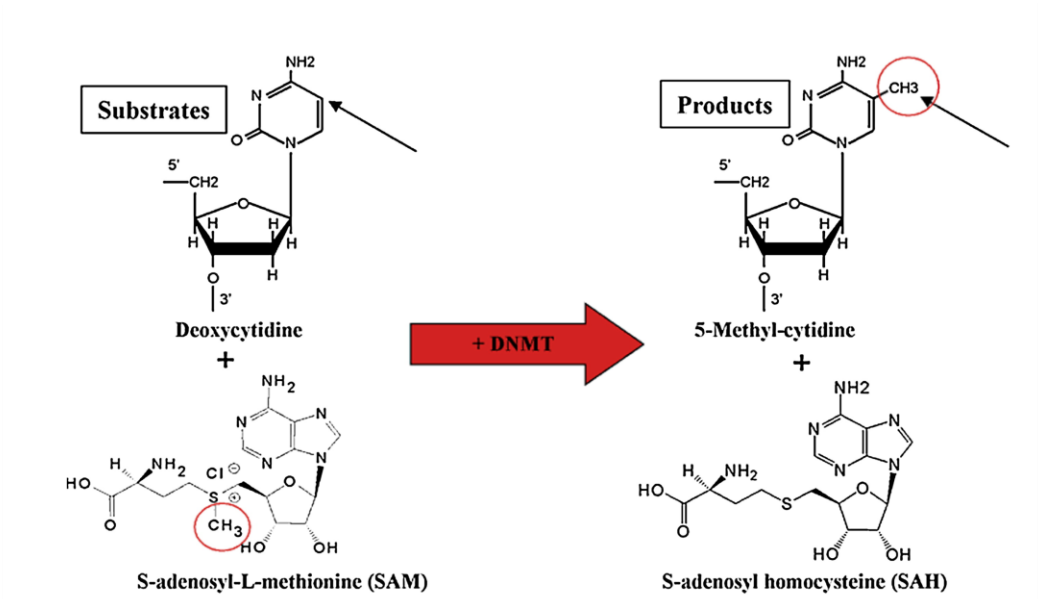


Figure 1: DNA methyltransferase reaction. Structure of cytosine before and after the transfer of a methyl group from the cofactor S-adenosyl-L-methionine, catalyzed by DNA methyltransferases. DNA methylation reaction involves DNA binding by DNMT, flipping the target cytosine out of the double helix, and formation of a transient covalent complex with the cytosine residue. Reprinted from *Jovana Jovanovic, et al, Molecular Oncology 4 (2010) 242-254*

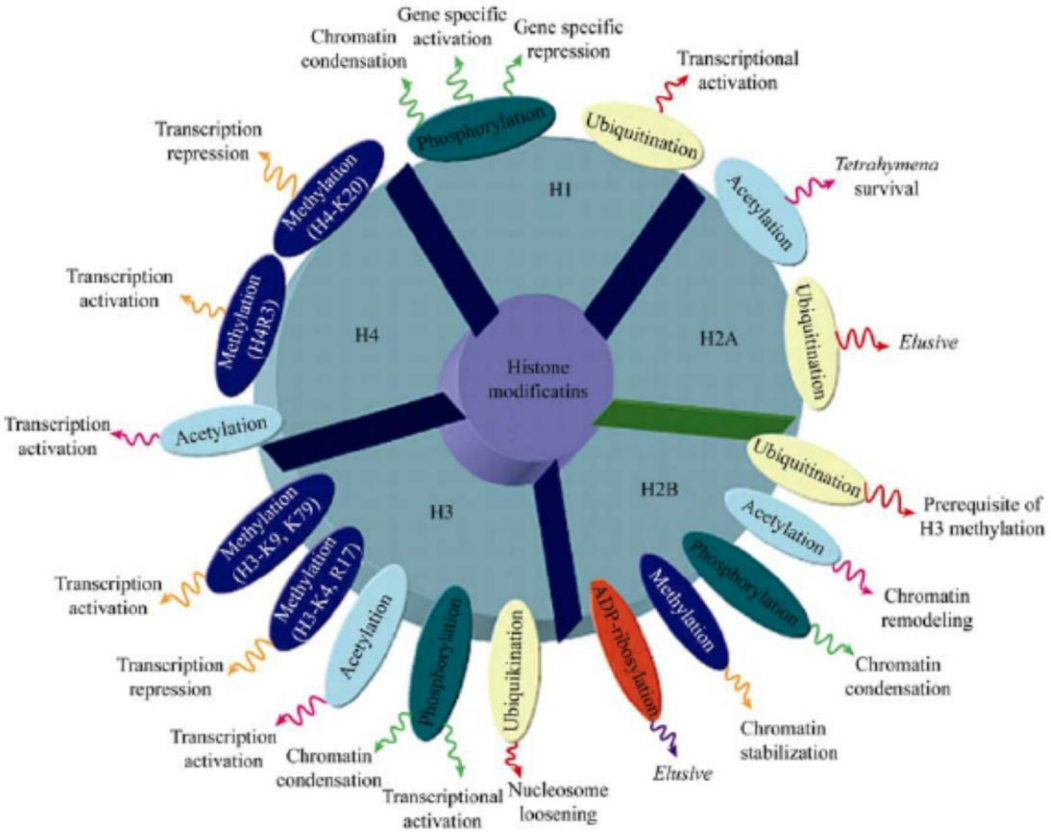


Figure 2: The pictorial representation of various post translational histone modifications and their biological roles. Post translational modifications of histones H1, H2A, H2B, H3 and H4 leads to transcriptional activation, transcriptional repression, nucleosome loosening, etc. Reprinted from *Anjana Munshi et al. Journal of Genetics and Genomics 36 (2009) 75-88.*

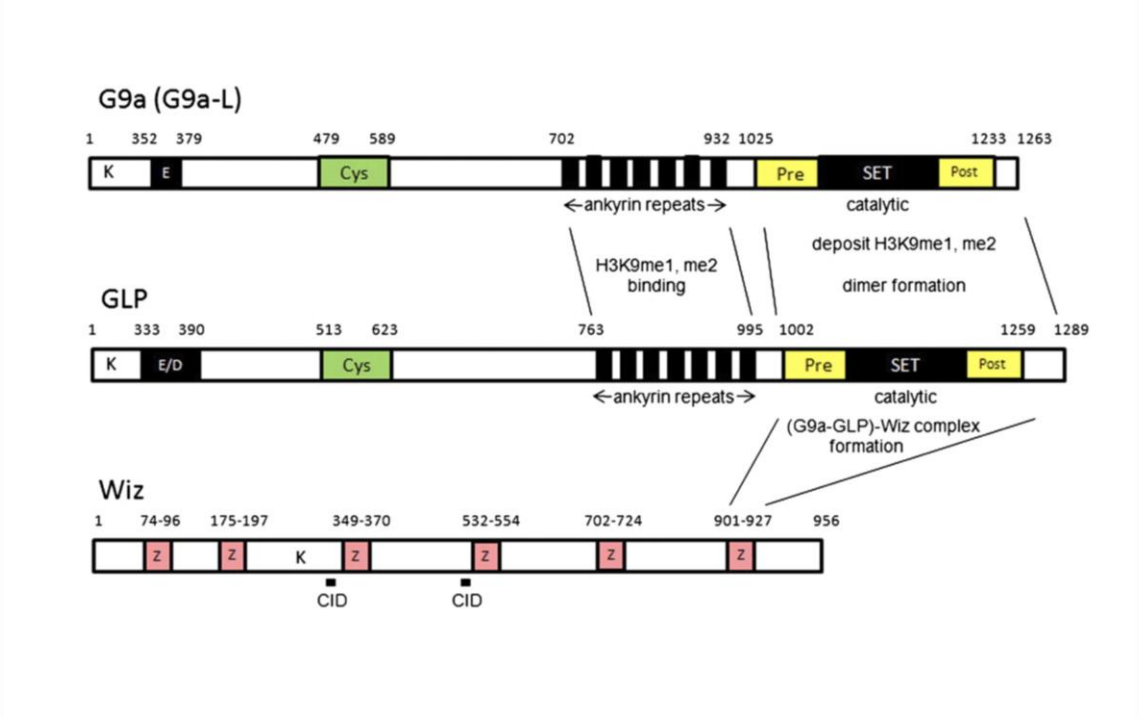


Figure 3: Domain organization of the core G9a complex components: G9a, GLP and Wiz. This figure shows the different functional domains of these three proteins, and its respective functions. G9a and GLP exhibit an overall sequence similarity, SET domain in two proteins is about 75% identical between the two proteins [41]. Ankryin repeats or chromodomain identify the methyl marks in its substrate peptides. Wiz is a multi-finger rich molecule that most stably interacts with the G9a-GLP heterodimer. Amino acid sequences of mouse mG9a-L, mGLP and mWiz. (K) Potential methylation sites by G9a or GLP (K167 and K239 in G9a-L, K154 and K206 in GLP, and K467 in Wiz). (e) Glutamate rich region; (E/D) Glutamate/Aspartate rich region; (Cys)- Cysteine rich region; (Pre) pre-SET domain; (SET) SET domain, (Post) post-SET domain; (Z) zinc finger motif; (CID) CtBP-interacting region. Reprinted from *Shinkai and Tachibana, Genes Dev. 2011 25:781-788.*

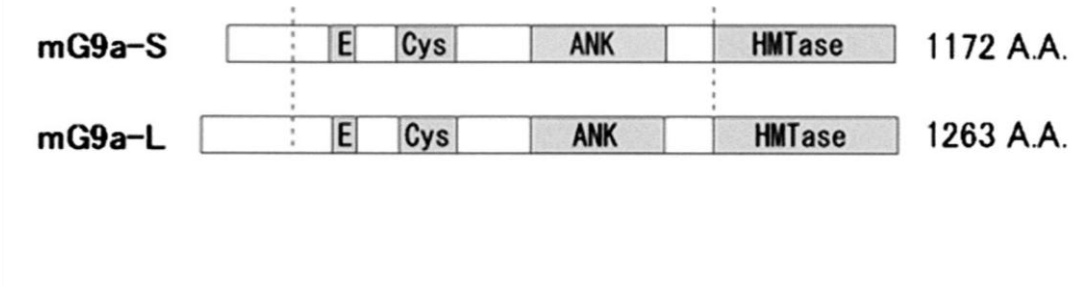


Figure 4: Schematic representation of the mouse *G9a* locus. Two splice variants of the murine G9a (mG9a-S and mG9a-L), ~ 165 and 140 kDa are shown here. (E) Glutamate rich region;(Cys) cysteine rich region; (ANK) ankyrin repeats; (HMTase) SET domain. Reprinted from *Tachibana et al. Genes and Development 16:1779-1791, 2002.*

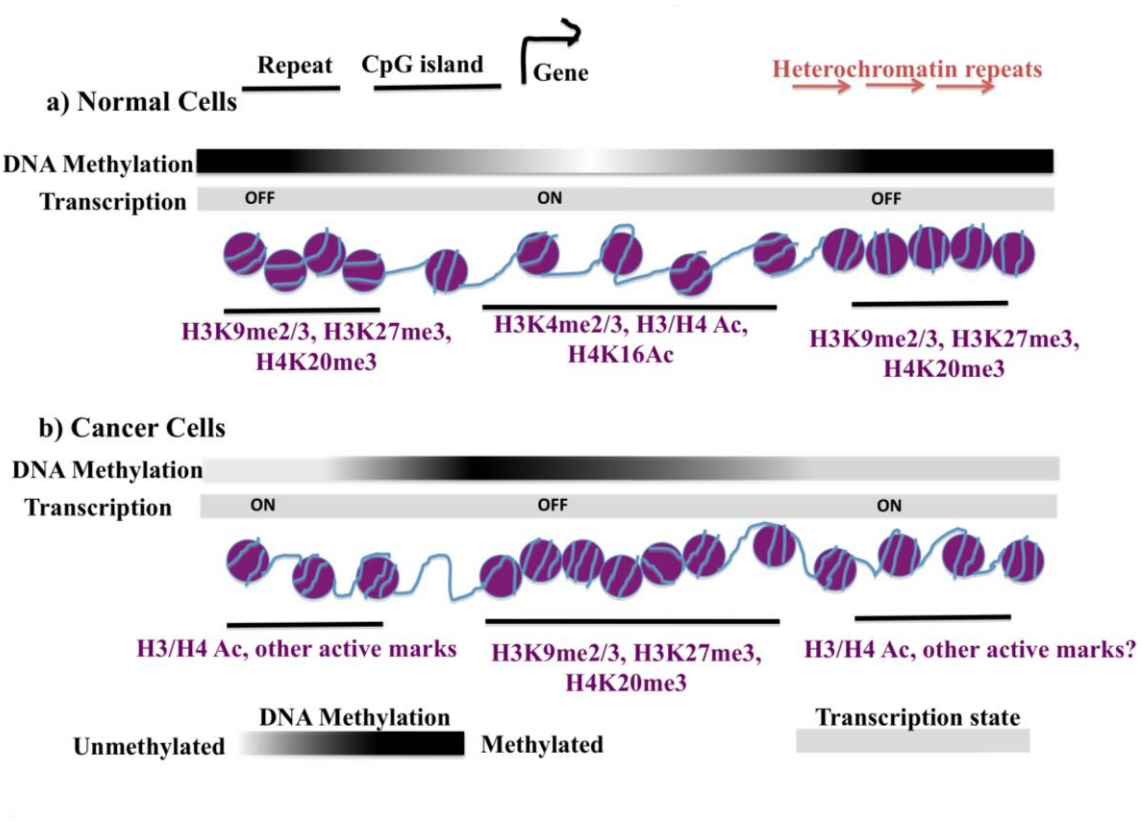


Figure 5: DNA methylation and histone modification patterns are altered in cancers. a) Approximately 70% of genes possess promoter associated CpG islands that mostly remain unmethylated in normal cells and gene bodies that are mostly methylated. Maintenance of an unmethylated promoter CpG island contributes to a high transcriptional potential and is associated with active histone modifications such as H3/H4 acetylation and methylation at H3K4. b) In cancer cells, dense hypermethylation of CpG island associated promoters and hypomethylation of gene bodies is observed. Densely methylated CpG islands are associated with chromatin compaction and repressed gene expression in association with repressive modifications including H3K9me2/3, H3K27me3 and/or H4K20me3. Figure and text adapted and modified from *Michael T. McCabe etal, Clin Cancer Res 2009; 15(12) June 15, 2009.*

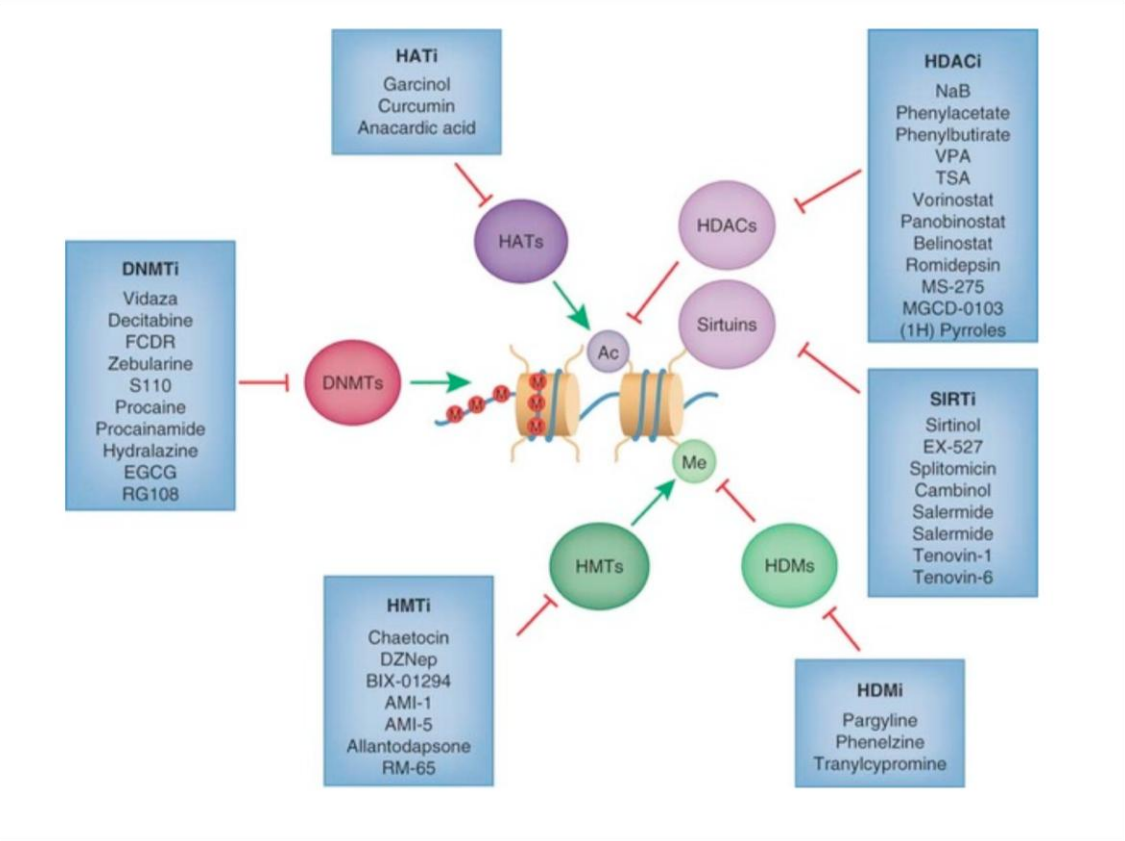


Figure 6: Epigenetic drugs for cancer therapy. Numerous compounds have been reported to be effective against cancer cells by inhibiting components of the epigenetic machineries. This figure illustrates the most important epigenetic drugs classified depending on their particular epigenetic targets. HATi is histone acetyl transferase inhibitor, HDACi is histone deacetylase inhibitor, SIRTi is sirtuin inhibitor, HDMi is histone demethyltransferase inhibitor, HMTi is histone methyltransferase inhibitor and DNMTi is DNA methyltransferase inhibitor. Reprinted from *Rodriguez et al, Nature Medicine, Volume 17, Number 3, March 2011.*

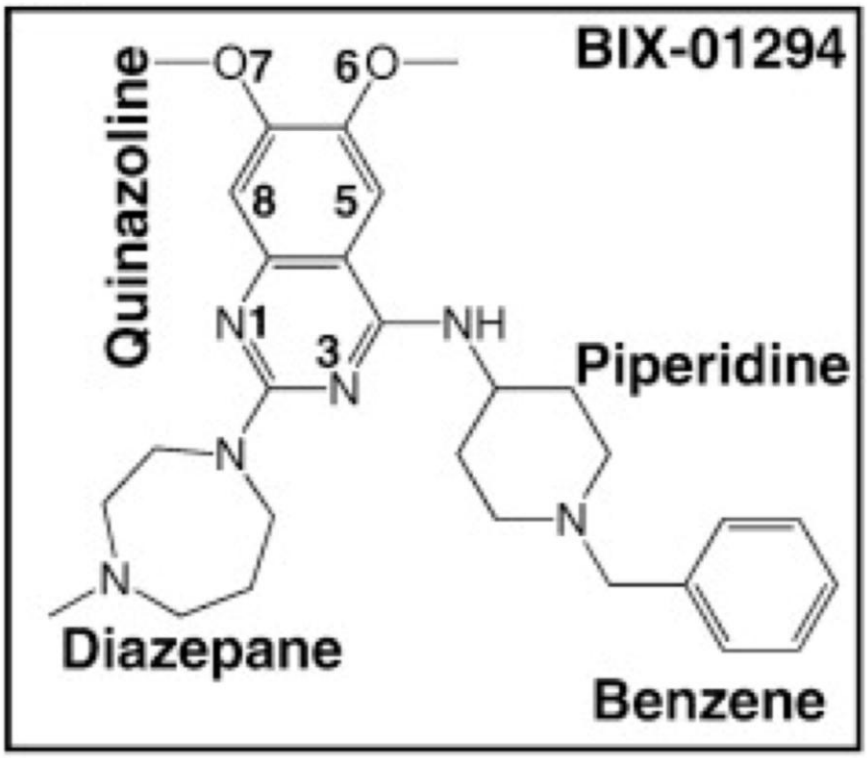


Figure 7: Chemical structure of the compound BIX-01294. It is a (1H-1,4-diazepin-1-yl-quinazolin-4-yl) amine derivative. Reprinted from *Chang et al, Journal of molecular Biology (2010), 400, 1-7.*

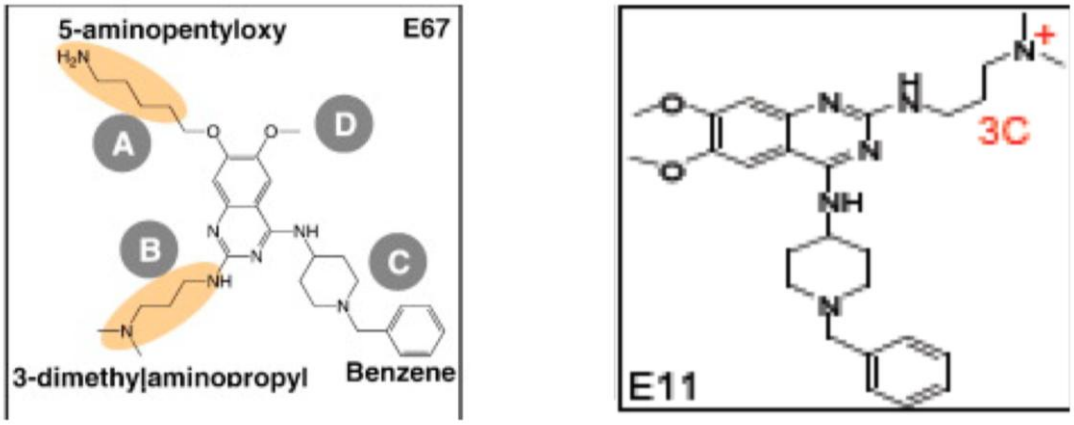


Figure 8: Chemical structures of E67 and E11 (BIX-01294 derivatives). The 5-aminopentyloxy moiety at site A of E67 is inserted into the target lysine-binding channel of GLP and appears to be optimal for inhibition. The three carbon aliphatic chain (3C) of the three-dimethyl amino propyl group in E11 appears to be optimal for charge-charge interaction of GLP and E11, reducing or increasing the chain length by one carbon resulted in less inhibition. Reprinted from *Chang et al, Journal of molecular Biology (2010), 400, 1-7.*

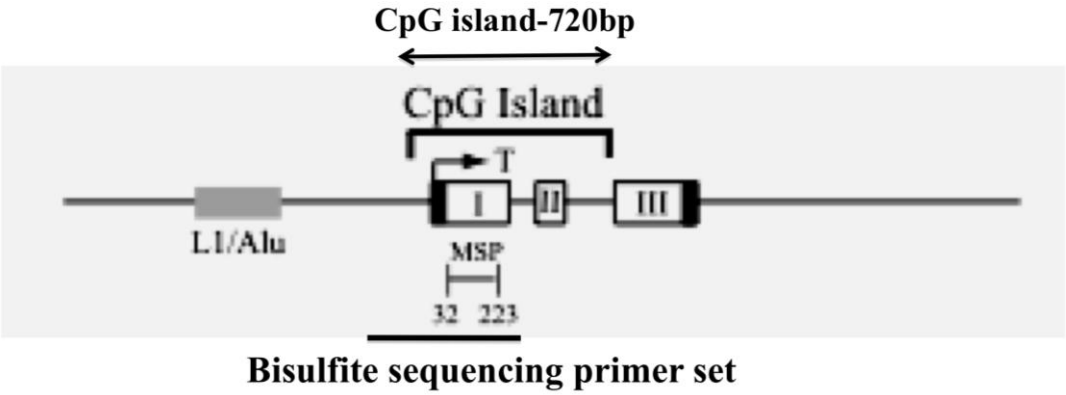


Figure 9: Diagram of the *TMS1* gene. *TMS1/ASC* locus on chromosome 16p11.2 encodes a 770bp mRNA. Open boxes indicate exons, arrow indicates transcription start site. Primer set used for bisulfite sequencing PCR in this study is indicated below the gene. CpG island is indicated above the genes. Reprinted and modified from *Kapoor-Vazirani, Cancer res 2008, 68: (16).*

CHAPTER 2

Materials and Methods

1. Cell culture:

MDA-MB231 cells were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. Cells were routinely passaged every three days at a split ratio of 1:10.

MCF10A cells were obtained from the Karmanos Cancer Institute and maintained in DMEM/F12 plus 5% fetal bovine serum, 20ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 2 mM glutamine.

2. Preparation of BIX-01294, E67, E11 and 5-azaCdR stocks:

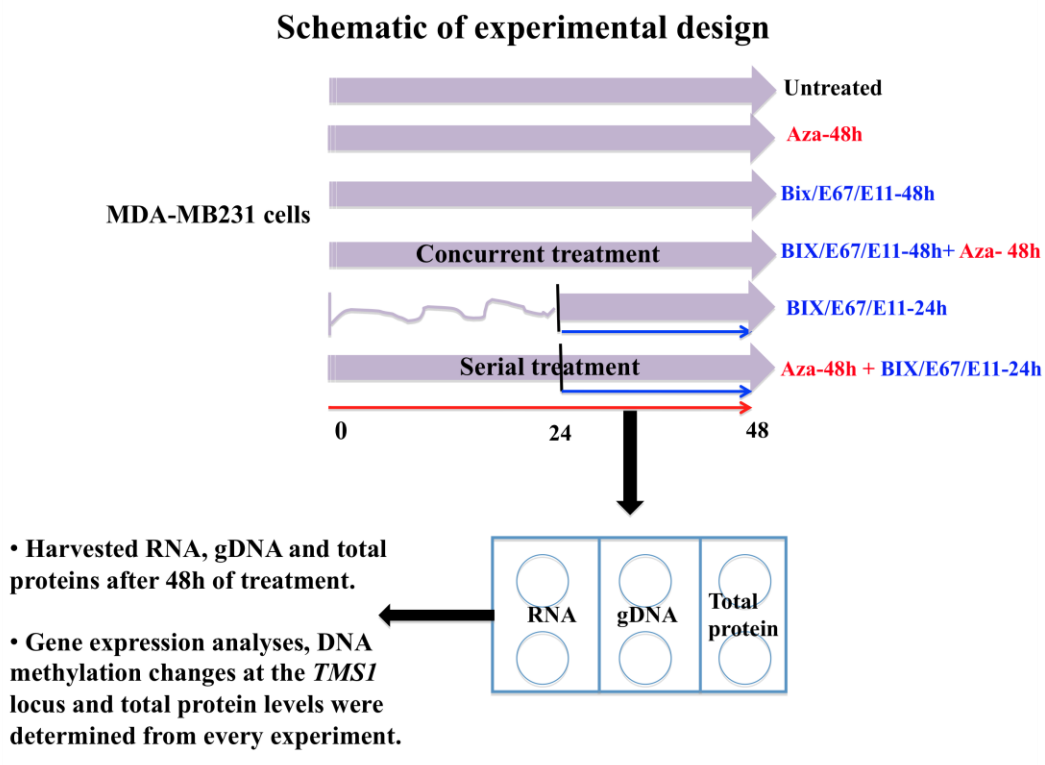
Dr. James Snyder, Director of Biostructural Research in the Department of Chemistry at Emory University, generously offered BIX-01294 and its analogs E67 and E11 for our studies. E67 and E11 were synthesized in his laboratory. 10mM stocks of these chemicals were dissolved in 100% DMSO following sterile aseptic techniques. Stock preparations were carried out in the tissue culture hoods. 5mM, 1mM, 0.5mM and 0.1mM stocks were prepared in 100% DMSO from 10mM stocks. A 1:1000 dilution of the above stocks were done in complete DMEM medium (final concentration of DMSO is 0.1% in cell culture medium) for the experiments in this thesis. These stocks were stored at -20°C.

0.5 μM of 5-azaCdR working solutions were prepared from frozen stocks of 5-azaCdR at 50mM concentration. 0.5 μM of 5-azaCdR is the working concentration that is routinely used in our laboratory.

3. Drug treatments:

MDA-MB231 cells (2 x 10^5) were seeded in 6-well plates on the day before drug treatment. Twenty-four hours later, cells were treated with 0.5 µM 5-azaCdR and/or with 4.0 µM BIX-01294, 10 µM E67 and 3.0 µM E11. For 5-azaCdR treatments, freshly diluted drug was added to the cells every day until the harvest. After 48 hours, genomic DNA, RNA and total proteins were harvested from untreated and treated cells as outlined in the schematic of experimental design above.

Figure 10:



4. Cell titer non-radioactive cell proliferation assay (MTS assays):

This is a colorimetric method for the determination of number of viable cells. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of formazan at 490nm can be measured directly from 96-well assay plates and is directly proportional to the number of living cells in culture. Four thousand MDA-MB231 cells/MCF10A cells were seeded per well in a total volume of 200 µl on 96-well plates. Following day, increasing doses of BIX-01294, E67 and E11 were administered to the cells as follows- 0.1µM, 0.33µM, 1.11µM, 3.33µM, 10µM and 30µM. After 48 hours, 40µl of activated MTS reagent [3-(4,5-dimethylthiazol-2-yl) was added to each well. MTS assays were done in quadruplicates.

5. Bisulfite sequencing PCR:

In single stranded DNA, sodium bisulfite preferentially deaminates cytosine residues to uracil compared to a very slow rate of deamination of 5-methylcytosine to thymine [91]. Genomic DNA (2µg) in a volume of 50µl was denatured by sodium hydroxide (0.2M final concentration) for 10 minutes at 37°C. Freshly prepared 30µl of 10mM hydroquinone (Sigma) and 520µl of 3M sodium bisulfite (Sigma) at pH 5, were added to the genomic DNA solution and mixed gently by pipeting. These samples were incubated at 50°C for 16 hours. Modified gDNA was purified using the Wizard DNA purification resin according to the manufacturer's protocol (Promega) and eluted into 50µl of nuclease free water pre-warmed at 65°C. Modification was completed by treatment with sodium hydroxide (final concentration being 0.3M) for 5 minutes at room temperature, followed by ethanol precipitation. Modified gDNA was resuspended in 30µl of nuclease free water and stored at -80°C.

For bisulfite sequencing, bisulfite-modified genomic DNA was amplified by PCR using primers that were designed to avoid potential methylation sites (CpGs) in order to amplify both the methylated and unmethylated gDNA. Amplification conditions are as follows [70]: 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulphate, 6.7 μM EDTA, 10mM β-mercaptoethanol, 6.7 mM magnesium chloride, 2 μM dNTP, and 1 μM of forward and reverse primer in a 50 μl reaction. A hot start was performed at 95°C for 5 minutes followed by 37 cycles of following PCR conditions- 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute with a final extension of 72°C for 10 minutes.

Reaction products were checked for accuracy on 1.5% agarose gel followed by purification using a PCR purification kit (Qiagen) in order to eliminate primers, dNTPs, and salts from PCR reaction before subcloning. Purified PCR pools were subcloned into the pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen). Manufacturer's instructions were followed for subcloning.

Ten to twelve subclones per PCR reaction were isolated for plasmid DNA preparation using the Qiaprep plasmid mini-prep kit (Qiagen) and sequenced at the Genewiz sequencing facility. Sequences were analyzed using BiQ analyzer, a software program that enables the visualization and quality control of DNA methylation from bisulfite sequencing. Primer pairs used in bisulfite sequencing were the following:

1) Forward:5'-TTGGTGTAAGTTTAGAGATAAGT-3', Reverse: 5'-ACCATCTCCTACAAACCCATA -3' and 2) Forward: 5'-GGTTTGGGGTTTTAATTTAGAG-3', Reverse: 5'-ACCATCTCCTACAAACCCATA -3'.

6. Reverse transcription and quantitative real-time PCR:

Total RNA from MDA-MB231 cells was isolated using the RNeasy kit (Qiagen) and pretreated with DNase1. Total RNA (3μg) was reverse transcribed into cDNA using random hexamer primers and Moloney murine leukemia virus reverse transcriptase [70]. Quantitative real-time PCR was performed in Bio-Rad CFX equipment using Sybr green PCR reaction mix. Reaction conditions used are as follows, **1.** 95°C for 3:00, 2. 95°C for 0:10, 55°C for 0:20 (55°C for TMS1 and beta actin, 60°C for ER and EGR1, 57°C for CDH1), repeat step 2, 44 more times; 3. 95.0°C for 0:10 and 4. Melt curve 55°C to 95°C: Increment of 0.5°C for 0:05.

Gene-specific primers used in this work are listed as follows**: 1).** ***TMS1*****:** Forward: TGGGCCTGCAGGAGATG, Reverse: ATTTGGTGGGATTGCCAG; 2). ***EGR1*****:** Forward: 5' to 3'- GACCGCAGAGTCTTTTCCTG, Reverse: 5' to 3'- AGCGGCCAGTATAGGTGATG; 3). ***ESR1*****:** Forward: 5' to 3'- TGAACCGTCCGCAGCTCAAGATC, Reverse: 5' to 3'- GTCTGACCGTAGACCTGCGCGTTG and 4). ***CDH1*****:** Forward: 5' to 3'- GAACGCATTGCCACATACAC, Reverse: 5' to 3'- AGCACCTTCCATGACAGACC.

7. Western blot analysis:

For total protein harvest, MDA-MB231 cells were scraped and pelleted in PBS and lysed in RIPA buffer (150mM sodium chloride, 1% NP40, Tris pH 8.0, 5mM EDTA) containing 1X protease inhibitors (Complete Protease Inhibitor Cocktail, Roche, Indianapolis, IN, USA). Cells were lysed in RIPA buffer, incubated on ice for 10 minutes with brief periodic vortexing of cells on table top Vortex Genie 2 and centrifuged at 16.1k xg at 4°C for 10 minutes. Supernatants were collected and

quantified to determine protein concentration using Bradford assay in the spectrophotometer. Total proteins (30µg) were denatured in 5x Laemmli buffer (0.05% Bromophenol blue, 10% SDS, 0.3M Tris, 25% β-mercapto ethanol, 50% glycerol), separated on 8% resolving SDS-Polyacrylamide gel, transferred to a PVDF membrane and blotted with the indicated primary antibodies. Proteins of interest were detected by chemiluminescence detection of horseradish peroxidase conjugated to the respective secondary antibodies.

Antibodies used were, αDNMT1 (rabbit polyclonal antibody directed at N-terminal 119 amino acids of full length human DNMT1), generated in our laboratory [92], αG9a (Rabbit polyclonal antibody, Millipore, Cat# 09-071) and αGAPDH (Abcam, mouse monoclonal antibody, cat# ab8245)

8. Acid extraction of proteins and immunoblot analysis of modified histones:

For the analysis of global modified histone levels, MDA-MB231 cells were subjected to acid extraction of proteins. Cells were scraped from the plate and suspended in ice-cold PBS and pelleted by centrifugation at 200 xg for 10 minutes at 4°C. Cell pellets were resuspended in lysis buffer (1.5mM $MgCl_2$, 10mM KCl, 10mM HEPES (pH 7.9), supplemented with PMSF (1.5mM) and DTT (0.5mM). Hydrochloric acid was then added to the resuspended cells to a final concentration of 0.2N. This was followed by incubation on ice for 30 minutes and centrifugation at 11,000 xg for 10 minutes at 4°C.

MDA-MB231 cells were subjected to acid extraction of proteins after 48 hours of respective treatments. Prior to electrophoresis, lysates were neutralized by the addition of 0.5 volumes of 1.5 mM Tris pH 8.8. These lysates were separated on 15% resolving

SDS-polyacrylamide gels, transferred to PVDF membranes and immunoblotted with the following antibodies- αH3 (Cell Signaling, catalog# 9715), αH3K9me2 (Millipore, catalog# 07441), αH3K9me3 (Abcam, catalog# ab8898), αH3K9me1 (Abcam, catalog# ab8896), αH3K27me2 (Millipore, catalog #07-452), αH3K4me2 (Cell Signaling, catalog# 9725s) and αH4 (Millipore, catalog# 05-858). Proteins of interest were detected by chemiluminescence detection of horseradish peroxidase conjugated to the respective secondary antibodies.

CHAPTER 3

Results:

1. Effect of BIX-01294 and its analogs on the growth of breast cells:

I started my thesis work by determining the IC_{50} of BIX-01294, E67 and E11 in MDA-MB231 and MCF10A cell lines. The half-maximal inhibitory concentration (IC_{50}) is a measure of effectiveness of a compound in inhibiting a biological or biochemical function. Our goal was to determine the relative growth inhibitory effects against cancer cells and normal cells. A dose response experiment was performed over a period of 48 hours using the following concentrations of BIX-01294 and its analogs - 0.1μM, 0.33μM, 1.11μM, 3.33μM, 10μM and 30μM. MTS assays were used to determine the cell growth in treated cells. GraphPad Prism 4 software was used to generate sigmoidal dose response curves and to determine IC_{50} values. In each experiment, % control was defined as the percentage of surviving drug treated cells relative to untreated control cells.

Our results show that in MDA-MB231 cells, the IC_{50} concentrations of BIX-01294, E67 and E11 from three independent experiments were 3.1 μM, 10.9 μM and 3.37 μM respectively (Figure 11). In MCF10A cells, the IC_{50} concentrations of BIX-01294, E67 and E11 from two independent experiments were 2.72 μM, 6.06 μM and 4.41 μM respectively (Figure 12).

Based on the average IC_{50} values, these results indicate that BIX-01294 and E11 have similar growth inhibitory effects and E67 has the least growth inhibitory effects in both the cell lines studied. Our results also suggest that these agents inhibit growth of both breast cancer cells and immortalized normal breast epithelial cells.

Given these results, the following concentrations of each drug (approximate average **IC_{50}** values) were used for rest of the experiments in this thesis work.

TABLE 1:

G9a/GLP inhibitors	**IC_{50} in MDA-MB231 cells**
BIX-01294-0129401294	**4.0 µM**
E67	**10.0 µM**
E11	**3.0 µM**

2. Treatment with BIX-01294 or its analogs has minimal effects on expression of epigenetically silenced genes in breast cancer cells:

Re-expression of epigenetically silenced genes can be achieved by 5-azaCdR mediated DNA demethylation. 5-azaCdR, a modified cytidine analog incorporates into genomic DNA during DNA synthesis and inhibits DNMT activity. For example, previous work has shown re-expression of epigenetically silenced tumor suppressor genes *MASPIN* and *Desmocollin 3* by 5-aza-CdR mediated DNA demethylation in breast cancer cells [84]. Furthermore, treatment of cancer cells with 5-aza-CdR restores *TMS1* expression suggesting an important role for DNA methylation in silencing of this gene [93].

To better understand the effects of these inhibitors on expression of epigenetically silenced genes in breast cancer cells, we studied the expression of promoter hypermethylated and epigenetically silenced *TMS1*, *ESR1* and *CDH1* genes in MDA-MB231 cells treated with these inhibitors. In addition, we also studied the induction of suppressed *EGR1* gene, in treated MDA-MB231 cells. We used IC_{50} concentrations of BIX-01294 and its analogs (Table 1) to understand whether these drugs by themselves or in combination with 5-azaCdR have any effect on the expression of epigenetically silenced genes. We used 0.5 μM of 5-azaCdR, which has previously been shown to effectively demethylate methylated genes in MDA-MB231 cells [67, 93].

As expected, treatment of MDA-MB231 cells with 5-azaCdR led to the up regulation of *TMS1* (50 fold up regulation), *ESR1* (12 fold up regulation) and *CDH1* (35 fold up regulation) expressions (Figures 13-15). Interestingly, 5-azaCdR treatment also caused an induction in *EGR1* expression (4 fold induction) (Fig 16). However, negligible

effects on the expression of these genes were observed when cells were treated with BIX-01294 or its analogs for 24 or 48 hours (Figures 13-16).

We maintained concurrent treatments of BIX-01294 or its analogs with 5-azaCdR (Figure 10) in order to study the effects from combination therapy of two classes of inhibitors. Based on our results from the MTS assays (Figures 11), growth inhibition occurs in cancer cells treated with BIX-01294 or its analogs. In order to avoid the growth inhibitory effects of these drugs in combination treatments with 5-azaCdR, we also maintained one set of experimental cells with serial treatments of these inhibitors (Figure 10). BIX-01294 or its analogs treated concurrently or serially with 5-azaCdR did not further induce expression of all the genes studied when compared to 5-azaCdR treatments alone (Figures 13-16). Interestingly, in concurrent treatments, E11 seemed to be antagonistic to the effects of 5-azaCdR on *TMS1* and *CDH1* expression (Figures 13 and 15). In addition to this, E67 and E11 seemed to be antagonistic to the 5-aza-CdR effects on *CDH1* expression in serial treatments (Figure 15).

Our data revealed a novel finding with respect to *EGR1* regulation. We found that there was an induction of *EGR1* in cells treated with BIX-01294, its analogs and/or 5-azaCdR. Treatment of cells with 5-azaCdR induced *EGR1* by 3 to 4 fold (Figure 16). Treatments with BIX-01294 or its analogs resulted in an average 2 fold induction of *EGR1* (Figure 16). Our combination treatments (concurrent and serial) showed an additive effect on *EGR1* expression by 1.3-2.3 fold in comparison to independent treatments (Figure 16). This data needs to be explored further in order to understand the mechanism of *EGR1* regulation by 5-azaCdR and/or BIX-01294 analogs.

Overall, these results suggest that BIX-01294 or its analogs have negligible effects on the expression of *TMS1*, *ESR1* and *CDH1* genes and thus likely do not play an important role in the epigenetic regulation of these genes. Induction of *EGR1* by BIX-0129 or its analogs or 5-azaCdR indicates the existence of an epigenetic mechanism in the suppression of this gene in breast cancer cells and needs to be explored further.

3. Effect of BIX-01294 and its analogs on DNA methylation of epigenetically silenced genes in breast cancer cells:

CpG rich *TMS1* promoter is aberrantly methylated and *TMS1* is silenced in MDA-MB231 breast cancer cells and in 40% of primary breast tumors (Figure 9). Treatment of MDA-MB231 cells with 5-azaCdR results in the demethylation of the *TMS1* CpG island and gene re-expression [93]. To address the question whether BIX-01294 and its analogs also affect DNA methylation of aberrantly methylated genes, we used *TMS1* as a model gene to study the effects of these drugs on DNA methylation. Cells were treated with BIX-01294, its analogs and/or 5-azaCdR (Figure 10) for 48 hours after which genomic DNA was harvested and subjected to bisulfite sequencing (Figures 17-19). Methylation status of 44 to 54 CpG sites in the *TMS1* promoter was studied.

Data from untreated and 5-azaCdR treatments are identical in all three treatments with BIX-01294 and its analogs because it was the same treatment that was used across all three treatments (Figures 17-19). Figure 17 shows the data from cancer cells treated with 4.0 μM BIX-01294 and 0.5 μM 5-azaCdR, either alone or in combination. Consistent with the role of 5-azaCdR in DNA demethylation, we saw approximately 30% demethylation in MDA-MB231 cells treated with 5-azaCdR (Figures 17-19). Treatment with BIX-01294 gave inconsistent results as trial-1 resulted in less than 4% CpG demethylation at 24 and 48-hour time points and trial-2 resulted in 5.8% CpG demethylation at 24 hour time point and 16.6% CpG demethylation at 48 hour time point. Cells treated concurrently with BIX-01294 and 5-azaCdR indicated that BIX-01294 antagonized 5-azaCdR mediated demethylation effects because the % CpG demethylation observed in combination treatments (5.2% CpG demethylation in trial-1,

18.9% CpG demethylation in trial-2, Figure 17) was less when compared to % demethylation seen in 5-azaCdR treatments alone (19.6% CpG demethylation in trial-1, 36.6% CpG demethylation in trial-2).

Figure 18 shows data from two independent experiments where cancer cells were treated with 10.0 μM E67 and 0.5 μM 5-azaCdR either alone or in combination. There were negligible effects on demethylation (less than 5% CpG demethylation on average) with E67 treatments at 24 hours and 48 hours. Cells treated concurrently with E67 and 5-azaCdR showed that E67 antagonized the demethylation effects of 5-azaCdR (~9% CpG demethylation is observed in concurrent treatment when compared to ~30% CpG demethylation in 5-azaCdR treated cells determined from two independent experiments). Serial treatments of 5-azaCdR and E67 were inconsistent because data from trial-1 indicated that E67 was antagonistic to the demethylation effects of 5-azaCdR (5.7% CpG demethylation in serial treatment versus 19.6% CpG demethylation with 5-azaCdR alone, Figure 18) whereas data from trial-2 indicated that E67 and 5-azaCdR were additive in their effects on DNA demethylation (45.1% demethylation in serial treatment versus 36.6% demethylation in 5-azaCdR treatment alone, Figure 18).

Figure 19 shows the data from two independent experiments where cells were treated with 3.0 μM E11 and/or 0.5 μM 5-azaCdR. On average cells treated with E11 for 24 and 48 hours showed partial demethylation. Concurrent treatments of E11 and 5-azaCdR have given us variable results, because data from trial 1 indicated that E11 and 5-azaCdR were antagonistic to each other in its demethylation effects (4.6% demethylation in concurrent treatment versus 19.6% demethylation with 5-azaCdR alone, and 10.8% demethylation with E11 alone). Data from trial-2 concurrent treatment

indicated that E11 and 5-azaCdR were additive in their demethylation effects (46.7% demethylation in concurrent treatment versus 36.6% CpG demethylation with 5-azaCdR, Figure 19).

Serial treatments of 5-azaCdR/E11 are variable at this time because data from trial-1 indicated that E11 was antagonistic to the demethylation effects of 5-azaCdR (19.6% CpG demethylation with 5-azaCdR alone and 12.8% CpG demethylation in serial treatment, Figure 19) and data from trial-2 indicated that E11 and 5-azaCdR were additive in their demethylation effects (40.9% CpG demethylation in serial treatment versus 36.6% CpG demethylation with 5-azaCdR alone).

Overall, our data from methylation experiments at the *TMS1* locus indicate that E11 might promote demethylation by itself. Cells treated with E67 alone showed no changes in the methylation status of *TMS1*. Antagonistic effects of BIX-01294 and E67 on 5-azaCdR mediated demethylation indicated that these drugs were interfering with the 5-azaCdR activity. Further experiments need to be conducted to draw conclusions from E11/5-azaCdR combination treatments and BIX-01294 only treatments. Percent CpG demethylation for every treatment group is mentioned in Figures 17-19. Table 2 is a tabulation of percent CpG demethylation at the *TMS1* locus from both the trials.

4. Effect of BIX-01294 and its analogs on G9a and DNMT1 protein levels:

It has been previously reported that 5-azaCdR treatment reactivated aberrantly silenced tumor suppressor genes and was associated with down-regulation of G9a in cancer cells [84]. To investigate the effects of BIX-01294 or its analogs on the total protein levels of G9a and DNMT, MDA-MB231 cells were treated with BIX-01294, its analogs, and/or 5-azaCdR as shown (Figure 10). Total cellular proteins were harvested, and subjected to western blot analyses using the antibodies against G9a, DNMT1 or GAPDH (control for loading).

We observed consistent results in our two independent experiments. While treatment with BIX-01294 or its analogs had no effect on G9a protein levels, treatment with 5-azaCdR either alone or in combination with BIX-01294 or its analogs seemed to down regulate total protein levels of G9a, although at this time we have not quantified G9a protein levels to solidify this conclusion (Figure 20) . There was no effect on DNMT1 protein levels in treatment with BIX-01294 or its analogs. In contrast, there was complete down-regulation of DNMT1 in treatment with 5-azaCdR either alone or in combinations with BIX-01294 or its analogs (Figure 20).

These results indicate that BIX-01294 or its analogs do not affect the translation of DNMT1 and G9a proteins in MDA-MB231 cells. However, 5-azaCdR promotes DNMT1 degradation by itself or in combination treatments with BIX-01294 or its analogs. This suggests a dominant DNMT1 inhibitory effect of 5-azaCdR over BIX-01294 and its analogs. 5-azaCdR may be exerting inhibitory effects on G9a protein levels that need to be confirmed.

5. Effect of BIX-01294 and its analogs on histone modifications:

It has peviously been shown that 5-azaCdR treatments lead to a global decrease in H3K9me2 levels in MDA-MB231 cells [84]. To determine whether treatment with BIX-01294 and its analogs also has similar effect on the global levels of H3K9me2 and other histone H3 modifications we treated MDA-MB231 cells as shown (Figure 10). After 48 hours, we isolated acid soluble histones and measured the changes in global histone modification levels by western blot analyses using antibodies against H3K9me1, H3K9me2, H3 K9me3, H3K27me2, H3K4me2, total H3 (loading control) and total H4 (loading control) (Figures 21-25).

Our data indicates that there was significant decrease ($P<0.05$) in H3K9me2 levels when MDA-MB231 cells were treated with BIX-01294 or its analogs (Figure 21). There was no effect on global H3K9me2 levels in cells treated with 5'azaCdR alone (Figure 21). Treatment of cells with 0.5μM 5-azaCdR antagonized the repressive effects of BIX-01294 or its analogs on H3K9me2 levels in several treatment groups (Figure 21).

Treatment of MDA-MB231 cells with BIX-01294 or its analogs alone or 5-azaCdR alone induced the global levels of H3K9me3, with the most robust effects seen with 5-azaCdR alone (Figure 22). Additive effects on H3K9me3 levels were observed in combination treatments (Figure 22). Our studies have revealed a novel observation where in global H3K9me3 levels are induced in 5'-azaCdR treated cells either alone or in combination with BIX-01294 or its analogs. There is some induction seen in cells treated with BIX-01294 and its analogs alone (Figure 22).

We next examined the global levels of H3K9me1 in cells treated with BIX-01294 or its analogs. As we have observed changes in H3K9me2 modifications with these

treatments, we asked if these changes are a result from corresponding changes in the mono methyl modification of H3K9. Our result from one experiment indicates that H3K9me1 levels remain unaffected in treated and untreated cells (Figures 23). This suggested that BIX-01294, its analogs and 5'-azaCdR does not affect the global mono methyltransferase activity of H3K9 in MDA-MB231 cells.

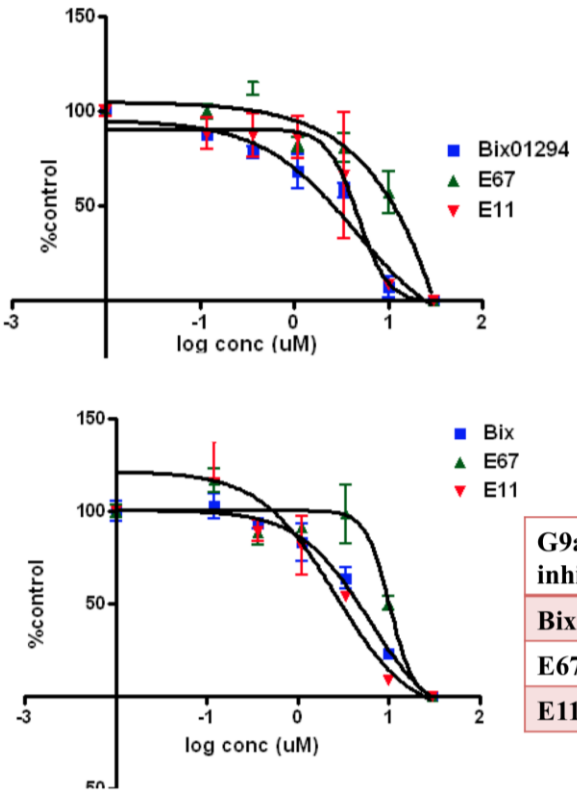
The next question we asked was if these drugs affected global levels of other prominent histone lysine methyl modifications. To this end we chose to study global changes in H3K27me2, which is a repressive mark associated with epigenetic gene silencing [78, 94]. We treated the cells in the same manner as in Figure 10 and subjected the acid extracted proteins to immunoblotting with an antibody specific to H3K27me2. Our results from two independent experiments indicated that there were no changes in this modification in treatment with BIX-01294 or its analogs either alone or in combination with 5'-azaCdR (Figure 24). This suggested that these small molecular inhibitors are specific in their activity towards histone H3K9 methyltransferases in cancer cells.

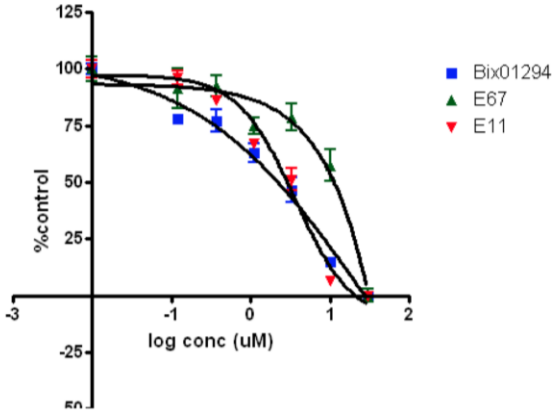
The final question we asked was if the decrease in global H3K9me2 levels in cells treated with BIX-01294 or its analogs were accompanied by a global increase in H3K4me2 levels, which is a histone modification associated with actively transcribing genes [93]. Our results indicated that overall there were no dramatic changes observed in H3K4me2 global levels after treatment with BIX-01294, its analogs or 5-azaCdR. Data from two independent experiments are shown in Figure 25.

In summary, our data indicates that treatment with BIX-01294 or its analogs lead to a global decrease in H3K9me2 global levels and global increase in H3K9me3 levels

without affecting other histone modifications studied. 5-azaCdR appears to antagonize the effects of BIX-01294 on H3K9me2 levels in some cases. 5-azaCdR treatment induces H3K9me3 levels without affecting other histone modifications..

Figure 11

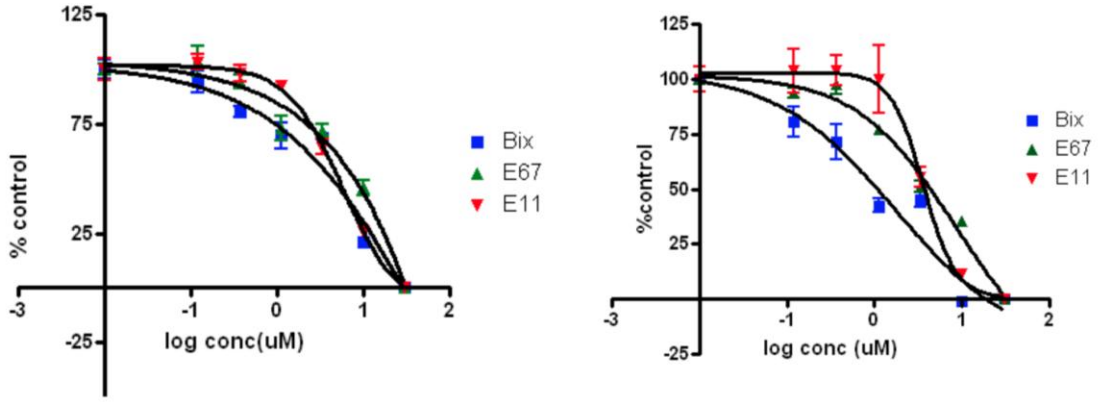




G9a inhibitors	Trial #1	Trial #2	Trial #3	Average (uM)
Bix01294	2.51	2.13	4.67	3.1
E67	11.48	11.22	10	10.9
E11	4.36	2.82	2.95	3.37

Figure 11: Effect of BIX-01294 and its analogs on the growth of breast cancer cells: MDA-MB231 cells were seeded in 96 well plates at 4000 cells/well. The following day, increasing doses of BIX-01294, E67 and E11 (0.1 μM, 0.33 μM, 1.11 μM, 3.33 μM, 10.0 μM and 30.0 μM) were added to the cells in quadruplicates. Untreated cells were maintained as controls. Measurements were performed after 48 hours. Each experiment was done in quadruplicates and Graph Pad PRISM 4 software was used to generate IC_{50} values for the drugs used. IC_{50} values of each drug in three independent experiments and its average values are tabulated in the table above. Results shown are from three separate growth assays in MDA-MB231 cells. Error bars are standard deviation of the mean of quadruplicate readings from each assay.

Figure 12



G9a inhibitors	Trial #1 (uM)	Trial #2 (uM)	Average (uM)
Bix01294	**4.36**	**1.09**	**2.72**
E67	**7.76**	**4.36**	**6.06**
E11	**5.12**	**3.71**	**4.41**

Figure 12: Effect of BIX-01294 and its analogs on the growth of normal immortalized breast epithelial cells: MCF10A cells were seeded in 96 well plates at 4000 cells/well and treated with BIX-01294, E67, and E11 as indicated in Figure 11. Measurements and quantifications were done in the same manner as indicated in Figure 11. Results shown are from two independent experiments. IC_{50} values of each drug in two independent experiments and its average values are tabulated in the table above. Error bars are standard deviation of the mean of quadruplicate readings from each assay.

Figure 13

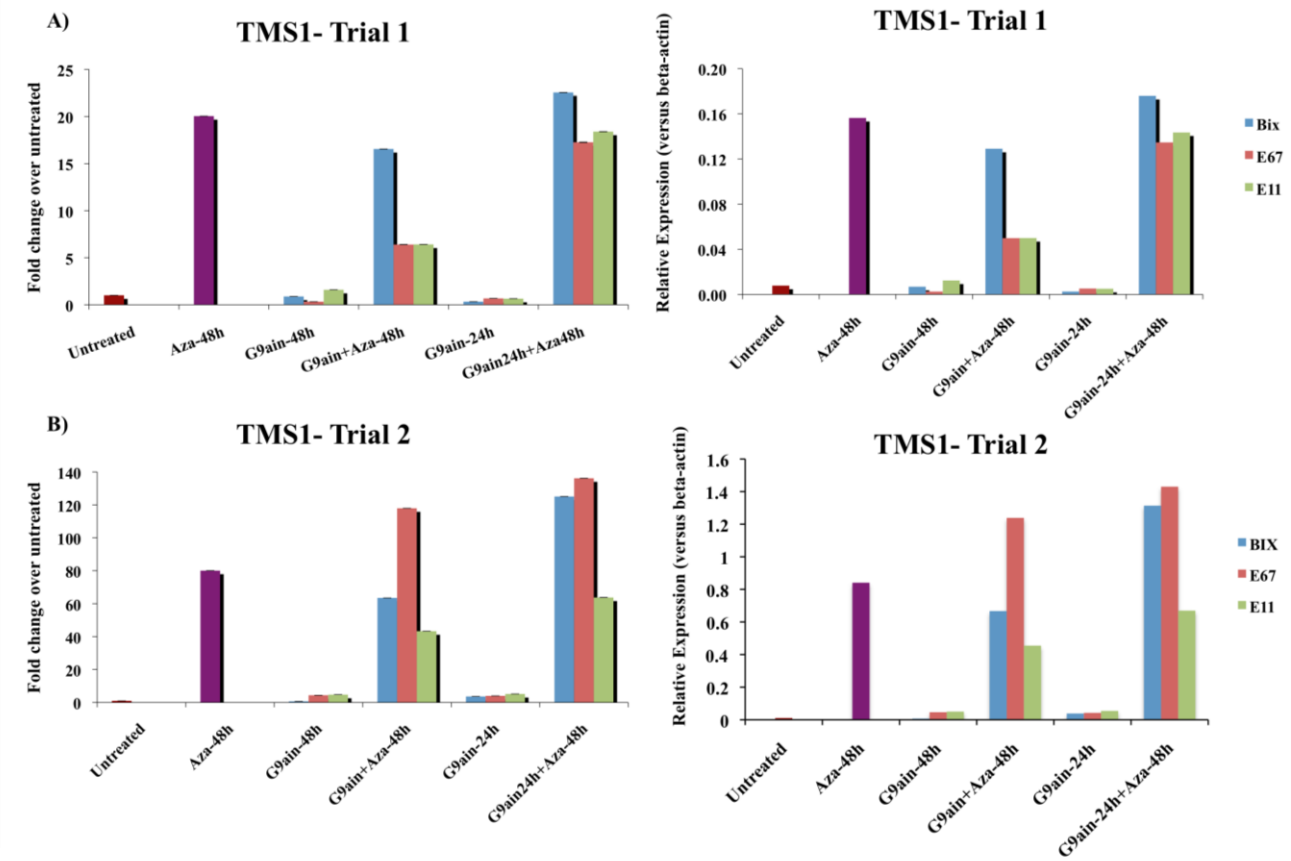

A)
TMS1- Trial 1
Fold change over untreated
25
20
15
10
5
0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain24h+Aza48h
TMS1- Trial 1
Relative Expression (versus beta-actin)
0.20
0.16
0.12
0.08
0.04
0.00
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h
Bix
E67
E11
B)
TMS1- Trial 2
Fold change over untreated
140
120
100
80
60
40
20
0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain24h+Aza-48h
TMS1- Trial 2
Relative Expression (versus beta-actin)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h
BIX
E67
E11

Figure 14

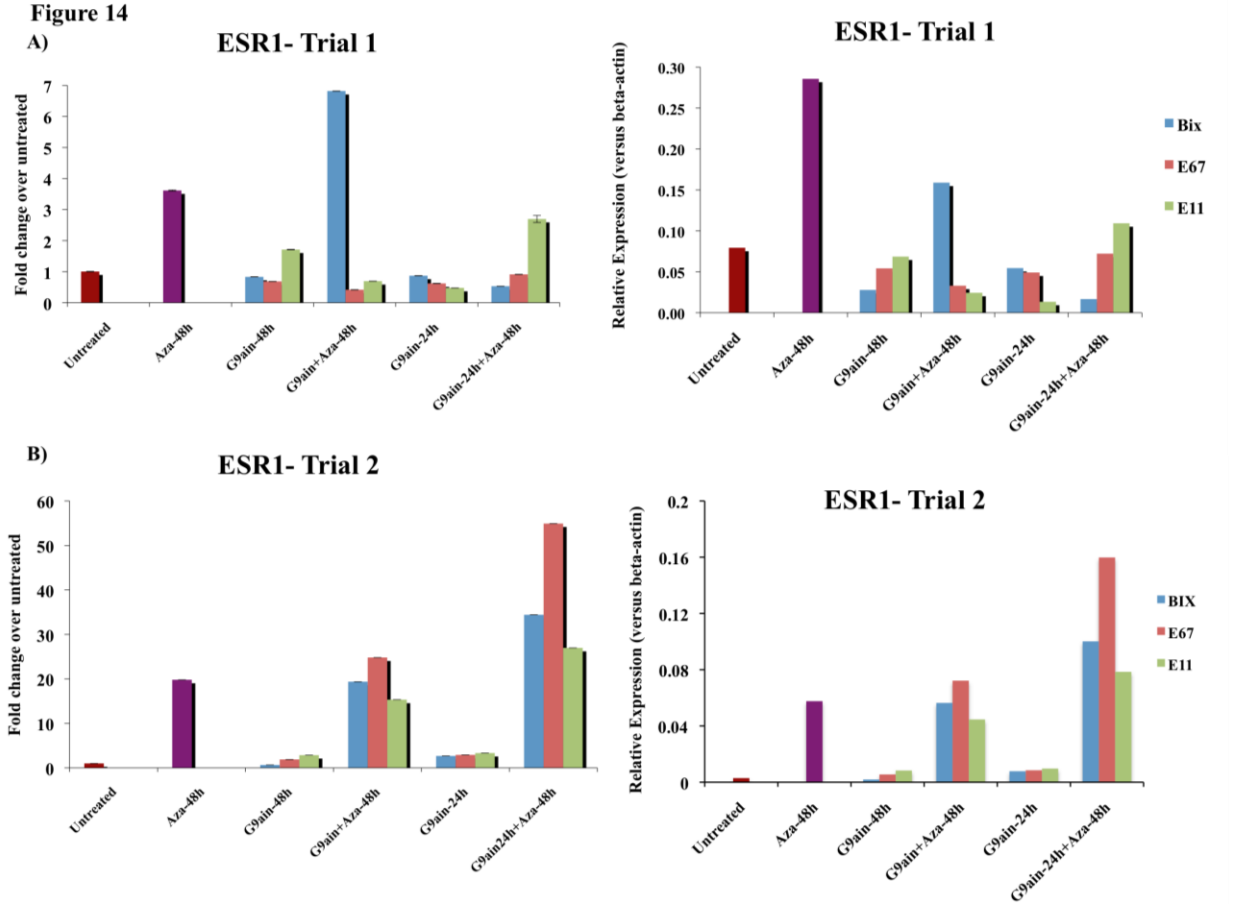

A)
ESR1- Trial 1
Fold change over untreated
0
1
2
3
4
5
6
7
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h
ESR1- Trial 1
Relative Expression (versus beta-actin)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Bix
E67
E11
B)
ESR1- Trial 2
Fold change over untreated
0
10
20
30
40
50
60
ESR1- Trial 2
Relative Expression (versus beta-actin)
0
0.04
0.08
0.12
0.16
0.2
BIX
E67
E11

Figure 15

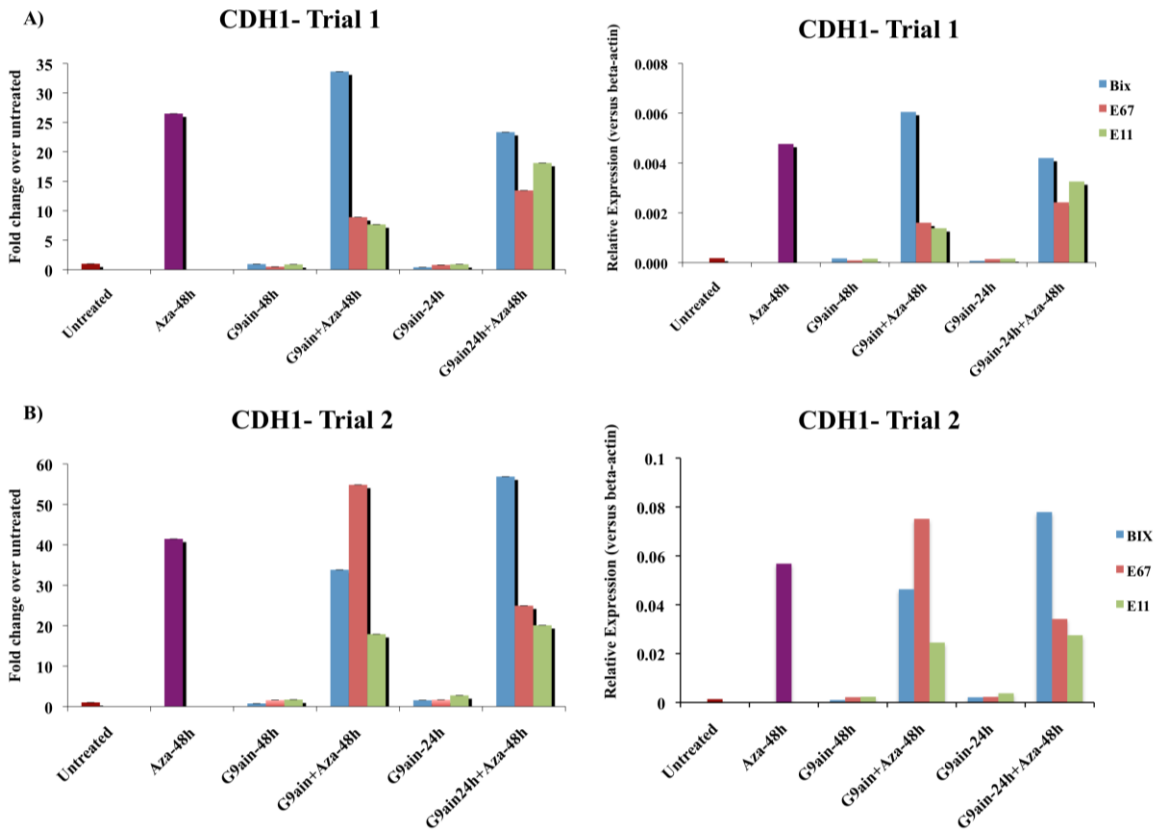
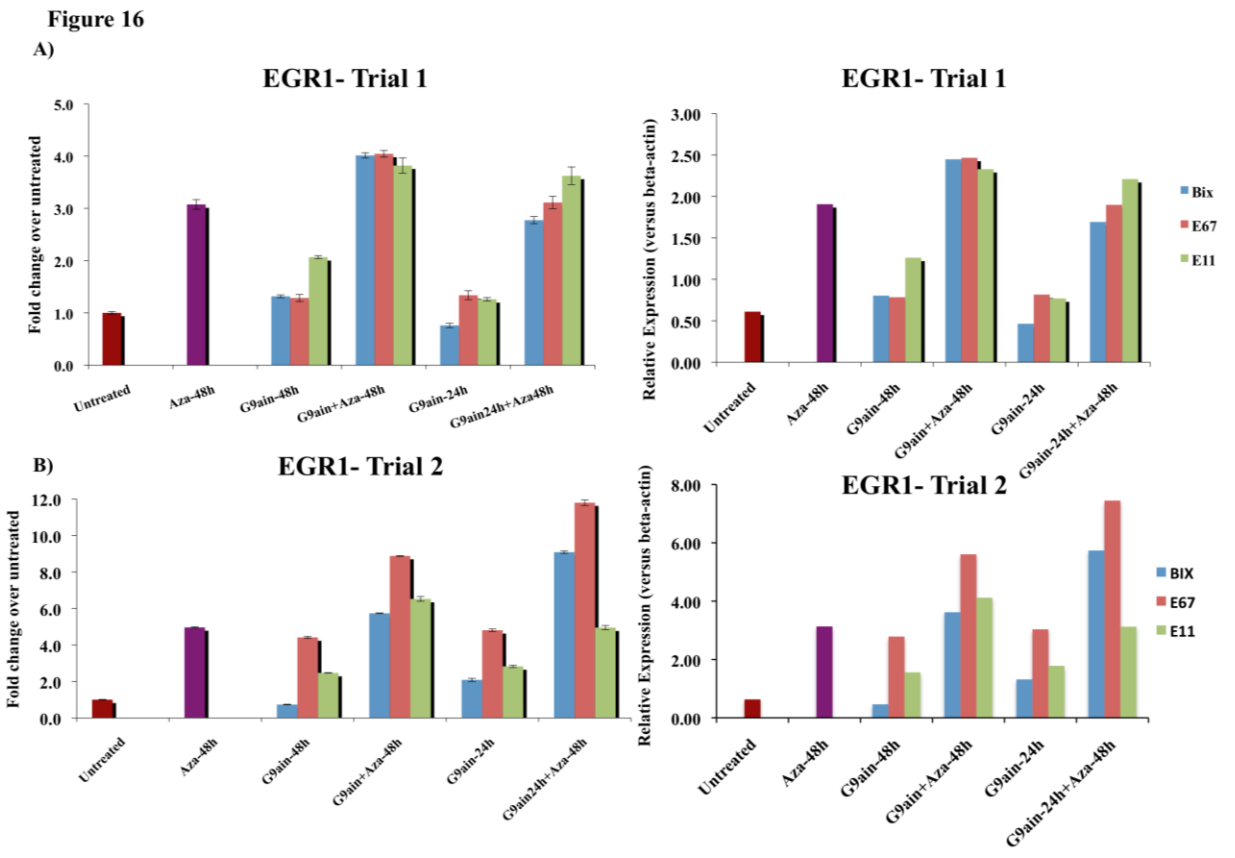

A)
CDH1- Trial 1
Fold change over untreated
35
30
25
20
15
10
5
0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain24h+Aza48h
CDH1- Trial 1
Relative Expression (versus beta-actin)
0.008
0.006
0.004
0.002
0.000
Bix
E67
E11
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h
B)
CDH1- Trial 2
Fold change over untreated
60
50
40
30
20
10
0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain24h+Aza-48h
CDH1- Trial 2
Relative Expression (versus beta-actin)
0.1
0.08
0.06
0.04
0.02
0
BIX
E67
E11
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h

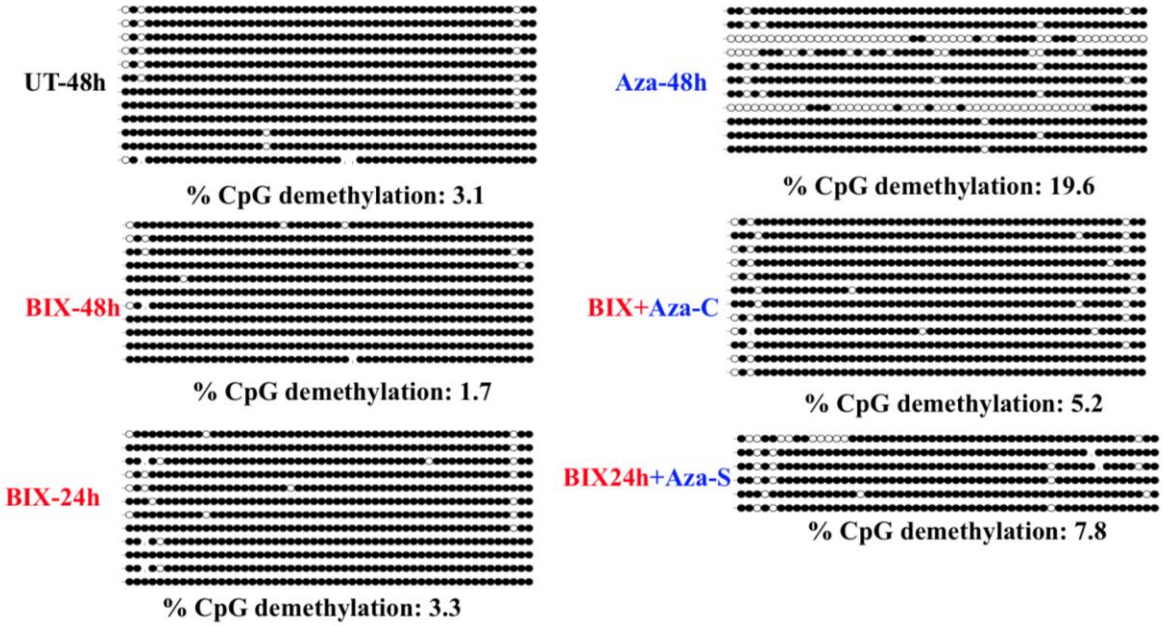
Figure 16


A)
EGR1- Trial 1
Fold change over untreated
5.0
4.0
3.0
2.0
1.0
0.0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain24h+Aza48h
EGR1- Trial 1
Relative Expression (versus beta-actin)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h
Bix
E67
E11
B)
EGR1- Trial 2
Fold change over untreated
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain24h+Aza-48h
EGR1- Trial 2
Relative Expression (versus beta-actin)
8.00
6.00
4.00
2.00
0.00
Untreated
Aza-48h
G9ain-48h
G9ain+Aza-48h
G9ain-24h
G9ain-24h+Aza-48h
BIX
E67
E11

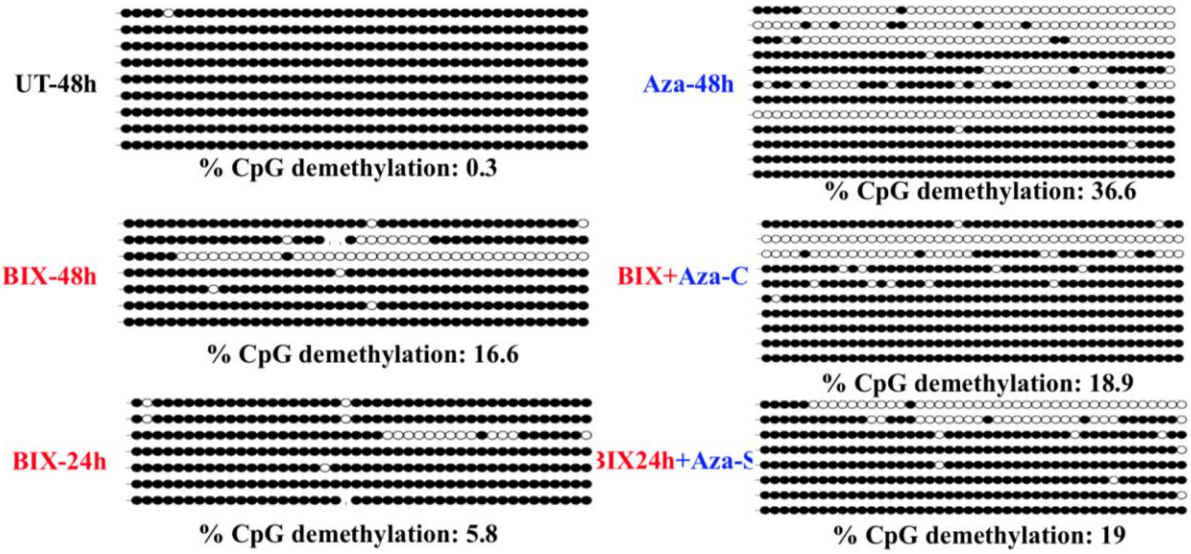
Figures 13, 14, 15, 16: Effects of BIX-01294, its analogs and/or 5-azaCdR on gene expression in MDA-MB231 cells: MDA-MB231 cells were seeded on 6-well plates and maintained as untreated (UT) or treated with BIX-01294/E67/E11 and/or 5-azaCdR as indicated. Total RNA was harvested 48 hours post-treatment, was reverse transcribed into cDNA and quantified by real-time PCR using primers for *TMS1* (Figure 13), *ESR1* (Figure 14), *CDH1* (Figure 15) and *EGR1* (Figure 16). Relative starting quantities were determined by comparison to MCF7 cDNA standard curve included in each run. Shown are the results from two independent experiments (a & b). Columns are triplicate values of relative mRNA expression of *TMS1, EGR1, ESR1* and *CDH1* compared with untreated after normalization to β-actin mRNA. Error bars are standard deviation of the mean of triplicate values from each experiment. Graphs on the right panels of a & b in figures 13-16 represents the normalized value of the respective gene over β-actin mRNA in every sample. Treatments at 24 hours and 48 hours are indicated in the respective sample labels. G9ain are BIX-01294/E67/E11, Aza is 5-azaCdR.

Figure 17

Trial 1:



Trial 2:



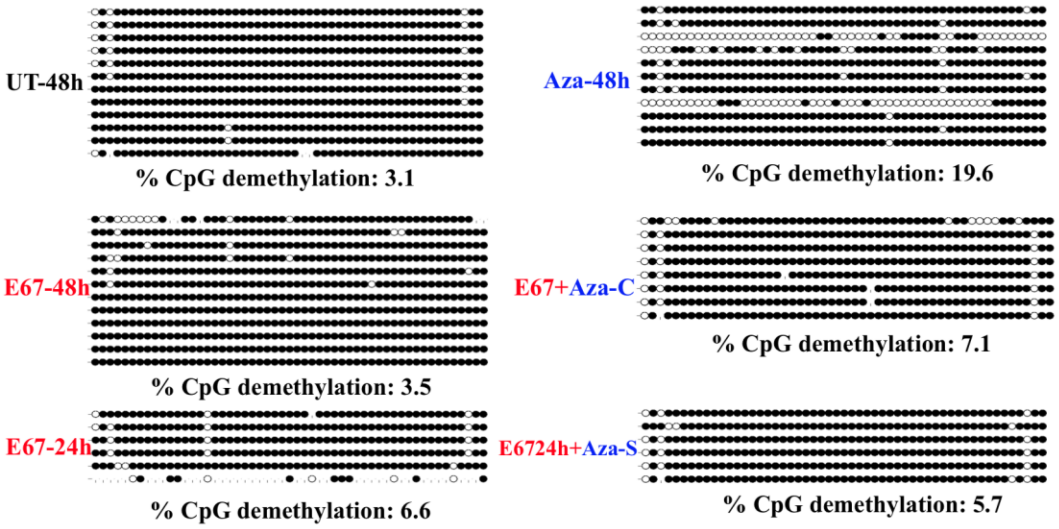
C- Concurrent treatment

S- Serial treatment

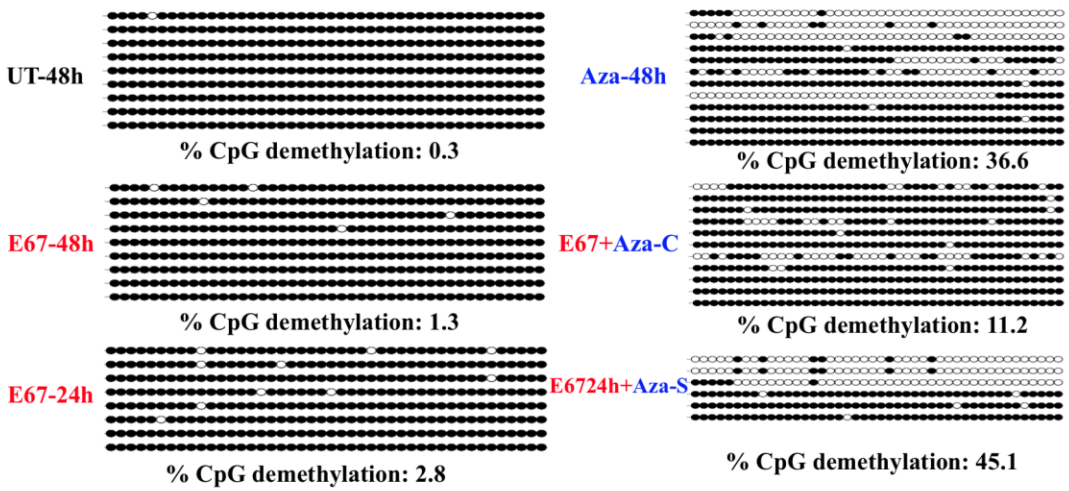
Figure 17: Effects of BIX-01294 and/or 5-azaCdR on DNA methylation at the *TMS1* locus: $2x10^5$ MDA-MB231 cells were seeded on 6-well plates. Following day, cells were treated for 48 hours with BIX-01294 and/or 5-azaCdR. Genomic DNA (gDNA) was isolated and subjected to bisulfite conversion. Bisulfite converted gDNA was used as a template for amplification in Bisulfite Sequencing PCR as mentioned in *Materials and Methods*. Amplified gDNA was then purified and sub cloned into TOPO TA vector; sequences from individual clones (6-12 clones per treatment group) were analyzed for changes in DNA methylation status at the *TMS1* locus in BiQ analyzer program. %CpG demethylation at *TMS1* gene locus is indicated below every treatment group. %CpG demethylation is the number of demethylated CpG sites over total number of CpG sites at that locus multiplied by 100. Results are shown from two independent experiments. Filled circles are methylated CpG sites; unfilled circles are unmethylated CpG sites. UT indicates untreated MDA-MB231 cells. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Figure 18

Trial 1:



Trial 2:

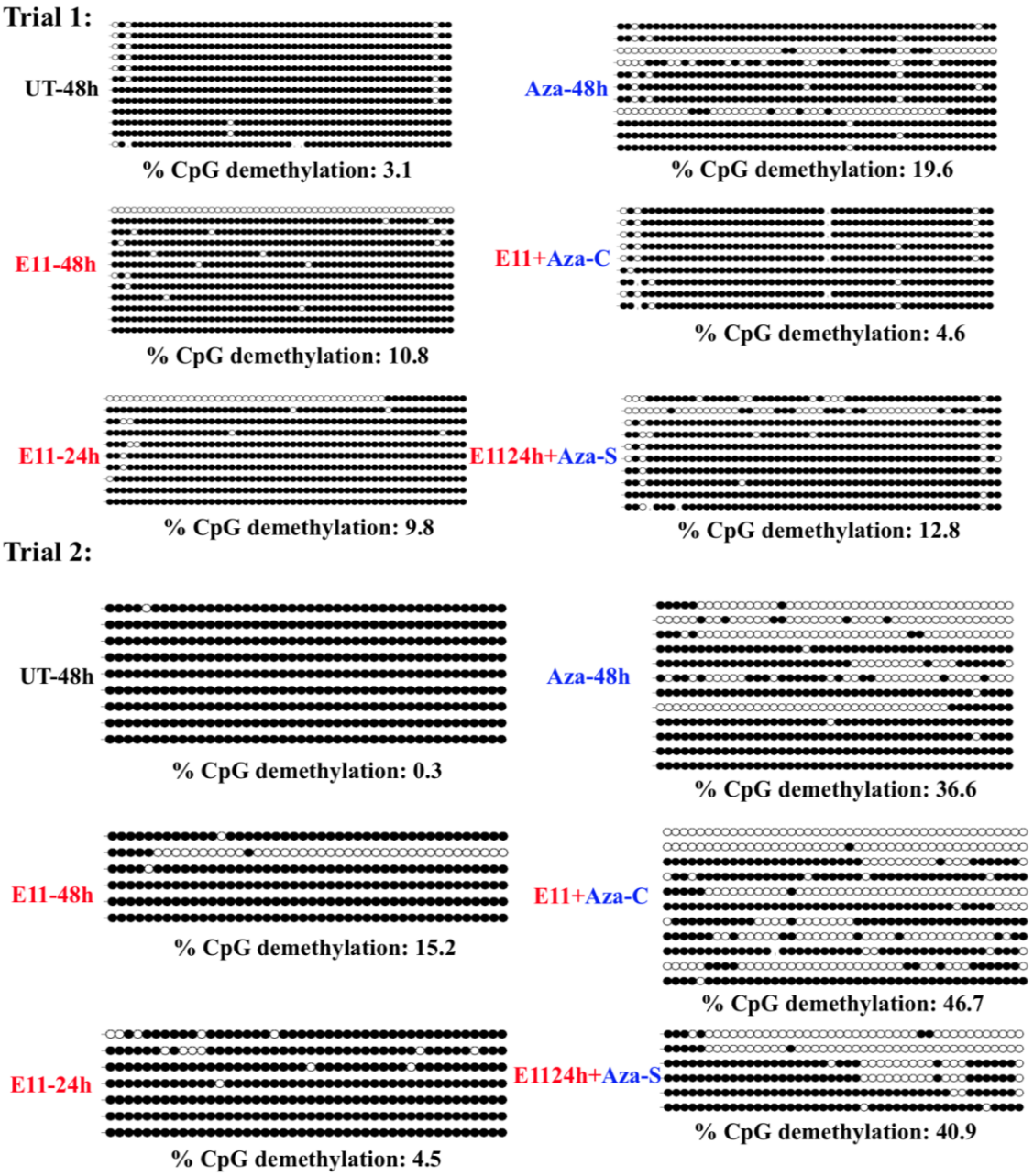


C- Concurrent treatment

S- Serial treatment

Figure 18: Effects of E67 and 5-azaCdR on DNA methylation at the *TMS1* locus: $2x10^5$ MDA-MB231 cells were seeded on 6-well plates. Following day, cells were treated for 48 hours with E67 and/or 5'azaCdR. Genomic DNA (gDNA) was harvested and subjected to bisulfite conversion. Bisulfite converted gDNA was used as a template for amplification in Bisulfite Sequencing PCR as mentioned in *Materials and Methods*. Amplified gDNA was then purified and sub cloned into TOPO TA vector; sequences from individual clones (6-12 clones per treatment group) were analyzed for changes in DNA methylation status at the *TMS1* locus in BiQ analyzer program. %CpG demethylation at *TMS1* gene locus is indicated below every treatment group. %CpG demethylation is the number of demethylated CpG sites over total number of CpG sites at that locus multiplied by 100. Results are shown from two independent experiments. Filled circles are methylated CpG sites; unfilled circles are unmethylated CpG sites. UT indicates untreated MDA-MB231 cells. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Figure 19



C- Concurrent treatment

S- Serial treatment

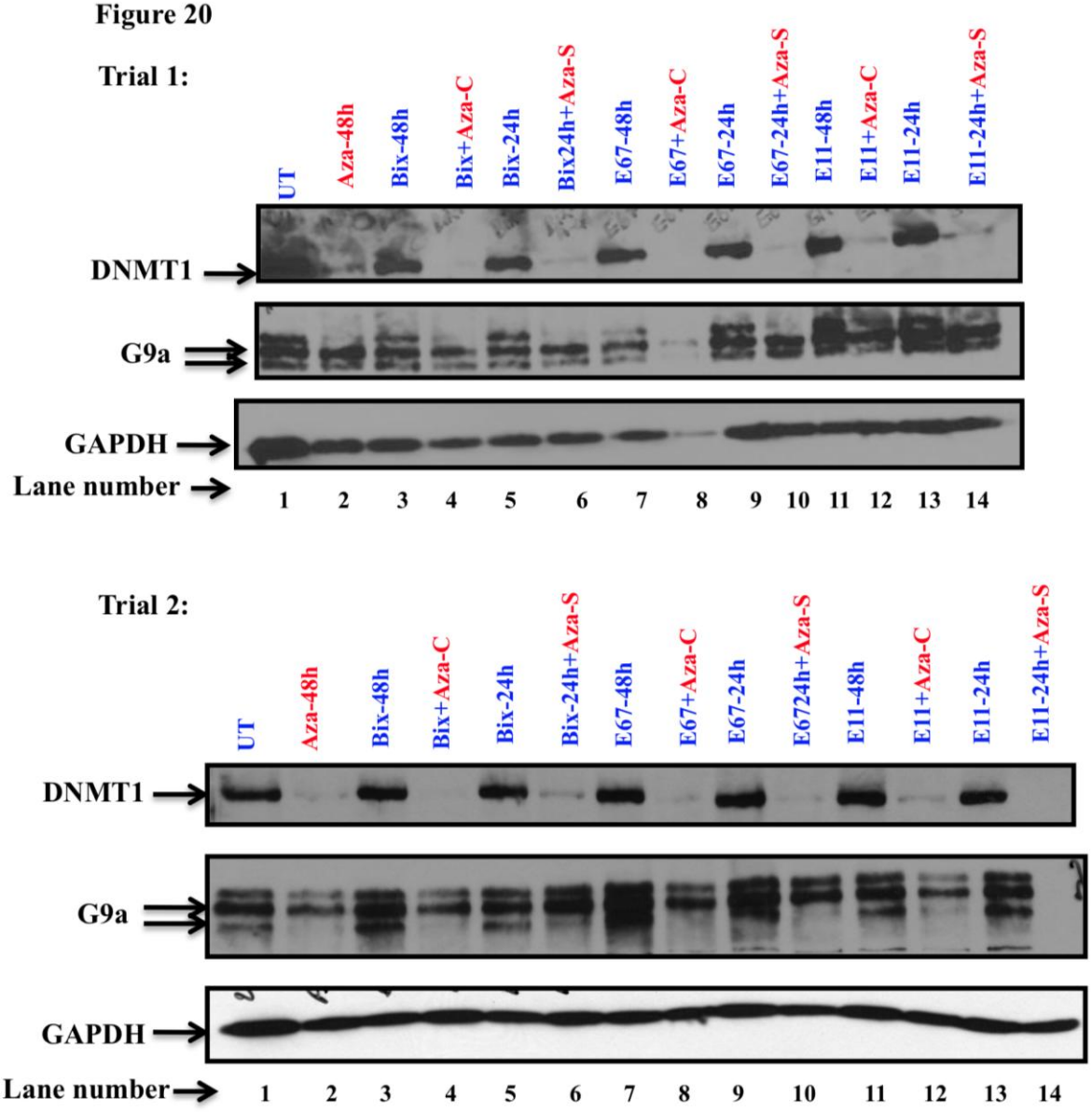
Figure 19: Effects of E11 and 5-azaCdR on DNA methylation at the *TMS1* locus: $2x10^5$ MDA-MB231 cells were seeded on 6-well plates. Following day, cells were treated for 48 hours with E11 and/or 5'azaCdR, by itself or in combinations as indicated. Genomic DNA (gDNA) was harvested and subjected to bisulfite conversion. Bisulfite converted gDNA was used as a template for amplification in Bisulfite Sequencing PCR as mentioned in *Materials and Methods*. Amplified gDNA was then purified and sub cloned into TOPO TA vector; sequences from individual clones (6-12 clones per treatment group) were analyzed for changes in DNA methylation status at the *TMS1* locus in BiQ analyzer program. %CpG demethylation at *TMS1* gene locus is indicated below every treatment group. %CpG demethylation is the number of demethylated CpG sites over total number of CpG sites at that locus multiplied by 100. Results are shown from two independent experiments. Filled circles are methylated CpG sites; unfilled circles are unmethylated CpG sites. UT indicates untreated MDA-MB231 cells. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Table 2:

Treatments	%CpG demethylation-Trial 1	% CpG demethylation-Trial 2
UT (Untreated)	3.1	0.3
Aza-48h	19.6	36.6
Bix-48h	1.7	16.6
Bix+Aza-C	5.2	18.9
Bix-24h	3.3	5.8
Bix24h+Aza-S	7.8	19
E67-48h	3.5	1.3
E67+Aza-C	7.1	11.2
E672-4h	6.6	2.8
E6724h+Aza-S	5.7	45.1
E11-48h	10.8	15.2
E11+Aza-C	4.6	46.7
E11-24h	9.8	4.5
E1124h+Aza-S	12.8	40.9

TABLE 2: Percent CpG demethylation at the *TMS1* locus in untreated and treated MDA-MB231 cells. Data from every treatment groups from two independent experiments shown in figures 7, 8 and 9 are tabulated above. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Figure 20



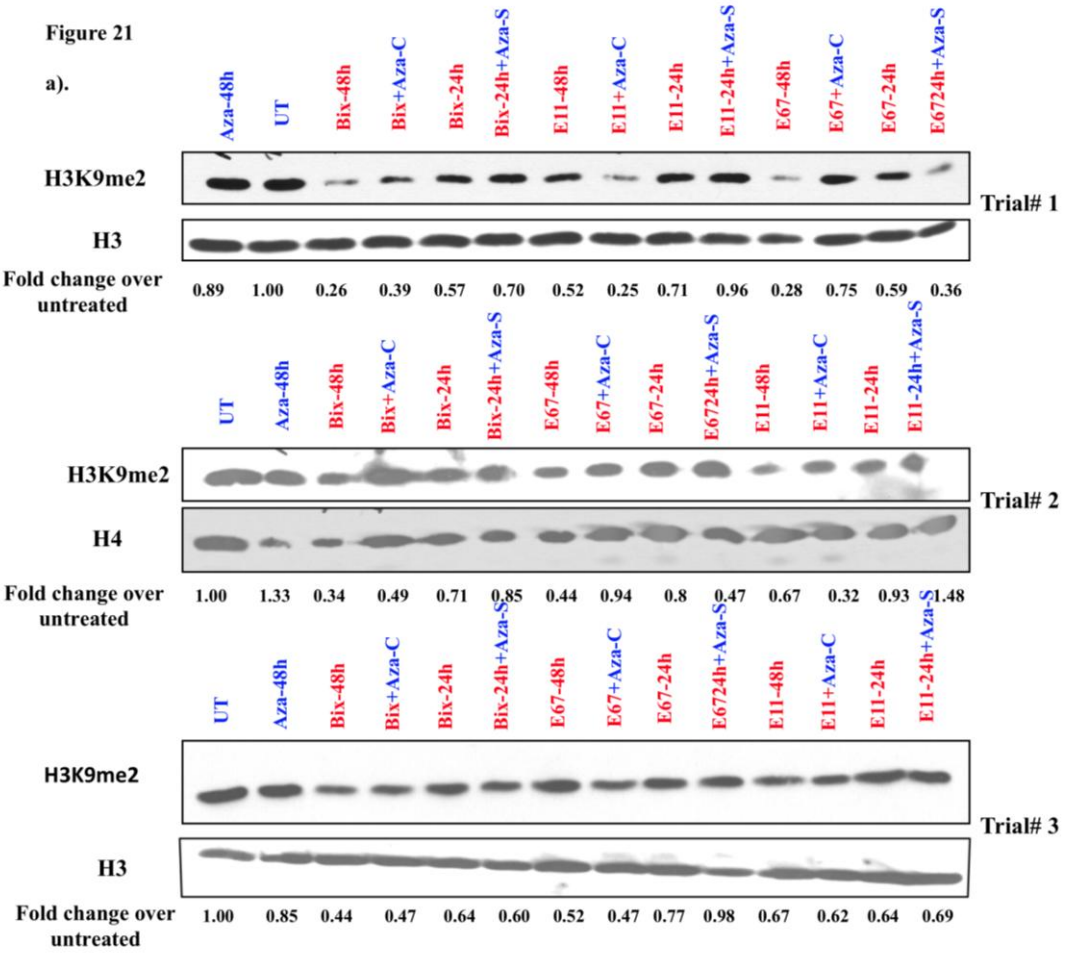
C- Concurrent treatment

S- Serial treatment

Figure 20: Effect of BIX-01294, its analogs and/or 5-azaCdR on G9a and DNMT1 protein levels in breast cancer cells: $2x10^5$ MDA-MB231 cells were seeded on 6-well plates and treated with BIX-01294, E67, E11, and/or 5-azaCdR for 24 or 48 hours. Total cellular proteins were isolated and separated on 8% resolving SDS-polyacrylamide gels, transferred overnight to PVDF membranes and subjected to western blot analyses using antibodies against DNMT1, G9a and GAPDH (loading control). Low DNMT1/G9a/GAPDH levels in lane 8 of trial 1 are because of insufficient loading of that protein sample. Lack of DNMT1/G9a signal in lane 15 of trial-2 is due to an unknown technical error that might have happened either during electrophoresis or transfer. GAPDH loading control in the same lane seems to be fine which indicates a technical fault during electrophoresis/transfer of high molecular weight proteins. Treatments at 24 hours and 48 hours are indicated in the respective sample labels. Results are shown from two independent experiments.

Figure 21

a).



C- Concurrent treatment

S- Serial treatment

b).

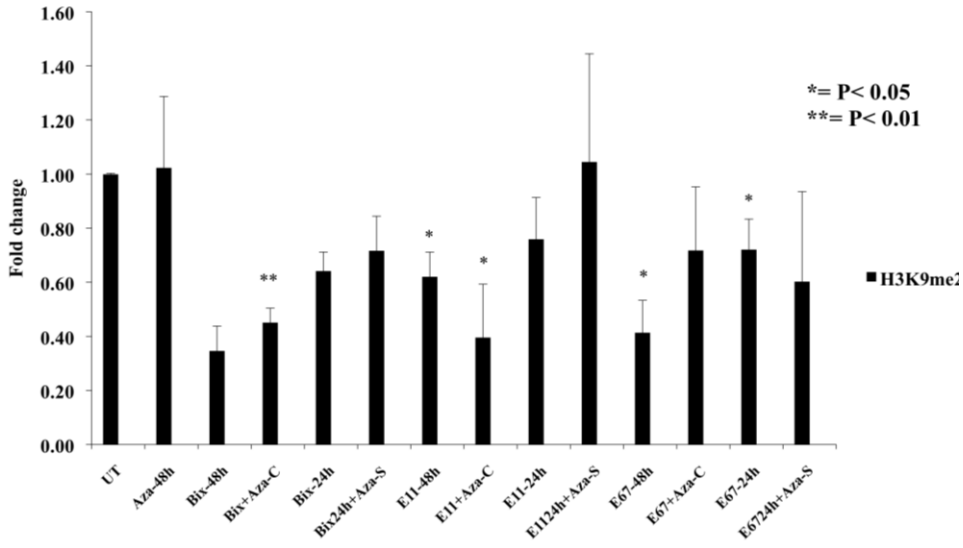
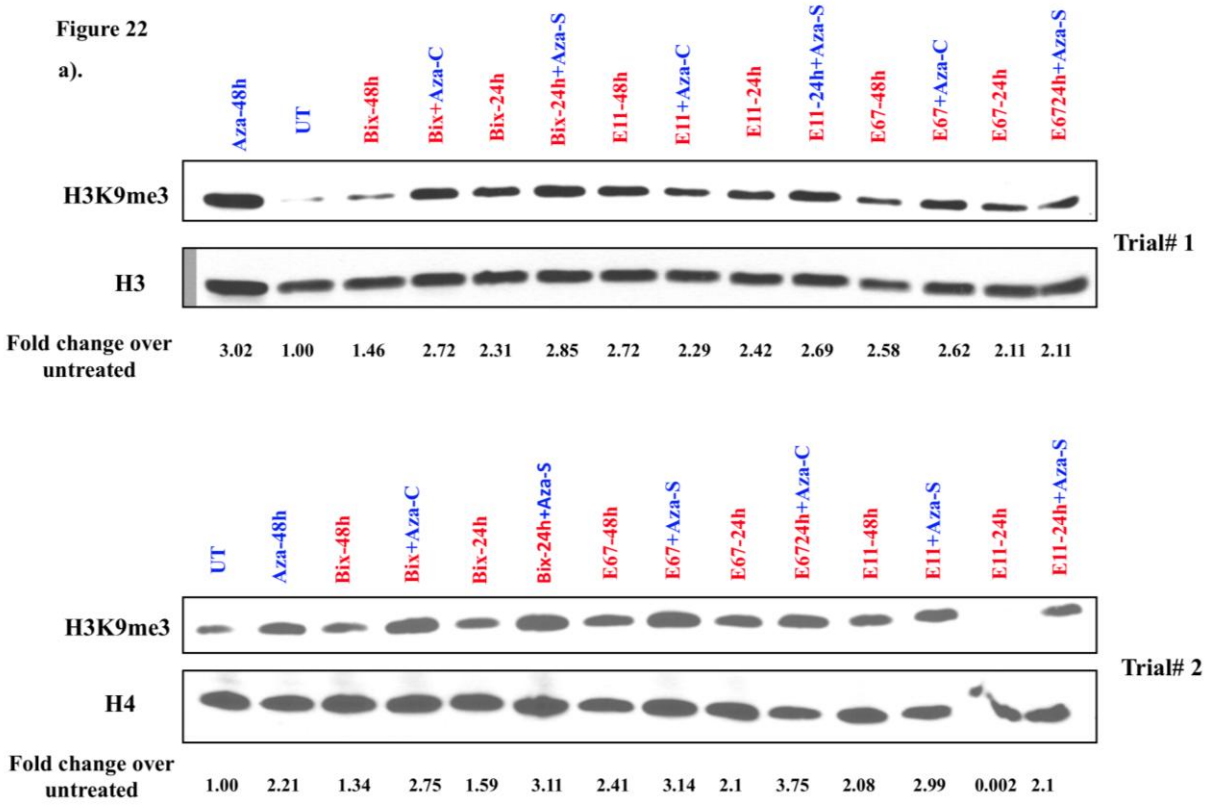


Figure 21: Effect on BIX-01294, its analogs and/or 5-azaCdR, on total levels of histone H3K9me2 modification in breast cancer cells. $5x10^5$ MDA-MB231 cells were seeded on 10cm culture dishes and treated with BIX-01294, E67, E11 and/or 5-azaCdR as indicated. Acid extraction of proteins was performed as mentioned in *Materials and Methods*. 10-20 µg of acid soluble proteins were separated on 15% SDS-polyacrylamide resolving gel and transferred onto PVDF membranes. Membranes were immunoblotted with antibodies against H3K9me2, total H3 and total H4 and signals were detected by chemiluminescence. Results are shown from three independent experiments in (a), fold change over untreated values is indicated below every experiment in (a). Mean of three experiments is shown in (b). Columns are mean fold change values from three independent experiments calculated by relative integrated density measurements of H3K9me2 compared with untreated after normalization to H3 or H4 integrated density measurements. Image J software program was utilized to quantify the immunoblots. Error bars are standard deviation of mean values. ** indicate $P<0.01$ and * indicate $P<0.05$ as determined by t-test. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Figure 22

a).


Aza-48h
UT
Bix-48h
Bix+Aza-C
Bix-24h
Bix-24h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E11-24h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S
H3K9me3
H3
Trial# 1
Fold change over untreated
3.02 1.00 1.46 2.72 2.31 2.85 2.72 2.29 2.42 2.69 2.58 2.62 2.11 2.11
UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix-24h+Aza-S
E67-48h
E67+Aza-S
E67-24h
E6724h+Aza-C
E11-48h
E11+Aza-S
E11-24h
E11-24h+Aza-S
H3K9me3
H4
Trial# 2
Fold change over untreated
1.00 2.21 1.34 2.75 1.59 3.11 2.41 3.14 2.1 3.75 2.08 2.99 0.002 2.1

b).

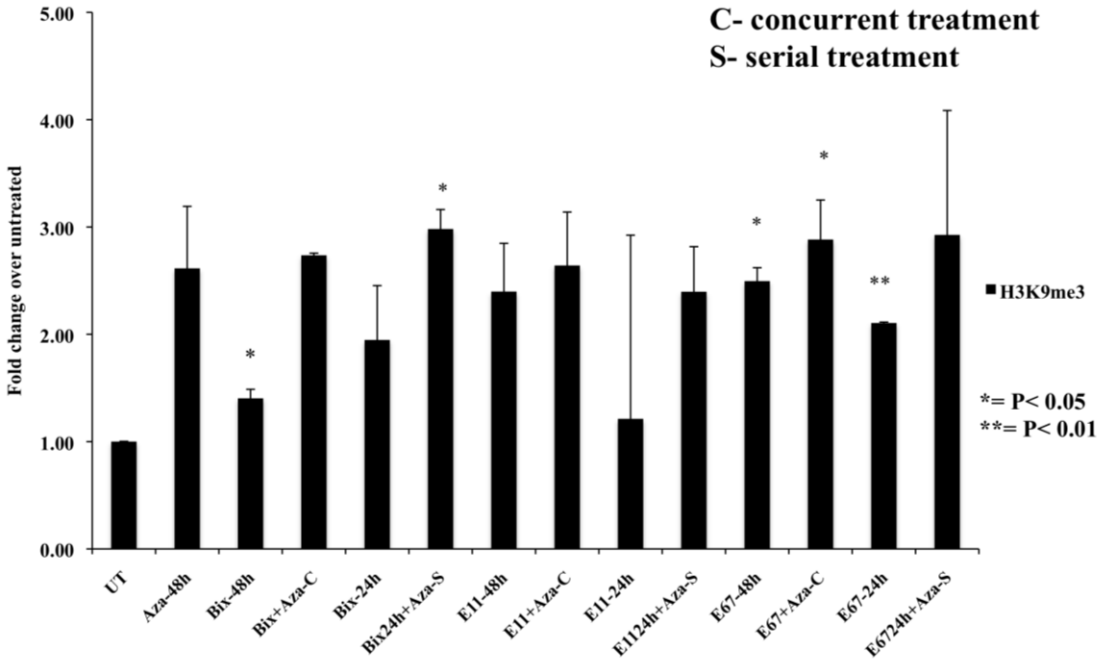
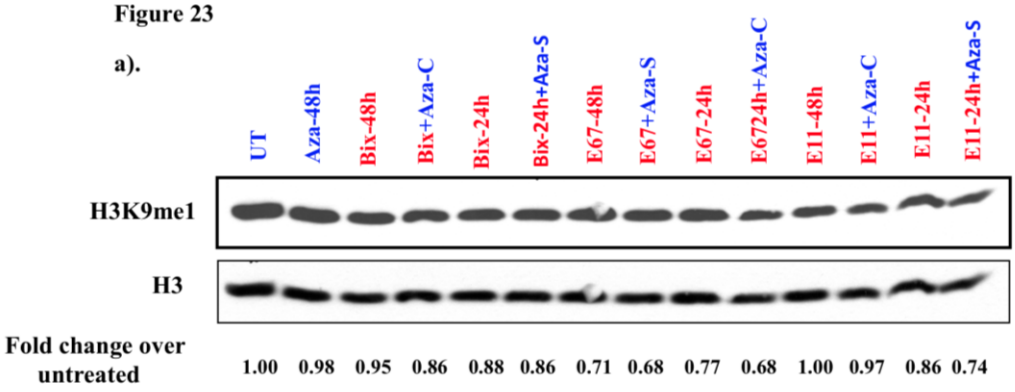

C- concurrent treatment
S- serial treatment
Fold change over untreated
5.00
4.00
3.00
2.00
1.00
0.00
H3K9me3
*= P< 0.05
**= P< 0.01
UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix24h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E1124h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S

Figure 22: Effect on BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K9me3 modification in breast cancer cells. $5x10^5$ MDA-MB231 cells were seeded on 10 cm dishes and treated with BIX-01294, E67, E11 and/or 5-azaCdR as indicated. Acid extraction of proteins was performed as mentioned in *Materials and Methods*. 10-20 µg of acid soluble proteins were separated on 15% SDS-polyacrylamide resolving gel and transferred onto PVDF membranes. Membranes were immunoblotted with antibodies against H3K9me3, total H3 and total H4 and signals were detected by chemiluminescence. Results are shown from two independent experiments in (a), fold change over untreated values is indicated below every experiment in (a). Mean of three experiments is shown in (b). Columns are mean fold change values from two independent experiments calculated by relative integrated density measurements of H3K9me3 compared with untreated after normalization to H3 integrated density measurements. Image J software program was utilized to quantify the immunoblots. Error bars are standard deviation of mean values. ** indicate $P<0.01$ and * indicate $P<0.05$ as determined by t-test . Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Figure 23

a).



Fold change over untreated	1.00	0.98	0.95	0.86	0.88	0.86	0.71	0.68	0.77	0.68	1.00	0.97	0.86	0.74

b).

C- concurrent treatment
S- serial treatment

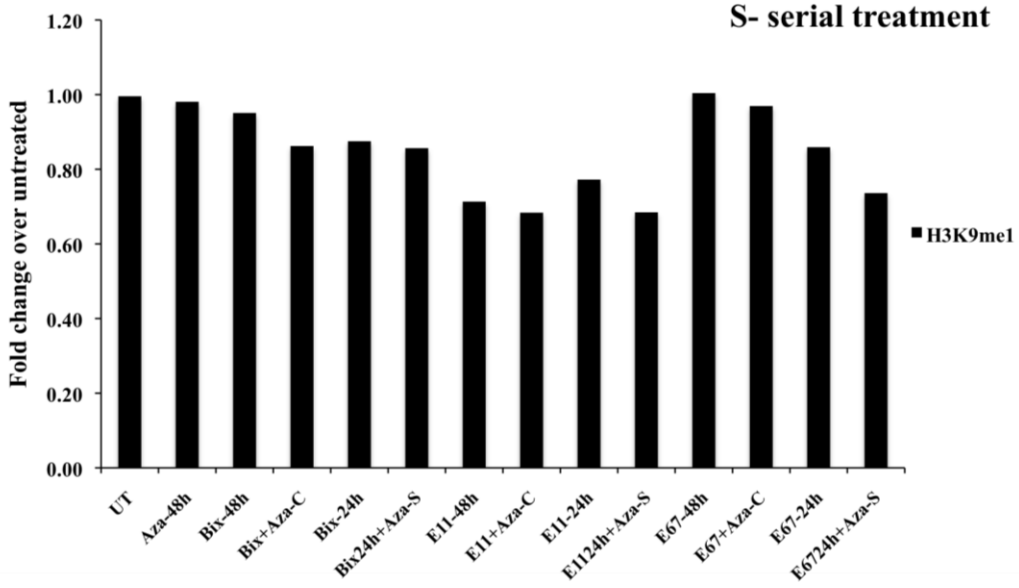
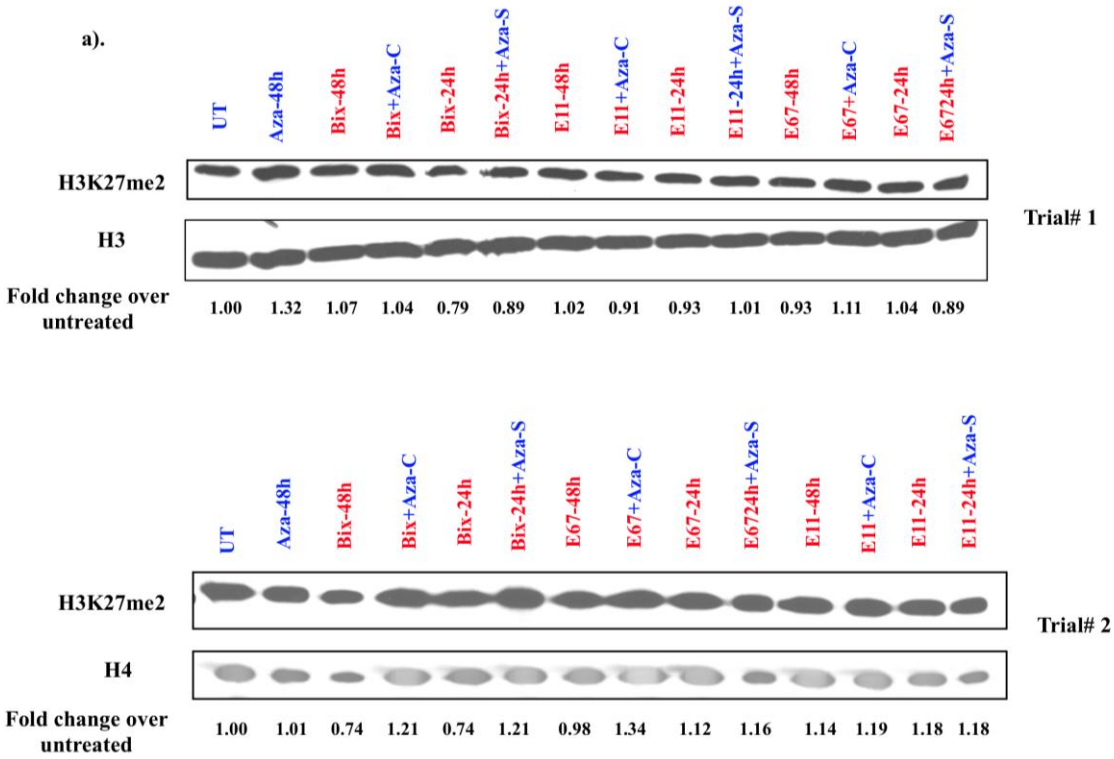


Figure 23: Effect on BIX-01294, its analogs, and/or 5-azaCdR, on total levels of histone H3K9me1 modification in breast cancer cells. $5x10^5$ MDA-MB231 cells were seeded on 10 cm dishes and treated with BIX-01294, E67, E11 and/or 5-azaCdR as indicated. Acid extraction of proteins was performed as mentioned in *Materials and Methods*. 10-20 µg of acid soluble proteins were separated on 15% SDS-polyacrylamide resolving gel and transferred onto PVDF membranes. Membranes were immunoblotted with antibodies against H3K9me1 and H3 and signals were detected by chemiluminescence. Results are shown from one experiment in (a), fold change over untreated values is indicated below this experiment in (a). Columns in (b) are graphical representation of fold change values calculated by relative integrated density measurements of H3K9me1 compared with untreated after normalization to H3 integrated density measurements. Image J software program was utilized to quantify the immunoblots. Treatments at 24 hours and 48 hours are indicated in the sample labels.

Figure 24

a).


UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix-24h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E11-24h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S
H3K27me2
H3
Trial# 1
Fold change over untreated
1.00 1.32 1.07 1.04 0.79 0.89 1.02 0.91 0.93 1.01 0.93 1.11 1.04 0.89
UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix-24h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E11-24h+Aza-S
H3K27me2
H4
Trial# 2
Fold change over untreated
1.00 1.01 0.74 1.21 0.74 1.21 0.98 1.34 1.12 1.16 1.14 1.19 1.18 1.18

b).

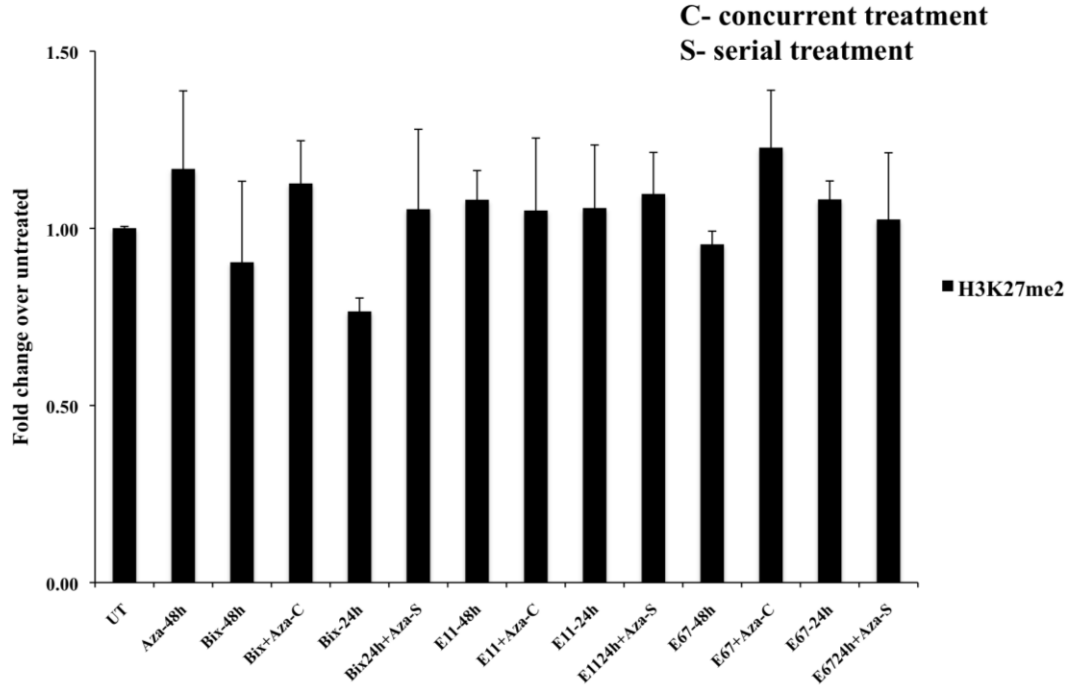
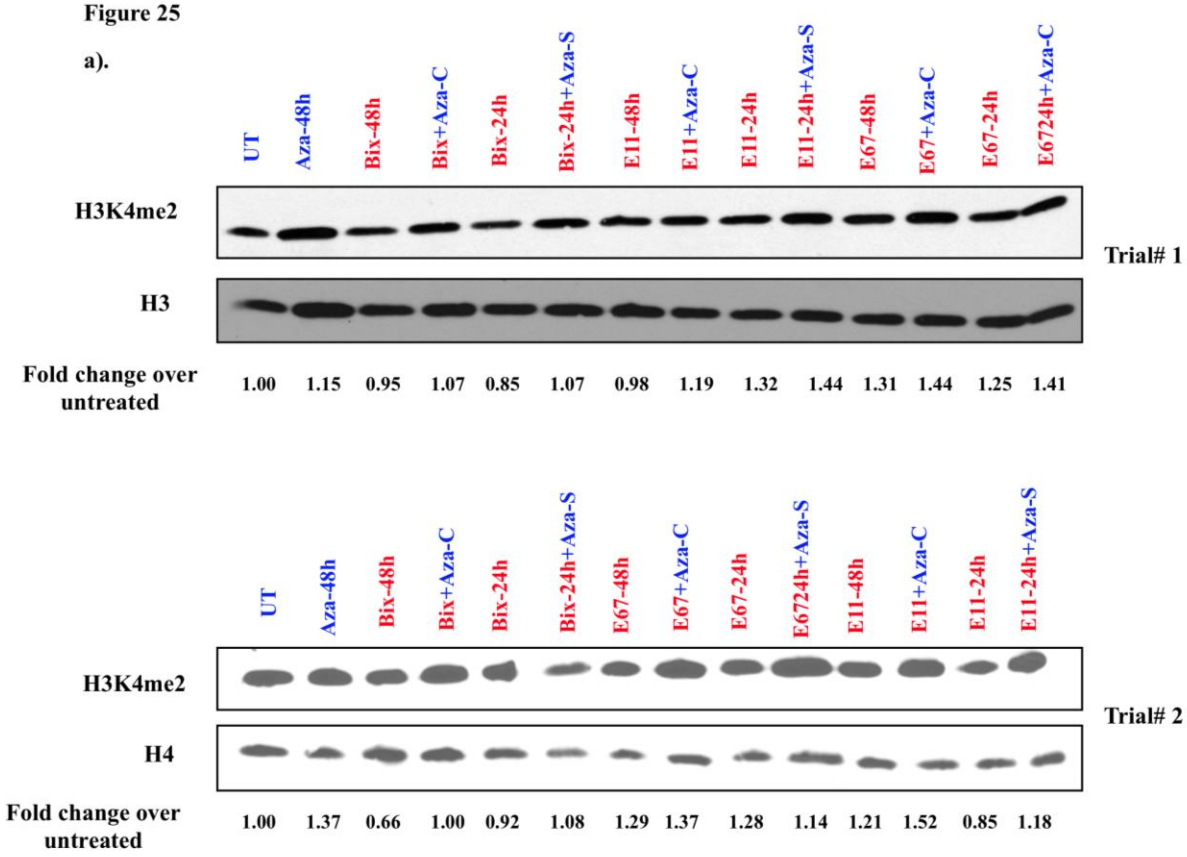

C- concurrent treatment
S- serial treatment
Fold change over untreated
1.50
1.00
0.50
0.00
H3K27me2
UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix24h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E1124h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S

Figure 24: Effect on BIX-01294, its analogs, and/or 5-azaCdR on total levels of histone H3K27me2 modification in breast cancer cells. 5 x10^5 MDA-MB231 cells were seeded on 10 cm dishes and treated with BIX-01294, E67, E11 and/or 5-azaCdR as indicated. Acid extraction of proteins was performed as mentioned in *Materials and Methods*. 10-20 µg of acid soluble proteins were separated on 15% SDS-polyacrylamide resolving gel and transferred onto PVDF membranes. Membranes were immunoblotted with antibodies against H3K9me3, total H3 and total H4 and signals were detected by chemiluminescence. Results are shown from two independent experiments in (a), fold change over untreated values are indicated below every experiment in (a). Mean of two experiments is shown in (b). Columns are mean fold change values from two independent experiments calculated by relative integrated density measurements of H3K27me2 compared with untreated after normalization to H3 integrated density measurements. Image J software program was utilized to quantify the immunoblots. Error bars are standard deviation of mean values. ** Indicate $P<0.01$ and * indicate $P<0.05$ as determined by t-test. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

Figure 25

a).


UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix-24h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E11-24h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-C
H3K4me2
H3
Trial# 1
Fold change over untreated
1.00 1.15 0.95 1.07 0.85 1.07 0.98 1.19 1.32 1.44 1.31 1.44 1.25 1.41
UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix-24h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E11-24h+Aza-S
H3K4me2
H4
Trial# 2
Fold change over untreated
1.00 1.37 0.66 1.00 0.92 1.08 1.29 1.37 1.28 1.14 1.21 1.52 0.85 1.18

b).

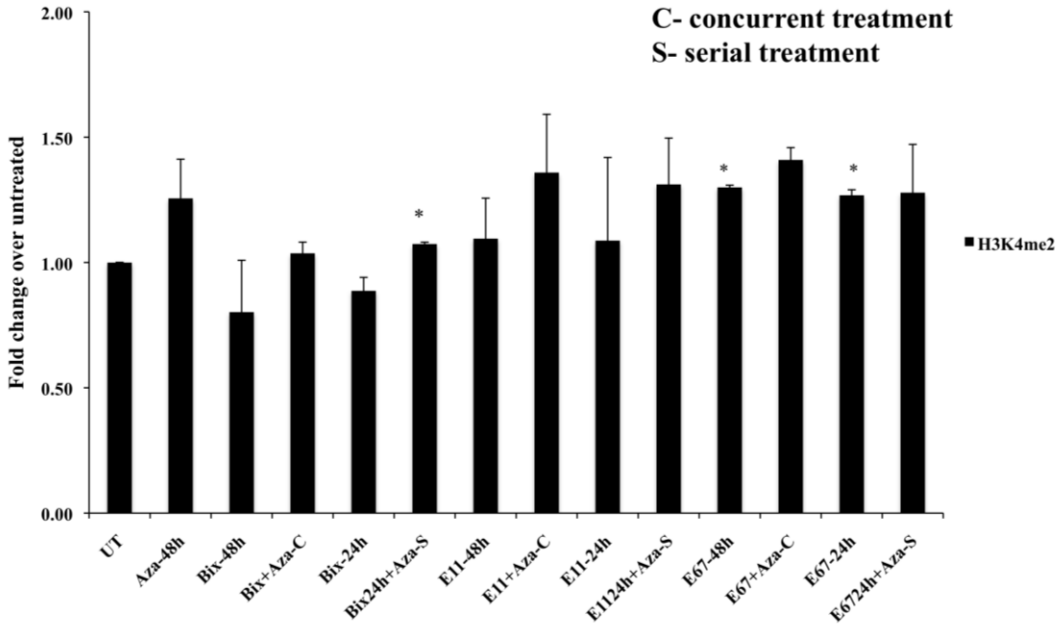

C- concurrent treatment
S- serial treatment
Fold change over untreated
2.00
1.50
1.00
0.50
0.00
H3K4me2
UT
Aza-48h
Bix-48h
Bix+Aza-C
Bix-24h
Bix24h+Aza-S
E11-48h
E11+Aza-C
E11-24h
E1124h+Aza-S
E67-48h
E67+Aza-C
E67-24h
E6724h+Aza-S

Figure 25: Effect on BIX-01294, its analogs and/or 5-azaCdR on total levels of histone H3K4me2 modification in breast cancer cells. 5 x10^5 MDA-MB231 cells were seeded on 10 cm dishes and treated with BIX-01294, E67, E11 and/or 5-azaCdR as indicated. Acid extraction of proteins was performed as mentioned in *Materials and Methods*. 10-20 µg of acid soluble proteins were separated on 15% SDS-polyacrylamide resolving gel and transferred onto PVDF membranes. Membranes were immunoblotted with antibodies against H3K4me2, total H3 and total H4 and signals were detected by chemiluminescence. Results are shown from two independent experiments in (a), fold change over untreated values are indicated below every experiment in (a). Mean of two experiments is shown in (b). Columns are mean fold change values from two independent experiments calculated by relative integrated density measurements of H3K4me2 compared with untreated after normalization to H3 integrated density measurements. Image J software program was utilized to quantify the immunoblots. Error bars are standard deviation of mean values. ** Indicate $P<0.01$ and * indicate $P<0.05$ as determined by t-test. Treatments at 24 hours and 48 hours are indicated in the respective sample labels.

CHAPTER 4

Discussion

The overall goal of this thesis was to characterize the *in vivo* effects of novel small molecular agents, BIX-01294 and BIX-01294 analogs (E67 and E11), in breast cancer cells. Specifically, I wanted to determine the *in vivo* effects of these agents on cell growth, gene expression, DNA methylation and histone modifications in breast cancer cells. Results from such studies can add use in the epigenetic therapy of cancer using these agents by themselves or in combination with clinically approved epigenetic therapeutic drugs such as 5-azaCdR. Our results indicate that BIX-01294, E67 and E11 inhibit the growth of breast cancer cells although to varying degrees. BIX-01294 and E11 were the most effective with IC_{50} values in the 2-4 μM range and E67 was slightly less effective with IC_{50} value close to 10 μM. We have also determined the effects of these agents on the expression of genes that are epigenetically silenced in breast cancer cells and on a transcription factor that is suppressed in breast cancer cells. Our results indicate that treatment of breast cancer cells with these agents has minimal effects on gene expression and little effect on 5-azaCdR induced re-expression of the same genes.

Our data further suggests that BIX and E67 antagonize 5-azaCdR induced DNA demethylation. Our results indicate that E11 may mediate minimal DNA demethylation. Our results also show that BIX-01294, E67 and E11 by themselves do not affect the protein levels of G9a and DNMT1. Finally, western blot analyses of several histone modifications indicate that BIX-01294, E67 and E11 significantly down regulated the global levels of histone H3K9me2. 5-azaCdR seemed to antagonize this effect in combined treatments with BIX-01294 or its analogs. Treatment with BIX-01294, E67, E11 or 5-

azaCdR also induced global H3K9me3 levels, and combination treatments of 5-azaCdR and BIX-01294, E67 or E11 have additive effects on H3K9me3 levels. These agents and 5-azaCdR do not have any effect on the global levels of histone H3K9me1, H3K27me2 and H3K4me2. This underlines the fact that these small molecular agents are specific to H3K9me2/me3 HMTases.

Here we have shown that BIX-01294 or its analogs E67 and E11 have growth inhibitory effects on cancer cells without any significant effects on gene expression. We do not know how these analogs lead to growth inhibition i.e is it because of killing the cells or because of inhibiting the cell division, in order to determine the mechanism of these analogs on growth inhibition, flow cytometry experiments need to be conducted. The effects on growth inhibition is consistent with previous findings, which showed that BIX-01294 has the greatest growth inhibitory effects *in vivo* and E67 has the least when compared to vehicle treated mouse ES cells [79]. When treated with 10 μM BIX-01294, there were no surviving cells after 24 hours whereas at the same concentration of E67, there was significant survival [79]. Similarly, our data agrees with these published results, as we also witness greatest growth inhibitory effects with BIX-01294 and E11, and the least effects with E67. Our results also indicate that these inhibitors are not specific in their growth inhibitory effects as similar effects are seen against normal breast epithelial cells. It is known that BIX-01294 and E11 occupy only part of the substrate peptide groove of GLP while leaving the target lysine channel open, so that they could be competed away by the substrate peptides relatively easily (indicated by their almost similar IC_{50} values and similar growth inhibitory effects) whereas E67 intercalates into the lysine-binding channel in addition to occupying the surface of the peptide-binding groove, so that each binding

event leads to effective inhibition of GLP (indicated by its higher IC_{50} value and reduced growth inhibitory effects) [79]. We conclude that the growth inhibitory effects and IC_{50} values of these agents in our study are related to the different modes of binding of these agents to the GLP.

Our gene expression studies reveal that treatment with BIX-01294 or its analogs alone does not significantly affect the expression of the four genes studied. It is known that the promoters of *TMS1, ESR1, CDH1* and *EGR1* in MDA-MB231 cells are tightly linked with H3K9me2 modification and DNA methylation [93] and after treatment with 5-azaCdR, there is a loss of DNA methylation and H3K9me2 accompanied by enrichment with H3K4me2 [67, 93]. Given the effects of the BIX-01294 or its analogs on global H3K9me2, we might expect that treatment with BIX-01294 or its analogs to induce expression of *TMS1, ESR1* and *CDH1*. Our results show that methylation is unaffected at the promoter of *TMS1* even after treatment with these analogs (Figures 17 and 18). E11 treatment demethylation is seen to a smaller extent (less than 10% demethylation, Figure 19). Our lab has recently published that Pol II (RNA polymerase II) does not bind to methylated promoter of *TMS1* and therefore there is no gene expression observed [95]. We extrapolate this finding with the results from gene expression experiments in this thesis and conclude that DNA methylation in the promoters of the genes studied prevent Pol II from binding and initiating transcription. Further experiments are necessary to determine whether there is an effect on H3K9me2 at the *TMS1* locus under these conditions.

Although our 5-azaCdR results are in agreement with the previously published results with respect to re-expression of *TMS1, ESR1* and *CDH1*, results from BIX-01294 or its analog treatments had little effects on gene expression. Recent studies by Shi et al [82]

have shown dedifferentiation of mouse embryonic fibroblasts into induced pluripotent stem cells by BIX-01294 and Bayk8644 (a calcium channel agonist) mediated reactivation of *Oct3/4* and *Nanog* genes. Here they demonstrated that treatment with BIX-01294 and Bayk8644 mediated DNA demethylation of *Nanog* promoter leading to gene activation. In our study, BIX-01294 and E67 had no impact on DNA methylation at the *TMS1* locus, and therefore no gene expression is seen with these treatments. We still need to find out the chromatin organization at this locus (and other genes studied), i.e. whether there is an impact on the local levels of H3K9me2. Although we see global effects on H3K9me2 modification, it does not indicate that this decrease is directly correlated to similar localized changes at the *TMS1* locus (or elsewhere).

The lack of effect on these epigenetically silenced genes may be because of one or more reasons. There is no effect on DNA methylation and these inhibitors may not lead to any changes in the chromatin organization at the promoters of the genes studied. As noted above, the genes studied in this thesis may not be the targets of these analogs; other gene targets could be identified by microarray experiments. Alternatively, these agents may not effectively remove all repressive marks at the gene loci (i.e. H3K9me3, H3K27me2/3) and down regulation of H3K9me2 alone may not be sufficient to induce gene expression. Further chromatin immunoprecipitation experiments will help to resolve the issue of changes in above histone modifications at the promoters of epigenetically silenced genes.

One novel finding in our expression studies is the induction of *EGR1* by 5-azaCdR treatment and BIX-01294 analog treatments. Unlike *TMS1, ESR1* and *CDH1, EGR1* is unmethylated and is expressed in MDA-MB231 cells. *EGR1* is a transcription factor that activates tumor suppressors such as PTEN, p53, etc [90]. It is noteworthy that polycomb-

group mediated histone H3 lysine 27 trimethylation, a key mark of genomic silencing is tightly correlated with *EGR1* repression [90]. Recent work by Su *et al* have shown that HDAC inhibitors efficiently remove histone H3 lysine 27 trimethylation marks and recruit acetyl groups to histone H3 tails to reactivate *EGR1* expression in synovial sarcoma cells [90]. We observe a 4-fold induction of this gene in 5-azaCdR treated breast cancer cells and an additive effect in combined treatments with BIX-01294 or its analogs. Although this induction is not huge, it will be worthwhile to explore the mechanisms of epigenetic regulation of *EGR1* in cells treated with BIX-01294, its analogs and/or 5-azaCdR. This information will be a valuable addition to existing mechanisms of *EGR1* epigenetic regulation [90].

In Neurospora, the H3K9 histone methyltransferase *DIM-5* is required for CpG methylation [96] and in Arabidopsis, H3K9 histone methyltransferase *KRYPTONITE* is required for CpNpG methylation [97] suggesting the existence of an evolutionarily conserved silencing pathway in which H3K9 methylation promotes *de novo* DNA methylation. Functional co-operation between G9a and DNMTs are well established in many mammalian studies where together they mediate *de novo* methylation and silencing of pluripotency genes during development [55, 63], they collaborate to mediate DNA and histone methylation during mammalian DNA replication [98] and promote gene silencing [52]. In most of these above studies, the data suggest that G9a mediates *de novo* DNA methylation independent of its catalytic activity and there is evidence from biochemical data that G9a recruits DNMTs by its ANK domain and not by its SET domain [55]. These data suggest that G9a mediated DNA methylation is not due to local effects on H3K9me2 modification but potentially G9a is required as a scaffold to

stabilize a complex with DNMT during replication, gene silencing and during programming of developmental genes. The integrity of G9a/DNMTs complex might be critical for DNA methylation.

Our data demonstrates that G9a and DNMT1 protein levels remain unchanged in cells treated with BIX-01294 or its analogs. In addition to this, there is also little to no change on DNA methylation at *TMS1* (Figure 17-19). This data suggests that in cells treated with BIX-01294 or its analogs, G9a can still function as a scaffold and maintain normal cooperation with DNMTs in establishing or maintaining DNA methylation of its target genes. This might explain the absence of changes in DNA methylation and therefore absence of gene expression. Unlike the finding from above studies where G9a knockouts or mutants were used, which would destroy the scaffolding function, our studies utilized chemical inhibition of G9a/GLP. This could also explain the differences in the findings because the mechanism of action of physiological inhibition of G9a and chemical inhibition of G9a are different.

One interesting finding from our studies is the antagonistic effects of BIX-01294 and E67 on 5-azaCdR mediated DNA methylation in cancer cells. We find 30% demethylation in 5-azaCdR treated cells, and only 10% or less demethylation in cells treated concurrently with 5-azaCdR/BIX-01294 and 5-azaCdR/E67 (Figures 17-18). Although there is complete loss of DNMT1 protein (Figure 20) in cells treated with 5-azaCdR either by itself or in combination with BIX-01294 and E67, we observe no changes in DNA methylation at the *TMS1* locus of cells treated with the above combination of inhibitors. These data suggest that DNMT1 can still function to maintain DNA methylation at this locus and that BIX-01294 and E67 are in some way inhibiting 5-

azaCdR mediated DNA demethylation. The above stated results are not directly comparable because the first effect is a global effect on DNMT1 protein levels and the second effect is a gene specific effect on DNMT1, but given that there is global loss of DNMT1, it is puzzling to see so much methylation at the *TMS1* locus in combination treatments. Plausible reasons for antagonism on DNA methylation by these agents may be explained in several ways. DNA demethylation by 5-azaCdR occurs during S-phase of the cell cycle and BIX-01294 or E67 inhibit the growth of breast cancer cells, so it is likely that in combination treatments, these agents inhibit the growth of cancer cells and that the alleles analyzed by BS sequencing at the *TMS1* locus are parental alleles where DNA demethylation is negligible because 5-azaCdR demethylates only newly synthesized DNA strands. Alternatively there could be different gene targets regulated by G9a/DNMT1 complex that needs to be determined. In addition to the above reasons, there could be complex chemical interactions between the compounds, i.e. between 5-azaCdR and BIX-01294/E67 that may interfere with the demethylation activity of 5-azaCdR at the *TMS1* locus in combination treatments.

Our studies indicate that BIX-01294 or it analogs do not have any effect on the global levels of G9a and DNMT1 in cancer cells (Figure 20). This indicates that these small molecule inhibitors exert their effects (i.e global down regulation of H3K9me2 levels) *in vivo* without altering the target protein levels. Data from Wozniak *et al* indicates dose-dependent reductions in G9a protein levels in 5-azaCdR treated MDA-MB231 cells with the highest doses (3 and 10 μM) resulting in an overall 75% decrease in G9a protein levels when treated for 4 days [84]. Although preliminary, our data suggest that there is some decrease in the global G9a protein levels in 5-azaCdR (0.5 μM)

treated samples either alone or in combination. Further experiments are required to solidify this conclusion. 5-azaCdR mediated reductions in G9a protein levels may be a result of degradation of DNMT1 and therefore destruction of the G9a/DNMT1 complex.

We have observed significant decrease ($P<0.05$) in the global levels of histone H3K9me2 in cancer cells treated with BIX-01294 analogs alone. This suggests that BIX-01294 or its analogs are inhibiting the global HMTase activity of G9a/GLP (Figure 21). This observation in our study is supported by the recent finding by Imai *et al* where they have shown that BIX-01294 reactivates HIV-1 provirus at the transcriptional level by down-regulating H3K9me2 levels [94]. On the other hand, we see in our study that there is no dramatic change in the global levels of H3K9me2 in cancer cells treated with 5-azaCdR by itself and some antagonism observed in combination treatments of 5-azaCdR and BIX-01294 or its analogs. This could be explained by the fact that we are using a lower concentration of 5-azaCdR (0.5 μM) in our experiments as compared to previous studies [78, 94] where a decrease in H3K9me2 was observed at 3.0 μM and higher concentration of 5-azaCdR in MDA-MB231 cells. Another study has shown no change in the global levels of H3K9me2 in T24 cancer cells when treated with 3.0 μM of 5-azaCdR [83]. In contrast, numerous studies have shown that treatment with 5-azaCdR lead to gene specific depletion of H3K9me2 [67, 93] consistent with the coupling of DNA methylation and H3K9me2 at individual loci. The absence of an effect on H3K9me2 at a global level may be due to high levels of H3K9me2 in other parts of the genome such as pericentric heterochromatin or endogenous retroviruses which may be unaffected by 5-azaCdR [35, 36]

A novel observation made in our study is the up regulation in H3K9me3 levels upon treatment of MDA-MB231 cells with BIX-01294, its analogs and/or 5-azaCdR. Our data is in contrast to those observed by Kubicek *et al* [78] which showed no changes in the H3K9me3 at the promoters of *Mage-a2* and some reduced H3K9me3 at the promoters of other G9a target genes- *Bmi1, Serac1* in BIX-01294 treated mouse ES cells [78]. The global effects that we are observing with H3K9me3 in our study may or may not be reflected at individual genes. We observed no dramatic changes in the global levels of H3K27me2 and H3K4me2 modifications. Absence of changes in the global levels of H3K27me2/H3K4me2 support the specificity of these agents only towards histone H3K9 methyltransferases and not any other methyltransferases, which is in agreement with the previously published results [78].

Overall, data from our study indicates that alterations observed in cells treated with BIX-01294 or its analogs are distinct from those observed in G9a knockout or mutant studies. We have shown that after treatment with BIX-01294 or its analogs the expression of tumor suppressor genes remains unchanged, DNA methylation remains unaltered and the G9a/DNMT1 total proteins are unaffected, in spite of the reduced catalytic activity of G9a (shown by reduced levels of H3K9me2). This indicates that there may be additional or different gene targets that are being regulated by this complex and/or by active G9a. G9a mutant/G9a knockout studies in cell lines indicate significant down regulation of G9a protein levels, and significant effects on DNA methylation of target genes [55, 99]. This explains the differences in effects between G9a knockout/ G9a mutant studies and chemical inhibition by small molecular agents in our studies.

Based on our observations, we propose a model (**Figure 26).** In untreated MDA-MB231 cells, the promoters of epigenetically silenced genes are DNA methylated and marked by H3K9me2, and together these epigenetic modifications lead to a closed chromatin which is inaccessible by RNA Polymerase II for gene transcription. In MDA-MB231 cells treated with BIX-01294 or its analogs, promoters of epigenetically silenced tumor suppressor genes are DNA methylated and there is global decrease in H3K9me2 levels, chromatin is closed and RNA polymerase II cannot initiate gene transcription because of inactive chromatin. In MDA-MB231 cells treated with 5-azaCdR, the promoters of epigenetically silenced tumor suppressor genes are hypomethylated and marked by H3K9me2, chromatin is open and RNA polymerase II is able to access the chromatin to initiate gene transcription. In MDA-MB231 cells treated with BIX-01294 or its analogs and 5-azaCdR, the promoters of epigenetically silenced tumor suppressor genes are hypermethylated and a global decrease in H3K9me2 levels are seen. RNA polymerase II can access the chromatin and initiate gene transcription.

Figure 26

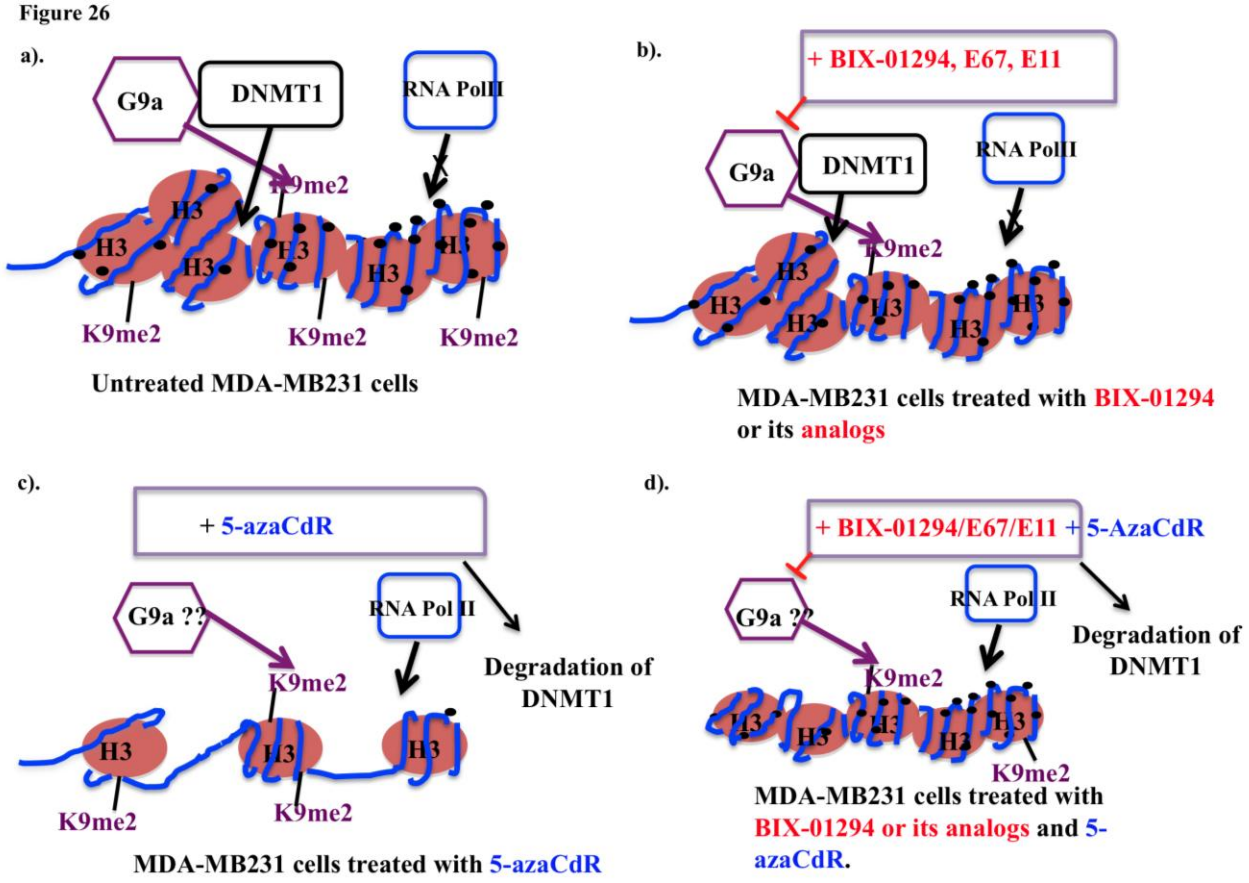

a).
G9a
DNMT1
RNA PolII
K9me2
H3
K9me2
K9me2
K9me2
Untreated MDA-MB231 cells
b).
+ BIX-01294, E67, E11
G9a
DNMT1
RNA PolII
K9me2
H3
MDA-MB231 cells treated with BIX-01294 or its analogs
c).
+ 5-azaCdR
G9a ??
RNA Pol II
Degradation of DNMT1
K9me2
H3
K9me2
K9me2
MDA-MB231 cells treated with 5-azaCdR
d).
+ BIX-01294/E67/E11 + 5-AzaCdR
G9a ??
RNA Pol II
Degradation of DNMT1
K9me2
H3
K9me2
MDA-MB231 cells treated with BIX-01294 or its analogs and 5-azaCdR.

Figure 26: Model for epigenetic regulation of BIX-01294, its analogs and 5'azaCdR at the promoters of epigenetically silenced tumor suppressor genes in MDA-MB231 cells- a) shows the chromatin organization in untreated MDA-MB231 cells. G9a/DNMT1 mediated promoter DNA hypermethylation and enrichment of H3K9me2 together leads to a closed chromatin organization inaccessible to RNA Polymerase II resulting in epigenetic silencing of tumor suppressor genes. b) shows the chromatin organization in MDA-MB231 cells treated with BIX-01294 or its analogs. Catalytic activity of G9a is decreased resulting in global decrease of H3K9me2 independent of its effects on DNA methylation. This happens without affecting the stability of G9a and DNMT1 protein levels. RNA Polymerase II is inaccessible and no reactivation of epigenetically silenced tumor suppressor genes is seen. c) shows the chromatin organization in MDA-MB231 cells treated with 5-azaCdR. 5-azaCdR mediated DNMT1 degradation leads to significant DNA hypomethylation and an open chromatin that is accessible by RNA Plolymerase II leading to reactivation of epigenetically silenced tumor suppressor genes. There is no dramatic reduction in global levels of H3K9me2; stability of G9a in these cells is yet to be determined. d). shows the chromatin organization in MDA-MB231 cells treated with BIX-01294 or its analogs and 5-azaCdR. There is no DNA demethylation seen in spite of 5-azaCdR mediated DNMT1 degradation. Global decrease in H3K9me2 levels is observed, RNA polymerase II mediated reactivation of epigenetically silenced genes is seen.

Future Directions:

Our cell growth assays reveal that these analogs are potent in their effects on cancer cells, although the mechanism of potency is undetermined because we do not know if these analogs are killing the cancer cells or inhibiting the cell division. In order to determine the mechanism of these analogs on growth inhibition, flow cytometry experiments need to be conducted.

GLP and Wiz exist as a heteromeric complex with G9a *in vivo* [41, 53] and interactions with these proteins are required for stability of G9a in the complex and also for stability of the heteromeric complex; G9a interacts with GLP and Wiz through its SET domain. Analogs used in this study bind to the SET domain of G9a/GLP and inhibit its enzymatic activity. Co-immunoprecipitation experiments may be performed with antibodies to G9a/GLP/Wiz in cells treated with these analogs to determine the stability of this complex and to determine the interactions between these proteins *in vivo*. Changes in global levels of Wiz and GLP in cells treated with these analogs can be studied by immunoblots. Results from these experiments will help to determine the specificity of these analogs towards G9a heteromeric complex.

Chromatin immunoprecipitation assays with antibodies against G9a, H3K9me2 and DNMT1 needs to be conducted to study the promoters of the genes studied in this work. Recent work has shown BIX-01294 induced down-regulation in the occupancy of G9a and H3K9me2 at HIV-1 proviral DNA [94]. As our results indicate no significant effects on gene expression in cells treated only with BIX-01294 or its analogs, we are curious to know if these inhibitors are causing any changes in the occupancy of the above mentioned proteins and/or in the levels of H3K9me2 at the gene promoters. We can also perform the

same experiments in the promoter of *Mage-a2*, a germ line specific antigen, which is a major G9a target gene [78]. This will serve as a positive control for BIX-01294 or its analogs induced effects in our studies. It will be worthwhile to study the DNA methylation status of *EGR1* in cells treated with both classes of inhibitors individually and in combinations. We are proposing this experiment because of induction of this gene in treated cells. It will be a novel finding because regulation of *EGR1* by 5-azaCdR and/or BIX-01294 analogs has not yet been studied.

Our study has focused only on expression of three epigenetically silenced genes in MDA-MB231 breast cancer cells. In order to understand global gene expression changes after treatment with BIX-01294, its analogs and/or 5-azaCdR, it will be worthwhile to pursue microarray studies using cells treated with the same drugs. This will lead us a path to focus on other epigenetically modified breast cancer associated genes that may be targets of the analogs being characterized.

It is known that G9a recruits DNMT3a/DNMT3b in order to establish *de novo* DNA methylation of embryonically silenced pluripotent genes in mouse ES cells during development [55] and in the silencing of MLV based exogenous retroviruses [100]. It will be worthwhile to study changes in the global levels of DNMT3a/3b in MDA-MB231 cells treated with BIX-01294, its analogs and/or 5-azaCdR. Along the same lines, we also propose to determine the changes in the global levels of UHRF1 in MDA-MB231 cells treated with BIX-01294, its analogs and/or 5-azaCdR. *UHRF1* is known to recruit G9a [101] and other chromatin modifying factors to the target gene promoters to establish gene silencing.

Overall, results from this thesis work and the above-mentioned experiments will help us to determine the molecular mechanisms of action of these analogs in breast cancer cells. Results from the proposed experiments will provide specific details about the effect of these agents on the stability of G9a/GLP/Wiz heteromeric complex in breast cancer cells that will be important in designing future experiments to explore factors that target the specificity of these agents only to histone H3K9 methyltransferases and not other SET domain containing histone methyltransferases. This is a novel work where *in vivo* characterization of these analogs in terms of its potency and epigenetic mechanisms of reactivation of silenced tumor suppressor genes in breast cancer cells will allow for the further development of epigenetic therapy of human breast cancer. This thesis work will allow us to use these analogs either by itself or in combination with 5-azaCdR and/or in combination with chemotherapeutic drugs for the epigenetic therapy of breast cancer.

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