EFFECT OF HDAC INHIBITORS ON THE EXPRESSION OF MBD PROTEINS CODING GENES IN BREAST CANCER

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Submitted by:

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CERTIFICATE

This is to certify that the thesis entitled "**EFFECT OF HDAC INHIBITORS ON THE EXPRESSION OF MBD PROTEINS CODING GENES IN BREAST CANCER**" submitted by Miss Namita Panigrahy, Roll No.411LS2059, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I, Namita Panigrahy, hereby declare that this project report entitled "**EFFECT OF HDAC INHIBITORS ON THE EXPRESSION OF MBD PROTEINS CODING GENES IN BREAST CANCER**" is the original work carried out by me under the supervision of Dr. Samir K. Patra, Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

Date: **Namita Panigrahy**

Place:

ACKNOWLEDGEMENT

Accomplishment is a word which always sounds good. It is really a pleasure to see things done in a perfect and orderly manner. I take this opportunity to offer my gratitude to every person who has been a part of this project.

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I am genuinely appreciative of all my batch mates for their suggestions and moral support during my work.

Last but not the least, I would not have been able to complete this project without the love and support of my parents whose unflinching faith in my abilities helped me to overcome many obstacles and march ahead in spite of failures.

CONTENT

ABBREVIATIONS

DNA…….Deoxy-Ribonucleic Acid
RNA…….Ribonucleic Acid
CpG…….Cytosine- Phosphate-Guanine
DNMT…….DNA Methyltransferase
MBD……Methyl-CpG-binding domain
MeCP2…….Methyl-CpG-binding protein 2
HDAC……..Histone Deacetylase
HDACi…….Histone Deacetylase Inhibitor
OD……Optical density
UV……Ultra Violet
DEPC……...Diethyl Pyrocarbonate
EDTA……...Ethylene Diamine Tetra Acetate
dNTP……....Deoxy Nucleotide Triphosphate
U……Unit
g……Gram

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ABSTRACT

Methyl-CpG binding domain (MBD) proteins are considered as the decoders of the methyl signature on DNA. This highly conserved family of DNA-binding proteins translates the information represented by methylation patterns into appropriate functional language. MBD proteins bind to the DNA around methylated cytosine bases located particularly in the promoter regions, preventing binding of transcription factors and RNA polymerase and thereby repress transcription. Region-specific DNA hypermethylation is considered as a characteristic hallmark of cancer. MBD proteins are held as the main culprits in perpetuating gene silencing mechanism by interacting with a number of protein partners such as HDACs, hence they are now considered as potential biomarkers for diagnosis and prognosis of malignant transformation. The present study will be conducted to analyse the gene expression status of the methyl-CpG-binding (MBD) proteins - MeCP2, MBD1, MBD2, MBD3 and MBD4 in breast cancer after treatment with HDAC inhibitors TSA and SFN. Study and characterization of the pattern of binding of the MBD proteins will provides a powerful set of epigentic markers for outlining the disruption of critical pathways in tumorigenesis. This study based on MBD protein distribution and occupancy in a gene and tumor type specific context will define a sensitive molecular detection strategy for virtually every human cancer type.

INTRODUCTION

Differential gene expression and functional state results essentially from epigenetic manipulations of the genetic information through temporal and spatial regulation of gene activity. The epigenetic mechanisms entail cellular processes that do not alter the genomic sequence and they are believed to elicit and sustain relatively persistent biological effects. DNA methylation, post-translational covalent histone modifications such as methylation, acetylation, phosphorylation, sumoylation etc., incorporation of histone variants, gene silencing mediated by non-coding RNAs, nucleosomal rearrangement, higher order chromatin remodeling and transcriptional feedback loops are the important protagonists who modulate the DNA and its associated histone proteins to effectively manage the gene expression circuitry. These molecular restructuring events function as an interface between the genomic content and the external environment. They act as a conduit for the efficient regulation of cellular functions by the environment-induced cues to establish steady internal physiological states. Thus, the epigenetic mechanisms work in a rigidly controlled manner to establish a stable cell destiny by eliciting distinct transcriptional response in different biological settings.

DNA methylation is one of the principal epigenetic enforcers participating in cell-specific repression of transcriptional activity. It is considered as a fundamental phenomenon orchestrating functional re-orientation of the genomic data. It basically acts as a gene silencing mechanism to turn off specific genes at crucial junctures during development and differentiation. In biochemical language, DNA methylation is a post-synthetic process of addition of methyl groups to the cytosine residues in the presence of a co-factor S-Adenosyl Methionine (SAM). The enzymes responsible for catalyzing this reaction are known as DNA methyltransferases (DNMTs). The methyl tags on the cytosines are however recognized and translated into readable genetic language by a family of proteins known as the Methyl-CpG-binding domain (MBD) proteins. MBD proteins bind to the DNA around methylated cytosine bases located particularly in the promoter regions, thereby preventing binding of transcription factors and RNA polymerase. They also interact with a number of protein partners and lead to transcriptional

repression and/or heterochromatin formation. Thus, MBD proteins are the readers of the methyl code propagated by the DNMTs, deciphering the methyl signature in a functional context.

Over recent years, it has been firmly established that epigenetic mechanisms play a crucial role in provoking deregulated gene function during initiation, maintenance and progression of cancer. Any malfunctioning in the epigenomic supervisory system eventually affects the way in which the genomic information is inferred, resulting in malignant transformation. Thus epigenetic and genetic processes join hands to disrupt the normal activity of crucial tumor suppressors, cell-cycle regulatory and other such necessary genes to stimulate tumorigenesis. Gene-specific regional hypermethylation and subsequent silencing of tumor suppressor genes is regarded as a characteristic hallmark of cancer. This hypermethylation induced silencing of tumor suppressor genes in cancer cells is mediated by MBD proteins which bind to the methylated promoters, preventing their transcriptional activation in time to stop cancer. Hence, targeting of MBD proteins for effectively thwarting the neoplastic progress is now considered a potential therapeutic alternative. In the context of the significance of the MBD proteins in the epigenomic landscape, the present study was designed to investigate the gene expression pattern and profiling of the family of MBD proteins - MeCP2, MBD1, MBD2, MBD3 and MBD4 in breast cancer. A comparative analysis of the expression levels of different MBD proteins after treatment with epigenetic drug TSA (Trichostatin) and SFN (Sulphoraphane) will be done to create a diagnostic profile in breast cancer. The project aims to provide a comprehensive knowledge about the mechanistic action of the HDAC inhibitors on the gene expression of MBD proteins so as to design novel strategies for effective therapeutic targeting in breast cancer. The project will help to create an MBD protein based epigenetic profile in a gene and tumor type specific context for diagnosis, detection and prognosis. Further investigations into the pattern of binding of the MBD proteins in a variety of different cancer tissues will offer new insights into the role of MBD proteins in malignant transformation and subsequently pave way for combating this prevalent and potentially devastating disease.

REVIEW OF LITERATURE

Epigenetics [‘Epi’-above/over/beyond; ‘above’-genetics] is the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence that result in cell specific functional variations in phenotype. These modifications consist of DNA methylation, Histone modifications and Non-coding RNA mediated gene silencing (Fig 1). The term ‘epigenesis’ which refers to hypothetical aspects of developmental biology and the strategy of genes was coined by C. H. Waddington in 1930. Epigenetic modifications are heritable, reversible covalent modifications that work in tandem to orchestrate the transcriptional activity of the genome in various biological settings. Recent studies show that the association of components of transcriptional regulatory machinery with target genes on mitotic chromosomes is a novel epigenetic mechanism that poises genes involved in key cellular processes, such as growth, proliferation, and lineage binder, for expression in progeny.

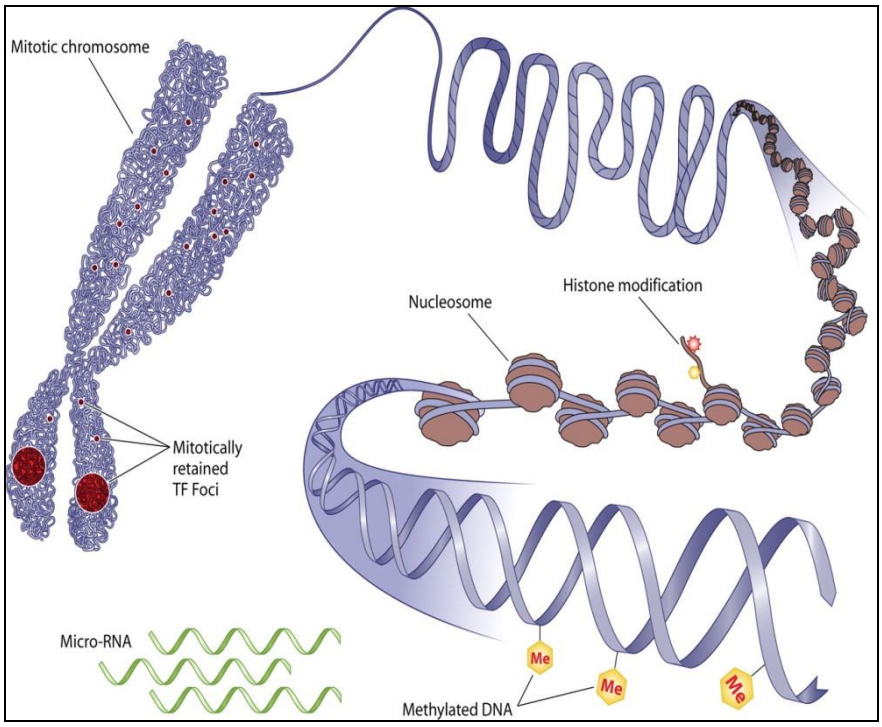


Figure 1: Mechanisms of inheritable epigenetics.

Shown here are some well-defined epigenetic mechanisms that include histone modifications, DNA methylation, and the noncoding RNA-mediated modulation of gene expression.

(Adapted from Zaidi et.al, Molecular and cellular biology, 2010)

DNA Methylation-The Epigenetic Writer:

DNA methylation is the first and most extensively researched epigenetic manipulation. DNA methylation frequently occurs in repetitive sequences, and helps to suppress the expression and mobility of 'transposable elements. DNA methylation patterns are recognized to be established and modified in response to environmental factors by a complex interplay of at least three independent DNA methyltransferases (DNMT) -- DNMT1, DNMT3A, and DNMT3B. DNMT1 is the most copious methyltransferase in somatic cells, localize to replication foci, has a 10–40-fold leaning for hemimethylated DNA and interacts with the proliferating cell nuclear antigen (PCNA). By preferentially modifying hemimethylated DNA, DNMT1 transfers methylation patterns to a newly synthesized strand after DNA replication, and hence is often referred to as the ‘maintenance' methyltransferase. DNMT3A and DNMT3B, known as the *de novo* methyltransferases add methyl marks to newly synthesized daughter DNA strands and faithfully propagate the methyl patterns across successive generations. DNA methylation, in general is a repressive mark on the genome playing crucial role during development and differentiation by influencing the transcriptional machinery and inducing gene silencing (Kar et al., 2012).

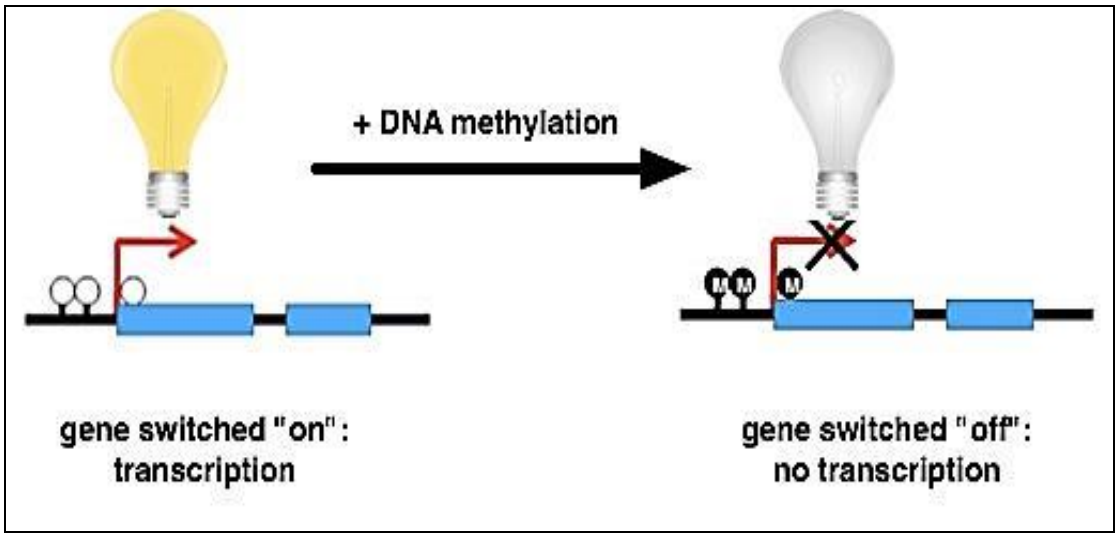


Figure 2: DNA Methylation and Gene Silencing.

The addition of methyl groups onto the cytosine residues in the promoter regions of the DNA prevents transcription factors from binding to it and thus leads to gene silencing.

(Adapted from Wade, Bio Essays, 2001)

Methyl-Binding Domain Proteins-The Epigenetic Readers:

The correlation between DNA methylation and transcriptional inactivity is well recognized. However, a causative role for CpG methylation in suppression of transcription has often been a subject for debate. Although many silenced genes are linked with dense CpG methylation, this epigenetic mark could be a downstream consequence of transcriptional inactivity rather than an active participant in the process of repression. However, the identification of a family of proteins that bind to DNA containing methylated CpG dinucleotides established a causative link between CpG methylation and repression of transcription. This family of proteins, known as Methyl-Binding Domain (MBD) proteins binds to methylated CpG islands and mediates transcriptional repression of affected genes. The MBD family of proteins consists of five members: MeCP2, MBD1, MBD2, MBD3 and MBD4 (Berger and Bird, 2005). All MBD proteins contain a similar methyl binding domain (MBD) of about 75 amino-acid residues (Fig 3). Sequence alignment of MBD proteins reveals several highly conserved residues on the interface between MBD and methylated DNA which are responsible for recognition of the methyl-CpG steps. Among these, MeCP2 is capable of binding to a single, symmetrically methylated CpG dinucleotide and binds to chromosomes at sites known to contain methylated DNA. MBD1 is a transcriptional regulator characterized by two or three CXXC domains that bind methylated CpG islands of the tumor suppressor genes and suppress transcription (Patra et al., 2001). MBD2 has been reported to have DNA demethylase activity along with repressor function (Bhattacharya et al., 1999). MBD4 is a repair enzyme of the DNA-glycosylase family and MBD3 apparently lacks specific methylated DNA-binding capability although it has a high degree of sequence similarity with MBD2. The finding that the hyper-methylation of promoters is associated with silencing of several genes in human prostate cancer is noted in the literature but the study of the respective enzymes and their relative levels, and repressor proteins is lacking.

MBD1-Methyl Binding Domain protein 1:

MBD1 is the biggest MBD family member and is known to act as a transcriptional repressor both *in vivo* and *in vitro* (Fig 3).Depending on the splicing isoform, it can connect

methylated as well as unmethylated DNA. Like the other family members, MBD1 associates with chromatin modifiers such as the Suv39h1–HP1 complex to enhance DNA methylation-mediated transcriptional repression (Zhang et al. 1999). The functional value of MBD1 was demonstrated in human HeLa cells, where MBD1 was shown to combine with the H3K9 methyltransferase SETDB1. During S phase the MBD1–SETDB1 complex is recruited to chromatin by the chromatin assembly factor CAF1 to establish new H3K9 methyl marks. An MBD1 mouse knockout has been obtained, but no severe developmental defects were found. MBD1-null mice had a normal morphology and appeared healthy, although they carried a number of minor neural defects like reduced hippocampal neurogenesis and had problems with spatial learning. Another interesting feature of this knockout was reduced genomic stability and an increase in expression of the intracisternal-a particle retrotransposon (Fujita et al., 2000).

MBD2- Methyl Binding Domain protein 2:

The second member of the MBD family, MBD2, is a 44-kDa protein which shares widespread sequence homology with MBD3. MBD2 is able to bind methylated CpGs *in vitro* and *in vivo* and confer DNA methylation mediated transcriptional silencing through its repression domain (Fig 3).The suppression established by MBD2 is responsive to HDAC inhibitors, in line with its association with HDAC1 and HDAC2 in the Mi-2/NuRD chromatin remodeling complex (Zhang et al. 1999). MBD2-null mice developed usually and remained viable and fertile, although MBD2-null mothers fail to nurture their pups properly. The association between the loss of MBD2 and the observed maternal behavior is unclear. MBD2, however, does play a role in helper T-cell differentiation. Normally, the induction of IL-4 during differentiation requires the GATA3 activator, yet, in MBD2-null mice GATA-3 is no longer needed for IL-4 induction and as a result IL-4 is ectopically expressed in undifferentiated helper T cells.

MBD3- Methyl Binding Domain protein 3:

MBD3 has extensive sequence similarity to MBD2 (Hendrich and Bird., 1998). It is expressed as several splice variants, some of which disrupt the MBD. MBD3 mRNA is expressed in the adult brain. In the brain, MBD3 expression can be detected in high levels in

neuroepithelial cells whereas MBD2 is barely detected (Jung et al., 2002). The protein has been identified as a component of the Mi-2/NuRD transcriptional co-repressor complex that includes Mi-2 ATPase, HDAC, and other proteins (Fig 3).Yet *in vitro,* mammalian MBD3 has little if any methyl-CpG-binding activity, likely because of amino acid substitutions within the MBD. MBD3 can be transiently induced by kindling or transient ischemia in rodent hippocampus, suggesting that these proteins may be involved in alteration of gene expression upon lesions (Francis et al, 2002). Therefore, the case for the *Xenopus* orthologue of MBD3 which contains a MBD with methyl-CpG-binding activity it is unlikely that mammalian MBD3 plays a role in methylation dependent transcription repression (Fraga et al., 2003). MBD3 is a necessary component for NuRD complex and involved in multiple gene regulatory pathways for embryogenesis.

MBD4- Methyl Binding Domain protein 4:

MBD4 includes a MBD similar to that of MeCP2, while the COOH-terminal domain is homologous to bacterial DNA repair enzymes. Although MBD4 is capable of binding to methyl-CpG sites, it has a high affinity for 5mCpG-TpG mismatched sites, and the DNA repair domain provides DNA *N*-glycosylase activity at G-T mismatches (Fig 3).So MBD4 is ideally suited to function in the repair of point mutations that result from spontaneous deamination of 5-methylcytosine to thymine (Hendrich et al., 1999). In addition, MBD4 (also known as MED1) binds to the MLH1 DNA mismatch repair protein *in vivo*. Expression of a MBD4 mutant missing the MBD induces microsatellite instability in cell lines; implicate MBD4 in this form of DNA repair as well (Bellacosa et al., 1999). Yet, in an *in vitro* assay, nuclear extracts containing MBD4 make mismatch repair independently of target CpG methylation status.

MECP2- Methyl CpG binding protein 2:

Methyl CpG binding protein 2 is a gene that provides instructions for making its protein product, MECP2, also referred to as MeCP2 (Amir et al., 1999). MECP2 appears to be necessary for the normal function of nerve cells. The protein seems to be mainly important for mature nerve cells, where it is there in high levels. The MeCP2 protein is likely to be occupied in turning off (repressing or silencing) of several other genes. This prevents the genes from production proteins when they are not needed. Within the brain, the MeCP2 protein is essential for the

function of nerve cells (neurons) and is present in high levels in mature neurons (Fig 3). This protein likely act a role in maintaining connections (synapses) between neurons, where cell-to-cell contact occurs. Many of the genes that are known to be regulated by the MeCP2 protein play a role in normal brain function, mostly the maintenance of synapses (Amir et al., 1999).

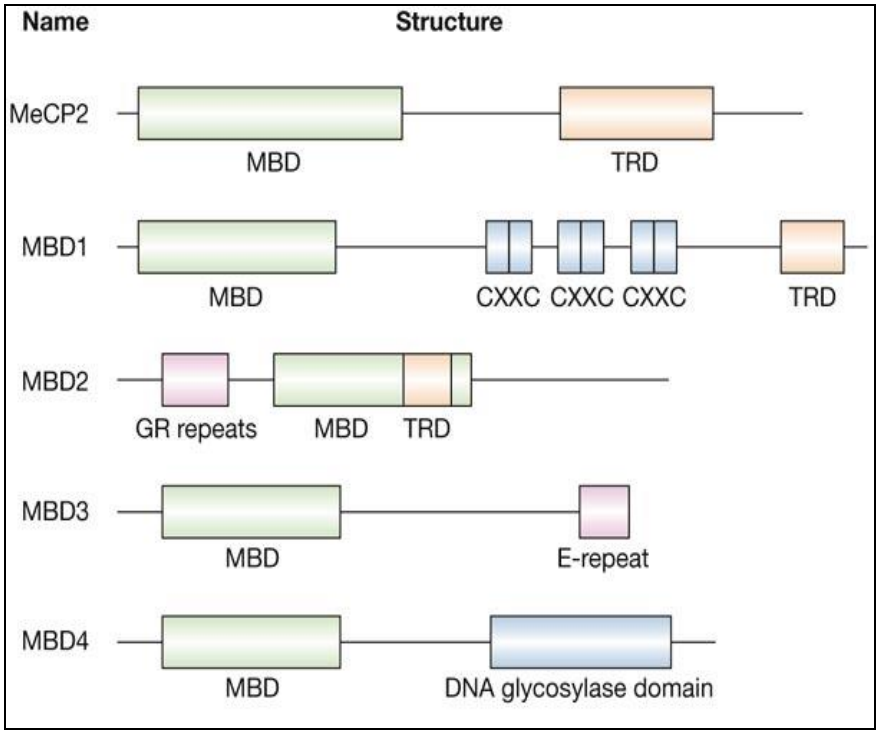


Figure 3: Schematic structure of the major MBD family members.

Only the general structure of the protein is shown—some have alternative isoforms. N-termini are on the left, C-termini are on the right. Abbreviations: MBD, methyl-binding domain; TRD, transcription repression domain; GR, alternating glycine and arginine repeats.

(Adapted from Sansom et al, Nature Clinical Practice Oncology, 2007)

MBD Proteins and Gene Silencing Mechanism:

The MBD sequence motif was defined by molecular analysis of prototype of MBD protein and MeCP2 (Nan et al., 1993). This MBD sequence motif was found to be both necessary and sufficient to be direct specific interaction with methyl-CpG-containing DNA fragments. It consists of around 70 amino acids and represents the sole sequence feature found in common all of the MBD family members. The single exclusion to this rule is case of the MBD2

and MBD3 which are nearly identical in amino acid sequences, and have common gene structures and are believed to have arisen from an ancient duplication in all the evolution of the vertebrate lineage. MeCP2 protein binds methylated CpG sites at gene promoters and promotes gene silencing by associating with Sin3A and Histone Deacetylases to form co-repressor complexes and through its interaction with CREB selectively regulates active BDNF gene transcription (Akbarian & Huang, 2009).

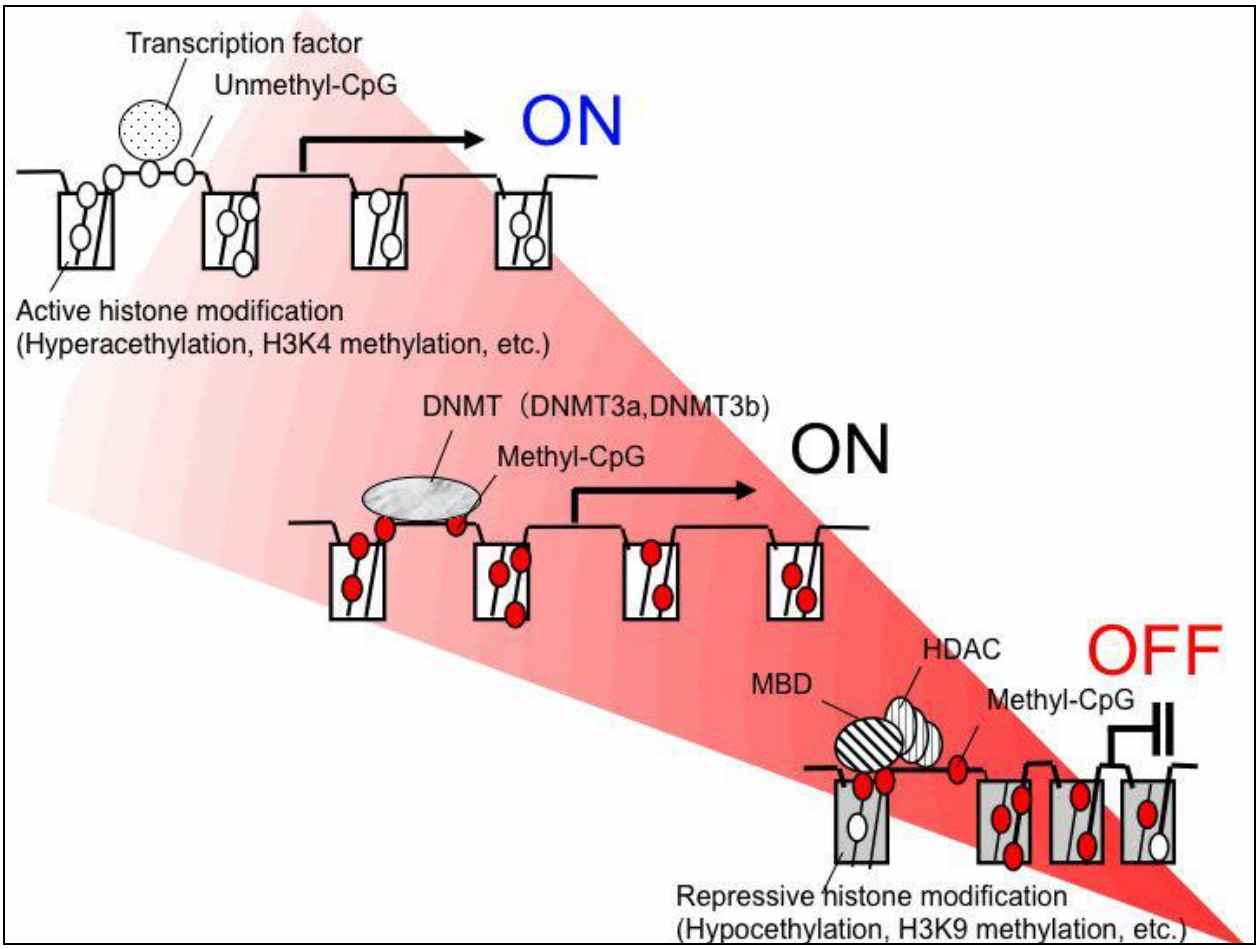


Figure 4: MBD Proteins Mediated Gene Silencing and Transcriptional inactivation.

MBD protein mediated gene silencing involves interaction of MBD proteins with different partners such as Histone Deacetylase (HDAC), Histone methyltransferases and chromatin remodelling complexes to form heterochromatin and prevent transcriptional activation of genes.

(Adapted from Kubota et al., The Mechanisms of DNA Replication, 2013)

Action of MBD proteins in promoting cancer via gene silencing:

It has recently been recognized that cancer is a manifestation of both abnormal genetic and epigenetic events (Jones, 2003). Deregulated epigenetic controls, which typically are represented by abnormal DNA methylation patterns such as global hypomethylation and region specific hypermethylation, are a trademark of most cancers. Aberrant hypermethylation of promoter CpG islands and the resulting transcriptional silencing is nowadays a widely accepted mechanism of inactivation of tumor suppressor genes in cancer that actively contributes to tumorigenesis. Treatment of cancer cells with the demethylating agent 5-aza-2′-deoxycytidine results in passive loss of CpG island methylation, MBD release, and gene re-expression, strengthening the notion that association of MBDs with methylated promoters is methylation-dependent (Fig 5). The existence of these profiles provides a powerful set of markers for outlining the disruption of critical pathways in tumorigenesis and for deriving sensitive molecular detection strategies for virtually every human tumor type (Wade, 2001).

The systematic study of DNA methylation patterns in human cancer cell lines has shown that these are appropriate models for this type of study as they show methylation patterns that resemble their corresponding tumor types. The selectivity of MBDs for methylated DNA and their transcriptional repression properties suggest that they could exert their function in methylated promoters (Fig 5).The occurrence of MBD proteins in the methylated promoter of a gene in cancer was first shown in 2000, when Magdinier and Wolffe (Magdinier and Wolffe, 2001) recognized MBD2 in the methylated promoter of p16INK4a in colon cancer cells. Human MBD genes are considered as housekeeping genes because they are widely expressed in somatic tissues. Given the epigenetic function of MBD proteins in regulating gene expression, MBDs may be concerned in cancer development by affecting the expression of cancer related genes. In fact, there is increasing evidence that aberrant expression of MBD proteins is associated with human cancers (Patra et al., 2003). The MBD2 gene is mapped to the conserved region within human chromosome 18q21. A recent finding also suggests that MBD2 has potential DNA demethylase activity (Bhattacharya et al., 1999), implying that it might reconcile gene activation in addition to transcriptional suppression. However, two subsequent studies could not demonstrate any demethylase activity of MBD2, and this inconsistency in the functions of MBD2 remains to be resolved. So many studies in human cancer research have demonstrated

that the MBD2 protein plays a role in tumorigenesis. Moreover, a significant reduction in MBD2 mRNA expression was found in human colorectal and gastric cancerous tissues (Kanai et al., 1999) and peripheral blood lymphocytes in bladder cancer patients, implying a protecting role for MBD2 in tumorigenesis. MBD2 protein expression and its demethylase activity were detected in normal human prostate tissue but not in cancerous tissue (Patra et al., 2002). These differences between types of cancers in the abundance of MBD2 levels may reflect different roles for MBD2 either in transcriptional repression or in the demethylation process.

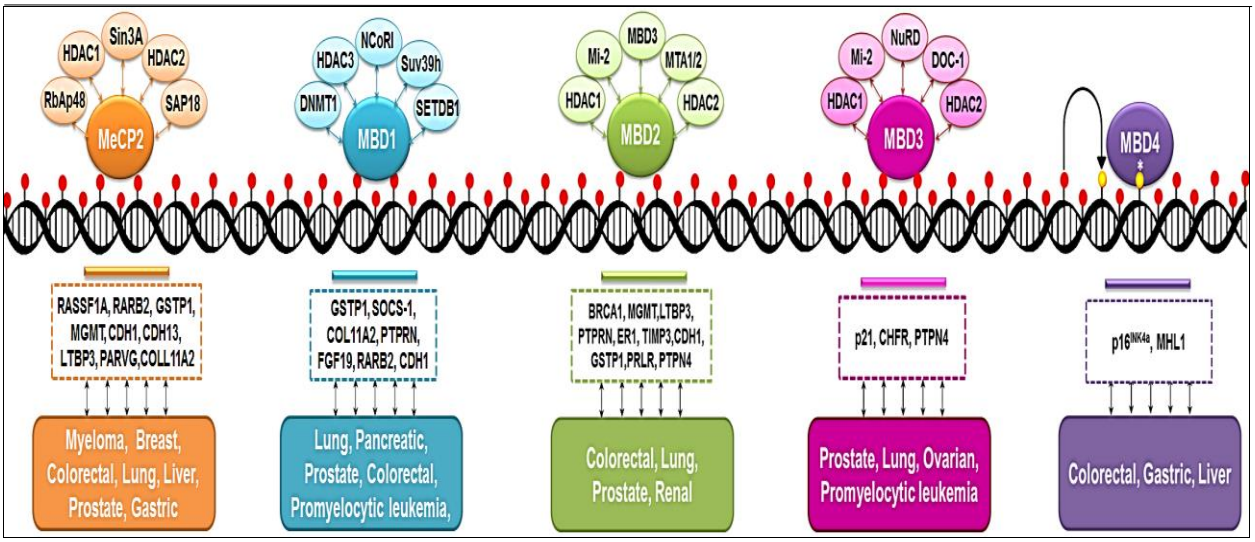


Figure 5: A simplified diagram depicting the modes of action of the different MBD proteins.

MeCP2, MBD2 and MBD3 interact with HDACs to bring about transcriptional repression whereas MBD1 binds with DNMT1 and acts to silence the hypermethylated promoter regions. MBD4 participates in DNA repair where it removes thymines (yellow dots) formed by oxidative deamination of methyl cytosines. MBD bind to the various hypermethylated promoters and are implicated in different MBD proteins thus form an important component of the epigenetic regulatory systems.

OBJECTIVES OF THE PROJECT

1. **Study of the gene expression status of the different MBD proteins - MeCP2, MBD1, MBD2, MBD3 and MBD4 in MDA-MB-231 breast cancer cell line.**

2. **Study of the effect of various epigenetic modulators such as Trichostatin A (TSA), and Sulforaphane (SFN) on the expression of the different MBD genes after 24 hours treatment at the transcriptome level.**

3. **Comparative analysis of the effect of the various epigenetic modulators on the different MBD proteins and on the survival of MDA-MB-231 breast cancer cells.**

MATERIALS AND METHODS

1. In vitro cell culture:

MDA-MB-231 human mammary tumor cells were cultured and maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) and 100 IU/mL Penicillin & 0.1 mg/mL streptomycin in a humified atmosphere of 5% CO_2 at 37 °C.

2. Drug treatment with AZA and SAM:

The cells (3×10^5/well) were grown in 6-well plates (40–50% confluent) and then treated with TSA (10,20,25,50,75,100,200,250,500 nM) and SFN (5,7,10,15,20,25,50,75,100 μM) mixed in DMEM supplemented with 5% FBS to determine the optimum doses for the drugs. Cells were then incubated at 37º C in complete growth medium for 24 and 48 hrs. Control cells were treated identically, except that no drugs were added to the cell medium.

3. Cell Viability Analysis by colometric MTT Assay:

Cell viability analysis and cytotoxicity studies were performed using the MTT assay based on the observation that the mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye. The drug-treated cells were washed in PBS, trypsinized and then seeded in 96-well plate at 105 cells/well (200 μl).100 μl of MTT solution (0.8 mg/ml) was then added to each well and the cells were incubated at 37º C for 4 hrs. Thereafter, DMSO was added to all wells and mixed thoroughly to dissolve the dark blue precipitate and incubated for 15 mins at room temperature. The absorbance of samples was determined at a wavelength of 590 nm. The extent of cytotoxicity was defined as the relative reduction of the optical density (OD), which correlated to the amount of viable cells in relation to cell control (100%). The cell viability was plotted in a graph and the IC50 was calculated accordingly to decide the optimum dosage of the drugs for further studies.

4. Flow Cytometry Analysis of the effects of epigenetic drugs on cell cycle (FACS):

Flow cytometry analysis of PI stained nuclei was done to assess the effects of epigenetic drugs TSA and SFN on the cell cycle distribution. TSA (100 and 250 nM) and SFN (10 and 20 μM) treated cells were incubated in DMEM with 5% FBS for 24 and 48 hrs. The cells were then trypsinized, collected by centrifugation, washed twice with PBS and then fixed in 90% ice-cold methanol. After incubation at -20℃ for 1 hr, cells were centrifuged and resuspended in PBS followed by treatment with RNaseA (500 U/ml) to digest the residual RNAs and stained with propidium iodide (50 ng/ml). Samples were incubated for 30 min at room temperature and cell cycle analysis was performed with a Becton–Dickinson fluorescence-activated cell sorter (FACS).

5. Chromatin condensation analysis after 24 hours drug treatment by Hoechst staining:

After treatment with drugs, the cells were stained with Hoechst 33342 stain (1 μg/ml) and incubated for 10 min at 37ºC and images were taken under UV filter using Epi-fluorescene Microscope at 400 X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm.

6. Extraction of Total RNA:

Total RNA was extracted from MDA-MB 231 cells by using the Trizol reagent. The drug treated cells (5-10 X 10^6 cells) were washed with 1 ml ice cold PBS, then trypsinized and then treated with 1 ml Trizol. 0.2 ml of chloroform (0.2 ml per 1 ml of TRI Reagent) was added to the tubes, shaken vigorously for 30 seconds by hand/vortex mixer and incubated at RT for 10 mins. The samples were centrifuged at 12,000 X g for 15 mins at 4 ° C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing the RNA. The upper aqueous phase was removed without disturbing the interphase and collected in a fresh tube. 0.5 ml isopropyl alcohol per 1 ml of TRI Reagent was added to the tubes. The tubes were then incubated at RT for 10 minutes and then centrifuged at not more than 12,000 x g for 10 minutes at 4 ° C. The supernatant was removed completely. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The pellet was washed with 1ml of 75% ethanol per 1 ml of TRI Reagent. The samples were mixed by vortexing and centrifuged at no more than 7,500 x g for 5 minutes at

4 ° C. The pellet was air-dried by keeping the RNA pellet containing tube opened in working bench for 15 mins. The RNA was dissolved in50 µl EPC-treated water by passing solution a few times through a pipette tip. The RNA was stored at - 20° C for further use or immediately processed for cDNA synthesis.

7. Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

The concentration of the extracted total RNA was quantified by measuring the absorbance at 260 nm in a spectrophotometer (ELICO, BL 200 Bio Spectrophotometer, double beam) and calculated by using the formula as given below:

$$\text{Total RNA (µg /ml)} = OD_{260} \times 40 \times \text{Dilution factor.}$$

8. Quantitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis:

The extracted RNA was run on a denaturing agarose gel and the quantity of RNA estimated from the band intensity. For denaturation gel (40 ml), 0.6 g agarose (Sigma), 28.8 ml dH_2O (Sigma), 7.2 ml formaldehyde (Sigma), 4 ml 10X MOPS buffer were mixed properly. About 2 µl (2µg) of the total RNA was mixed with 18 µl 1X Reaction Buffer (2µl of 10X MOPS Buffer, 4 µl formaldehyde, 10 µl formamide (Sigma) ,2 µl 0.2 mg/ml Etbr (Sigma)) and incubated at 55 °C for 1 hr. It was then cooled on ice and loaded in the wells of the denaturing gel.

9. First strand cDNA synthesis:

Total RNA (4 µg) was used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA was incubated with 1 µl of oligo $(dT)_{18}$ primers (100 µM, 0.2 µg/µl) and 12 µl of nuclease-free water at 65 °C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spin down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of Ribolock^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid^{TM} M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 hr at 42°C. Heating at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0} C for further use.

10. Gene-specific semi-quantitative PCR for amplification of the desired genes:

The PCR reaction mixtures, in a 25 µl volume, contained 17 µl of dH_2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of $MgCl_2$ (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) of MBD1, MBD2, MBD3, MBD4, MeCP2 and 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications of MBD1, MBD2, MBD3 and MBD4 were performed in a thermal cycler (Biorad) by initial denaturation at 94° C for 1 min, followed by 30 cycles of denaturation at 94° C for 20 secs, annealing at 57 ° C for 20 secs, and extension at 72° C for 30 secs, followed by an final extension step at 72° C for 5 mins. The constitutively expressed housekeeping gene, β-actin was used as a positive control to ensure high quality. RT-PCR products were then analyzed by 1% agarose gel electrophoresis containing ethidium bromide (0.05%). The primer sequences used for the PCR reaction are shown in Table 1.

Table 1.Table showing the forward and backward primers and their amplicon sizes.

PRIMER	TYPE	SEQUENCE	AMPLICON (bp)
MBD1	***Forward***	5’ CCTGGGTGCTGTGAGAACTGT 3’	107
	Reverse	5’ TTGAAGGCAATTCTCTGTGCTC 3’	
MBD2	***Forward***	5’ AGGTAGCAATGATGAGACCCTTTTA 3’	116
	Reverse	5’ TAAGCCAAACAGCAGGGTTCTT 3’	
MBD3	***Forward***	5’ CCGCTCTCCTTCAGTAAATGTAAC 3’	101
	Reverse	5’ GGCTGGAGTTTGGTTTTCAGAA 3’	
MBD4	***Forward***	5’ AGACCCGCCGAATGACCT 3’	144
	Reverse	5’ GCACCAAACTGAGCAGAAGCG 3’	
MeCP2	***Forward***	5’ TGACCGGGGACCCATGTAT 3’	145
	Reverse	5’ CTCCACTTTAGAGCGAAAGGC 3’	
β-ACTIN	***Forward***	5’ CTGGAACGGTGAAGGTGACA 3’	140
	Reverse	5’ AAGGGACTTCCTGTAACAACGCA 3’	

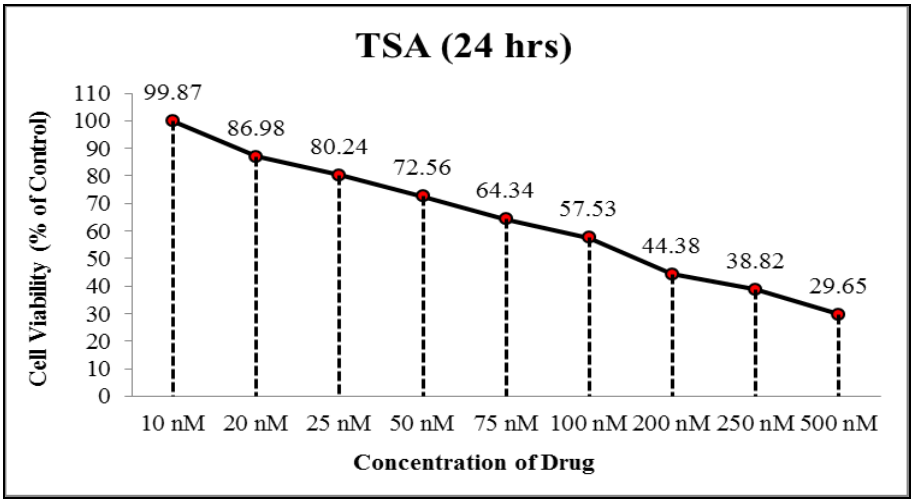
11. Relative Gene Expression Analysis after drug treatment by Real-Time PCR:

Quantitative estimation of the expression of the MBD genes after drug treatment was done via real-time PCR analysis. cDNA was used to analyze the expression of MBD genes along with β-Actin as a house keeping gene.

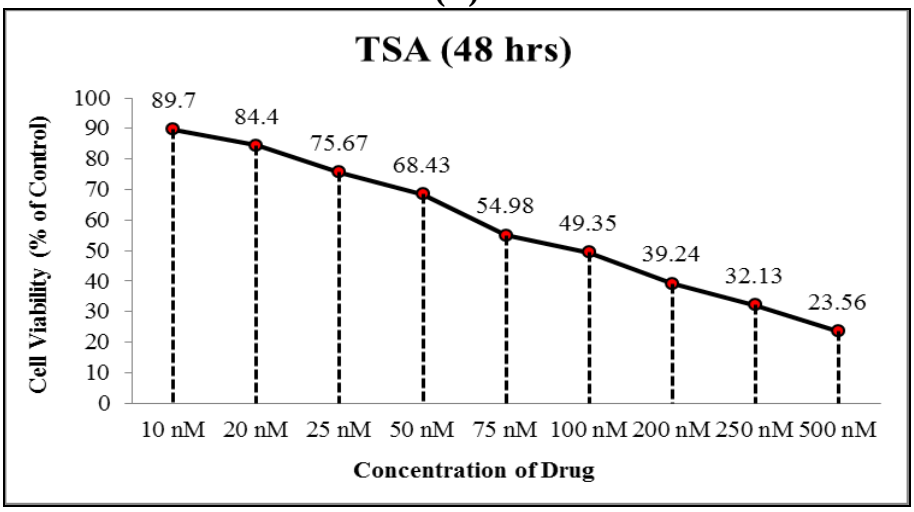
RESULTS AND DISCUSSION

1. **Cell Viability Analysis by colometric MTT Assay:**

Cytotoxicity studies were performed to determine the effect of epigenetic drugs such as TSA and SFN (inhibitors of Histone Deacetylases (HDACs)) on the expression of MBD genes. In order to determine the optimum dosage, different concentrations of the drugs were considered and the treatment was done for different time intervals. The results obtained from the MTT assays are given below:

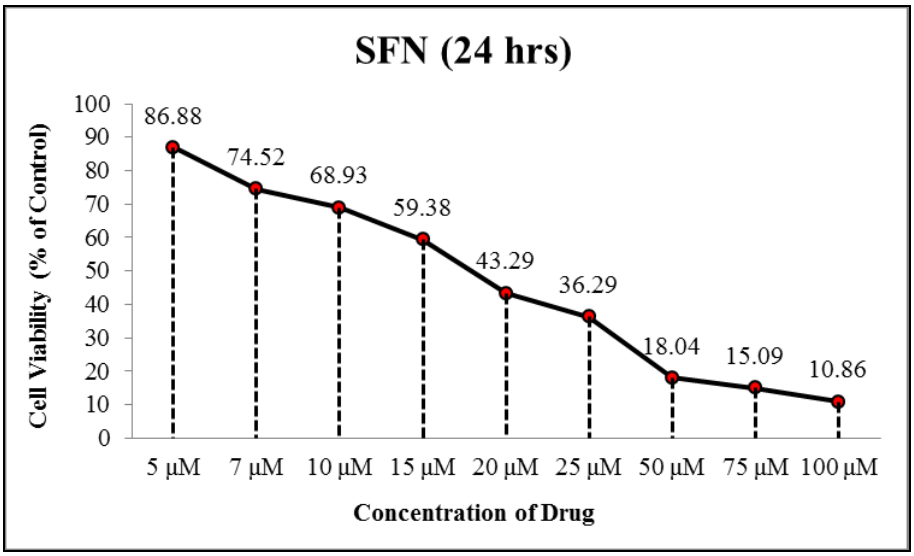


(A)

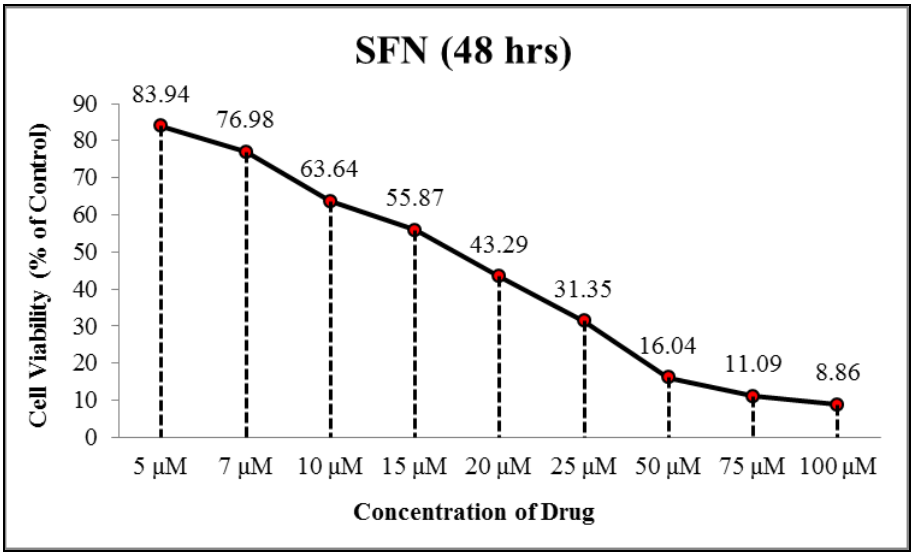


(B)

Figure 6: The effect of different concentrations of drug TSA (10,20,25,50,75,100,200,250,500 nM) at different time intervals of 24 hrs (A) and 48 hrs (B) on the survival of MDA-MB-231 breast cancer cells.



(A)



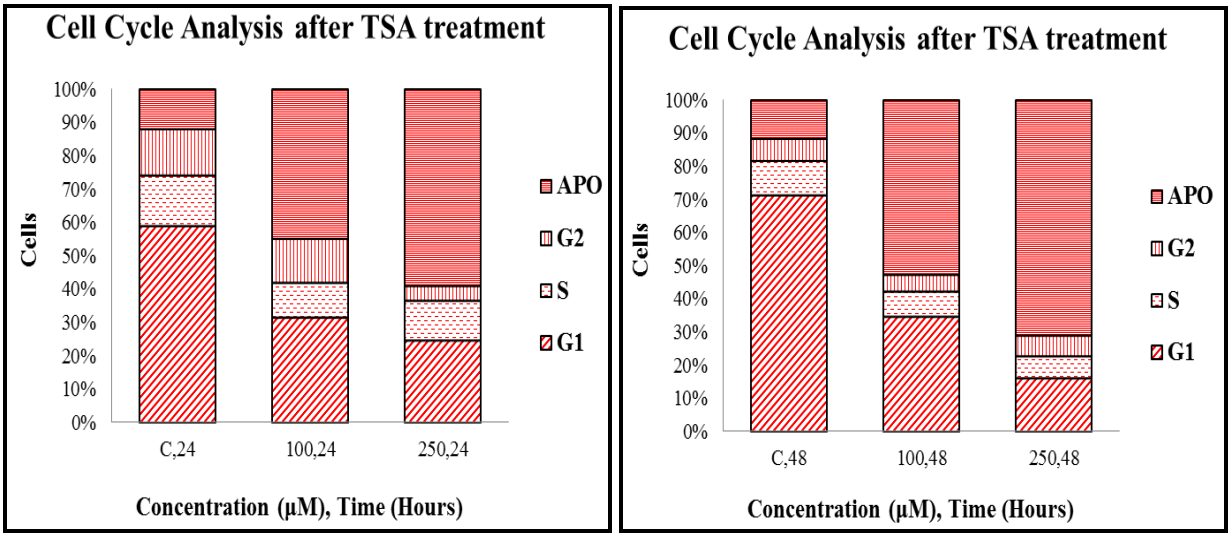
(B)

Figure 7: The effect of different concentrations of drug SFN (5, 7,10,15,20,25,50,75,100 µM) at different time intervals of 24 hrs (A) and 48 hrs (B) on the survival of MDA-MB-231 breast cancer cells.

In general, cell survival levels declined progressively with an increased dose of both the epigenetic drugs (Fig 6 & 7). Treatment with increasing doses of both TSA and SFN showed a drastic decline in cell survival. From this assay, 250 nM of TSA and 10 µM of SFN were chosen as the optimum doses for further MBD expression studies.

2. Flow Cytometry Analysis of the effects of epigenetic drugs on cell cycle (FACS):

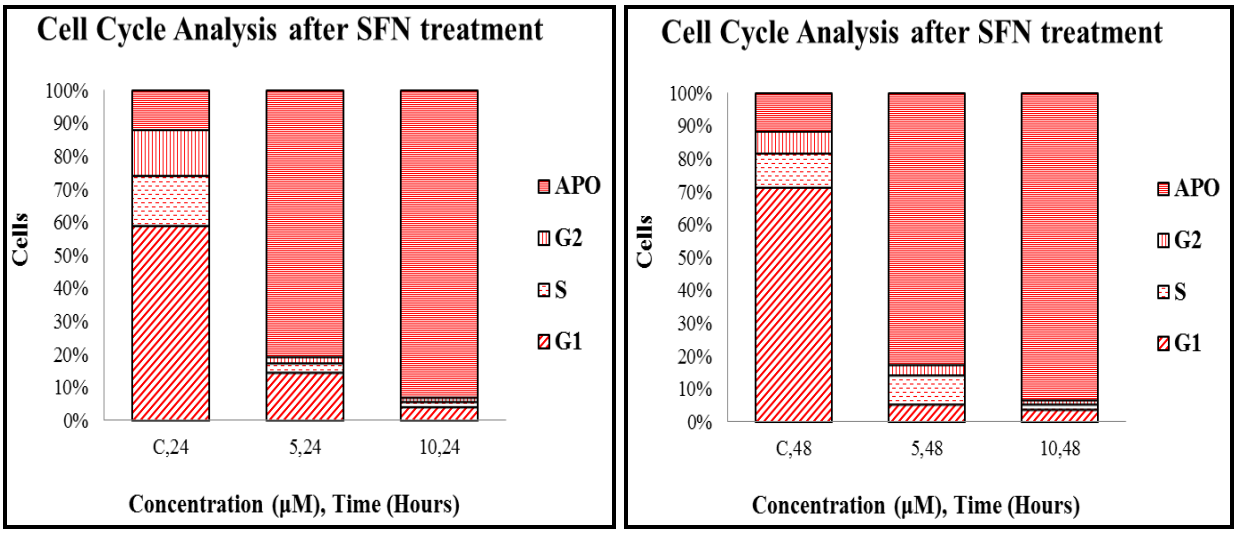
Flow Cytometric analysis was done to assess the effect of the epigenetic drugs on the cell cycle and consequently co-relate these effects to cancer progression. HDAC inhibitors TSA and SFN are known to effectively inhibit the action of HDACs and result in transcriptional activation. The results of the FACS study are represented below (Fig 8 & 9). As shown below, breast cancer cells were arrested in different phases in a dose and time dependent manner. In comparison to control, cells treated with TSA and SFN, exhibited reduction in G1-phase cells, decreased percentage of G2 population and drastic increase in apoptotic cells. Thus, the HDAC inhibitors TSA and SFN induce differentiation, growth arrest and apoptosis in MDA-MB-231 cells.



(A) **(B)**

Figure 8: Cell cycle distribution of MDA-MB-231 cells after treatment by two different doses of TSA (100 and 250 nM) for different time intervals of 24 hrs (A) and 48 hrs (B).

The alterations in the cell cycle distributions were distinct at different time intervals (24 & 48 hrs) as well as at low versus high doses. At a low dose of TSA (100 nM), there is a decrease in G_1 phase cells, S and G_2 populations but increase in apoptotic cell percentage. At higher dosage (250 nM), there is a drastic decrease in G_1, S, G_2 populations while apoptosis is greatly elevated. Thus TSA affects all the stages of cell cycle, arresting cell progression in each successive stage and ultimately increasing the rate of apoptosis of the cell population.



(A) **(B)**

Figure 9: Cell cycle distribution of MDA-MB-231 cells after treatment by two different doses of SFN (5 and 10 µM) for different time intervals of 24 hrs (A) and 48 hrs (B).

The alterations in the cell cycle distributions were distinct at different time intervals (24 & 48 hrs) as well as at low versus high doses. At a low dose of SFN (5 µM), there is a decrease in G_1 phase cells, S and G_2 populations but increase in apoptotic cell percentage. At higher dosage (250 nM), there is a drastic decrease in G_1, S, G_2 populations while apoptosis is greatly elevated. However, the effect of these drugs increases with time interval, the decrease in G1 phase cells is more after 48 hrs treatment; however, there was little effect on the other cell populations with increased time. Thus SFN is found to be a potent inhibitor of cell cycle progression, greatly increasing apoptosis. MBD proteins are known to interact with HDACs to repress transcriptional activity via heterochromatin formation. As the HDAC inhibitors effectively trap HDAC and prevent them to associate with MBD proteins, there is a possibility that the action of MBD proteins can be disrupted. Hence, treatment with HDACi affects the activity of MBD proteins.

3. Chromatin condensation analysis after drug treatment by Hoechst staining:

Nuclear chromatin condensation in drug treated MDA-MB-231 cell by Hoechst staining is a visually detectable assay to quantify the amount of apoptotic cells after drug treatment. The blue-fluorescent Hoechst 33342 brightly stains the condensed chromatin of apoptotic cells and less brightly stains the normal chromatin of live cells (Fig 10 & 11). After FACS analysis, the drug treated cells were stained with Hoechst dye to estimate the effectiveness of each drug and the rate of apoptosis inflicted by each drug. The results of the Hoechst staining assay are given below:

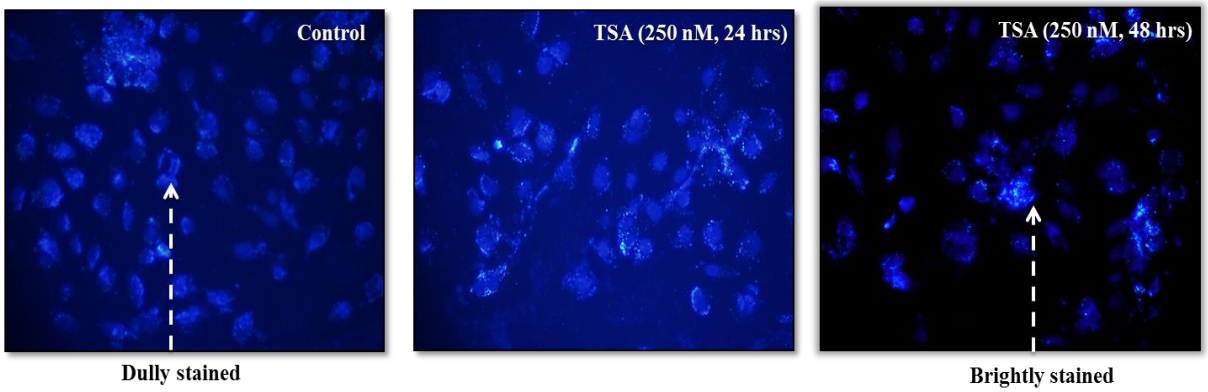


Fig 10: Nuclear chromatin condensation in TSA treated MDA-MB-231 cell after 24 & 48 hrs.

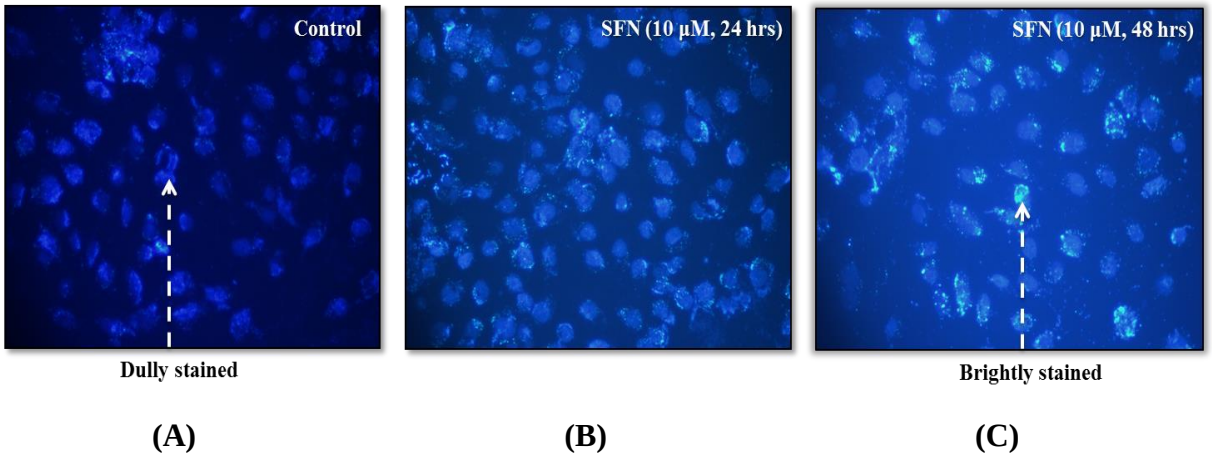


Fig 11: Nuclear chromatin condensation in SFN treated MDA-MB-231 cell after 24 & 48 hrs.

4. Relative Gene Expression Analysis after drug treatment by RT-PCR:

In general, there is over-expression of all the MBD genes in cancer. It is well documented that MeCP2, MBD2 and MBD3 interacts with HDACs in gene silencing, hence they showed decreased expression in relation to normal expression of MBD1 and MBD4 after treatment with HDAC inhibitor TSA and SFN. MBD2 is mainly involved in DNA repair; hence its expression is mostly uniform upon treatment with various epigenetic drugs.

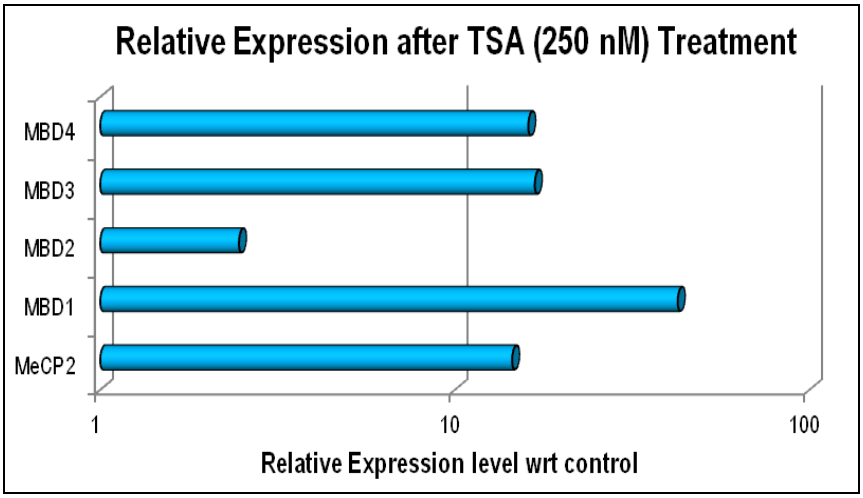


Figure 12: Relative expression of the different MBD genes with respect to β-actin (a house keeping gene) in TSA treated MDA-MB 231 cells.

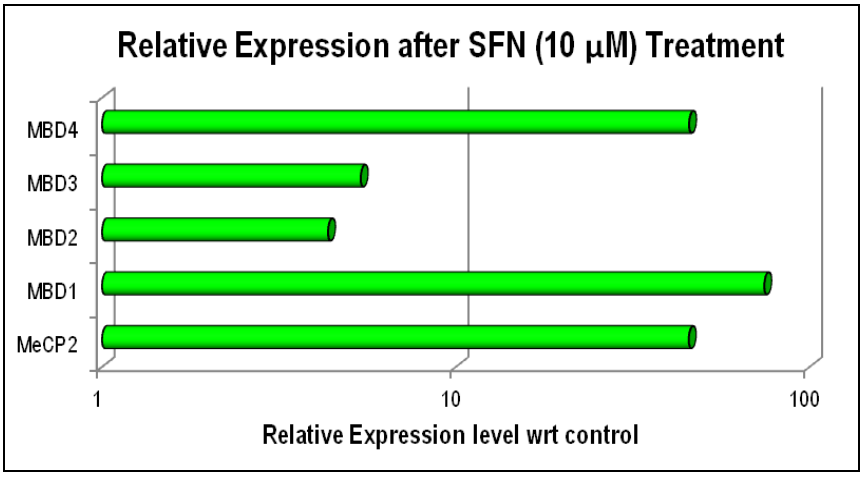


Figure 13: Relative expression of the different MBD genes with respect to β-actin (a house keeping gene) in SFN treated MDA-MB 231 cells.

CONCLUSION

In this study, we have shown the effect of different epigenetic modulators such as TSA and SFN on the expression of different members of the MBD group of proteins. MBD proteins interact with HDAC to bind to the methylated cytosine bases in CpG islands of the hypermethylated promoter regions and inhibit transcriptional activity. Hence it can be safely assumed that inhibiting MBDs can provide a means of negating its effect on transcriptional activity. Hence, inhibiting the HDACs by HDAC inhibitors such as TSA and SFN will serve as a means to restrict MBD proteins and help in transcriptional reactivation of silenced genes such as tumor suppressors, cell cycle regulatory and apoptosis inducing genes. The turning on of crucial tumor suppressor genes may be an efficient means to counteract malignant transformation; hence MBD proteins can be regarded as novel prognostic targets for cancer therapy.

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