Université de Montréal

PRECLINICAL EVALUATION OF 5-AZA-2'-DEOXYCYTIDINE, AN INHIBITOR OF DNA METHYLATION, FOR THERAPY OF BREAST CANCER.

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Thèse présentée à la Faculté des études supérieures
En vue de l'obtention du grade de
Philosophiæ Doctor (Ph.D.)
En Pharmacologie

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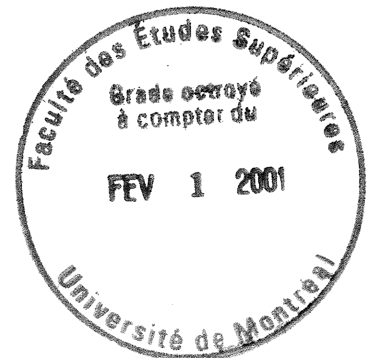

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Cette thèse intitulé:

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Présentée par

Veronica Bovenzi

A été évaluée par un jury composé des personnes suivantes:

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SUMMARY

Breast cancer is the second most common cause of cancer death among women in the United States. Chemotherapy and hormonal therapy have proven to be effective in early breast cancers, but most women with metastatic disease that does not respond to hormonal therapy have only a limited life expectancy with the current conventional therapy. There is an urgent need to develop new approaches for therapy of advanced breast cancer.

The identification of many tumor suppressor and cancer-related genes that are silenced by aberrant methylation in breast cancer make it interesting to investigate the potential of therapy with demethylating agents, such as 5-Aza-2'-deoxycytidine (5-AZA-CdR) for this disease. Aberrant DNA methylation of promoter regions of cancer-related genes leads to silencing of their expression. Demethylation may restore the tumor suppressor functions and arrest tumorigenesis.

We investigated the *in vitro* antineoplastic activity of 5-AZA-CdR, an analog of 2'-deoxycytidine, on the human MDA-MB-231 breast carcinoma cell line. We found that this analog was able to inhibit cell growth and clonogenic potential of these tumor cells. The concentrations used in our assays were in the same range of the plasma levels of 5-AZA-CdR used in pilot clinical studies with responses in patients with cancer.

An interesting approach to increase the therapeutic efficacy of an anticancer drug and to avoid drug resistance, is to use a combination of agents whose mechanisms of action are different. We investigated the combination of 5-AZA-CdR with a new class of antineoplastic drugs, the histone deacetylase inhibitors (HDIs), such as trichostatin A (TSA). These latter agents maintain histone acetylation, leading to less compact, transcriptionally active chromatin. HDIs also induce terminal cellular differentiation. We investigated the antineoplastic action of combination of 5-AZA-CdR and TSA on the MDA-MB-231 breast carcinoma cell line. Our results show a synergistic effect of the two drugs on the inhibition of clonogenic potential of these tumor cells.

In order to understand the mechanisms underlying this interaction, we studied the effect of 5-AZA-CdR and TSA on the expression of two tumor suppressor genes, the retinoic acid receptor β (RARβ) and the estrogen receptor (ER) gene. Both genes have been extensively reported to be silenced by aberrant methylation in breast cancer. We showed by RT-PCR that exposure of MDA-MB-231 tumor cells to 5-AZA restored expression of these two genes by demethylation of their promoter

regions. TSA is able to restore expression of RARβ, but not that of ER, suggesting the existence of different mechanisms of epigenetic silencing for different genes. Combination of the two agents appeared to induce a more extensive reactivation of expression of the two genes studied.

This study supports future investigations on the use of 5-AZA-CdR in the therapy of breast cancer. The efficacy of this cytosine nucleoside analog can be increased by the combination of other agents, such as HDIs, to induce a higher expression of cancer-related genes. This new approach to breast cancer therapy may be effective for women with advanced breast cancer. In addition, this therapy has the potential to re-express the ER gene and possibly reestablish responsiveness to antiestrogen therapy with tamoxifen.

RÉSUMÉ

Le cancer du sein représente la deuxième cause de mortalité chez la femme aux États-Unis. La chimiothérapie et la thérapie hormonale se sont révélées efficaces pour le traitement du cancer précoce du sein, mais beaucoup de femmes atteintes de ce type de cancer développent des métastases qui ne répondent pas à la thérapie hormonale ou autres traitements conventionnels et par conséquent ont une espérance de vie limitée. Pour ces raisons, il demeure impératif de développer des nouvelles approches pour la thérapie du cancer du sein rendus à un stade avancé.

Plusieurs gènes suppresseurs de tumeurs et gènes reliés au cancer sont supprimés par une hyperméthylation de l'AND dans differents types de, notamment le cancer du sein. De ce fait, nous avons envisagé le potentiel thérapeutique d'agents inhibiteurs de la méthylation telle, la 5-Aza-2'-déoxycytidine (5-AZA-CdR), au niveau du cancer du sein. La méthylation des îlots CpG situés dans la région promotrice est associée à la répression transcriptionelle des gènes. L'inhibition de la méthylation de l'ADN peut amener à la reprise des fonctions suppressives de tumeurs de certains gènes reliés au cancer.

Pour ce faire, nous avons évalué l'activité anti-néoplasique de la 5-AZA-CdR, un analogue de la 2'-déoxycytidine, chez une lignée cellulaire humaine de carcinome du sein, MDA-MB-231. Nous avons montré que cet analogue induit une forte inhibition de la croissance cellulaire et du potentiel clonogénique de ses cellules tumorales. Fait à noter, les concentrations actives de 5-AZA-CdR utilisées dans nos expériences sont conforment à celles retrouvées dans le plasma sanguin de personnes cancéreuses participant à des essais cliniques pilotes.

Une approche intéressante d'accroître l'efficacité thérapeutique et d'éviter la résistance aux médicaments anti-cancéreux se veut l'administration combinée de différentes classes d'agents dont le mécanisme d'action diffère. Nous avons donc étudié l'effet concerté de la 5-AZA-CdR avec la Trichostatin A (TSA), un inhibiteur de la déacétylase des histones (HDI) appartenant à une nouvelle catégorie d'agents anti-néoplasiques. Ce dernier composé est responsable du maintien du niveau de l'acétylation des histones, ce qui amène une chromatine de structure moins compacte

et transcriptionnellement plus active. À l'instar de la 5-AZA-CdR, les HDIs favorisent la perte de la capacité de prolifération des cellules tumorales en induisant leur différenciation. Nous avons investigué l'activité anti-néoplasique conjointe de la 5-AZA-CdR et de la TSA chez des cellules de carcinome du sein MDA-MB-231. Les résultats ont montré un effet synergique de ces deux agents au niveau de l'inhibition du potentiel clonogénique de ces cellules cancéreuses.

Afin d'élucider le mécanisme moléculaire d'interaction entre ces deux agents anti-néoplasiques, nous avons étudié les effets de la 5-AZA-CdR et de la TSA, pris individuellement ou mis ensemble, sur l'expression de deux gènes suppresseurs de tumeurs soit le gène codant pour le récepteur de l'acide rétinoïque β (RARβ) et celui du récepteur à l'estrogène (ER). Il est connu que l'expression de ces deux gènes peut être supprimée par l'hyperméthylation de l'ADN dans leur région promotrice dans un haut pourcentage des cancers du sein et chez les cellules de carcinome du sein MDA-MB-231. Nous avons donc incubé ces cellules avec les agents ci-haut mentionnés et par la suite, l'ARN totale a été extraite afin d'effectuer un PCR à transcriptase inverse (RT-PCR). Les résultats ont montré que la 5-AZA-CdR peut réactiver l'expression des deux gènes étudiés alors que la TSA ne rétablit que l'expression du gène codant pour le RARβ. Cependant, l'administration concomitante de deux agents semble entraînée un niveau d'expression plus élevé des deux gènes étudiés.

À partir de biopsies de tumeurs du sein, nous avons analysé l'état de méthylation du gène du RARβ par la technique du PCR (*polymerase chain reaction*) spécifique pour la méthylation (MSP). Cette technique expérimentale prend avantage des différences existant entre des séquences méthylées et non après traitement avec le sodium bisulfite. En bref, l'ADN génomique est traité avec le sodium bisulfite ce qui amène à la conversion des cytosines en uraciles par un processus de désamination tandis que les cytosines méthylées demeurent inchangées. Après le traitement avec le bisulfite, la séquence à analyser est amplifiée par PCR en utilisant deux couples d'amorces, l'une spécifiques à l'ADN méthylé et l'autre spécifique à l'ADN non méthylé. Au moyen de cette technique, des bandes spécifiques pour l'ADN méthylé ont été observées chez 30% des biopsies du sein examinées.

La technique de l'extension des amorces par un seul nucléotide qui est sensible à la méthylation (Ms-SNuPE) est couramment utilisée pour la quantification de l'état de méthylation de l'ADN au niveau de sites spécifiques de certains gènes. Cette technique permet aussi de quantifier l'inhibition de la méthylation de l'ADN produit par des agents comme la 5-AZA-CdR. Dans ce travail, une modification a été élaborée dans le but de simplifier cette téchnique. Brièvement, après traitement avec le bisulfite l'ADN est amplifié au niveau de la séquence cible en utilisant des amorces spécifiques pour l'ADN modifié. Cet ADN amplifié est utilisé dans la réaction de SNuPE. Les amorces utilisées pour cette réaction sont choisies afin qu'elles terminent juste avant le site d'incorporation choisi pour l'analyse de la méthylation. L'ADN amplifié et purifié est incubé avec soit du dCTP ou du dTTP radioactif, en présence de l'ADN polymérase et des amorces spécifiques. Dépendant de l'état de méthylation, une molécule de cytosine ou thymidine sera incorporée si le site est méthylé ou non, respectivement. La technique de quantification de la radioactivité incorporée a été modifiée, en replaçant l'électrophorèse par gel suivie par la quantification des bandes, par une mesure directe de la radioactivité incorporée dans l'ADN. Nous avons verifié la reproductibilité de cette technique modifiée et nous l'avons utilisée pour la quantification de l'inhibition de la méthylation du gène pour l'RARβ produite par des différentes concentrations de 5-AZA-CdR chez des cellules de carcinome du sein MDA-MB-231 et des cellules d'adénocarcinome du côlon DLD1. Nous avons montré une diminution dose-dépendante de la méthylation de deux sites spécifiques au niveau du gène du RARβ suivant le traitment par la 5-AZA-CdR.

En conclusion, les études préliminaires sur l'efficacité *in vitro* de la 5-AZA-CdR chez les cellules de carcinome du sein montrent que cet agent a un potentiel contre ce type de cancer. La réactivation de plusieurs gènes suppresseurs de tumeurs semble être un des effets les plus importants causés par cet analogue. A partir de nos résultats, ils nous est permis de croire que la combinaison de la 5-AZA-CdR avec d'autres classes d'agents anti-néoplasiques, particulièrement les inhibiteurs de la

déacétylase des histones (ex. TSA), serait souhaitable dans le but d'augmenter l'efficacité du traitement contre le cancer du sein.

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ABBREVIATIONS

AC	Adriamycin, cyclophosphamide
APL	Acute promyelocytic leukemia
5-AZA-CdR	5-aza-2'-deoxycytidine
5-AZA-dCDP	5-AZA-2'-deoxycytidine-5'-diphosphate
5-AZA-dCMP	5-AZA-2'-deoxycytidine-5'-monophosphate
5-AZA-dCTP	5-AZA-2'-deoxycytidine-5'-triphosphate
5-AZA-dUMP	5-AZA-2'-deoxyuridine-5'-monophosphate
5-AZA-UdR	5-AZA-2'-uridine
bp	base pair
BRCA	breast cancer susceptibility gene
CAF	Cyclophosphamide, adriamycin, fluorouracil
CMF	Cyclophosphamide, methotrexate, fluorouracil
CR deaminase	Cytidine deaminase
DAP-kinase	Death Associated Protein kinase
DMTase	DNA methyltransferase
EGF	Epidermal Growth Factor
EGF-R	EGF-receptor
ER	Estrogen receptor
G-CSF	Granulocyte- colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GST	Glutathione S-Transferase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HER/neu	Oncogene belonging to the EGF-R family
HIC	Hypermethylated in Cancer
hMLH1	human MutS homologue gene
IGF2	Insulin-like growth factor
LOH	Loss of heterozygosity
5-MC	5-methylcytosines

MDGI	Mammary-derived growth inhibitor
MDBP1 and 2	Specific methylated DNA binding proteins
MeCP1 and 2	methylated CpG binding proteins
MDa	megadalton
MGMT	O^6-methyl-guanine-methyltransferase
MSP	Methylation sensitive PCR
Ms-SNuPE	Methylation sensitive single nucleotide primer extension
PML gene	Promyelocytic leukemia gene
RAR	Retinoic Acid Receptors
RARE	Retinoic Acid Response Element
SWI/SNF	Switching/sucrose nonfermenting
TAF	Transcription associated factor
TFIID	Transcription factor
TGFβ	Tumor growth factor β
TSA	Trichostatin A

Dedicated to the memory of Massimo and Raffi

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PART 1: LITERATURE REVIEW

CHAPTER 1: BREAST CANCER

1.1 Incidence and etiology

Breast cancer is the most frequently diagnosed cancer in American women, and the second most frequent cause of cancer death (Parker et al. 1996). Over the past few decades, there has been a steady increase in the incidence of this disease. The incidence of breast cancer varies among different populations: women in the United States and in Western Europe have a higher incidence than women do in most other parts of the world. The age specific incidence of breast cancer rises with age up to the time of menopause, and then slows to one sixth of that seen in premenopausal period. Many factors increase a woman's risk to develop breast cancer. The most important of these risk factors is family history, particularly if more than one member of the same family has developed breast cancer at an early age. Genetic analysis has shown linkage to germ line mutations in particularly one gene, BRCA1 (see page 6).

Breast cancer is clearly related to hormone exposure. Numerous studies have linked its incidence with age at menarche, age at first pregnancy and age at menopause. The effect of exogenous hormones on breast cancer risk has been extensively studied, but has not been clearly established.

1.2 Familial breast cancer

A family history of breast cancer has long been recognized as a major risk factor for the disease. About 5 to 10% of all breast cancer are due directly to genetic factors. The identification in 1990 of the BRCA1 gene that, when mutated, is associated with an extremely high risk of breast cancer has provided new understanding of breast cancer risk (Miki et al. 1994). Genetically transmitted breast cancer should be suspected in women with multiple relatives (>3) with breast cancer. In most women with family history of breast cancer in which the disease is not linked to germ-line mutations, there is a decreased risk for other members of the family.

1.3 Growth factor pathways in the normal and malignant breast tissue

The natural products of the mammary epithelial cells, colostrum and milk, are abundant in growth factors. Growth factors are necessary in newborn development and in normal mammary growth. They may play an important role in breast tumorigenesis. Estrogens and progestins regulate certain growth factors of the epidermal growth factor (EGF) and tumor growth factor β (TGFβ) families. Overexpression of these growth factors has been reported during malignant transformation (Hynes and Stern, 1994; Travers et al., 1988).

Study of EGF overexpression has led to new understanding of breast cancer. Gene amplification and overexpression of EGF receptor-related HER/neu protein has been observed in 25% of human breast cancers (Hynes and Stern, 1994). Other growth factors such as TGFβ are present in normal and malignant breast tissue. TGFβ family members produce negative proliferating effects on murine mammary epithelial tissue (Dickson and Lippman, 1996). Paradoxically, TGFβ production increases during tumorigenesis and can even stimulate tumor growth (Stampfer et al 1993).

1.4 Genetic alterations in breast cancer

Cancer is thought to progress by accumulation of multiple genetic changes (Fearon and Vogelstein, 1990). Several specific mutations have been frequently identified in breast cancer. The most common genetic abnormality in cancer appears to be loss of heterozygosity (LOH) at multiple loci (Reid et al. 1995). At the present time, LOH of BRCA1, Rb1 and p53 genes have been frequently observed in breast cancers.

The second most common genetic alteration occurring in breast cancer is gene amplification of proto-oncogenes. The most studied amplified gene in this type of cancer are the EGF receptor (EGF-R) family and the nuclear transcription factor c-myc.

Also observed in breast cancer is the loss of expression of some tumor suppressor genes, even in the absence of chromosomal alterations (Domann et al. 2000). The primary mechanism responsible for loss of their expression is DNA methylation. Methylation of cytosines in their promoter regions results in silencing of many cancer-related genes in malignant tissue. For example, some of the genes that are silenced by this mechanism in breast cancer are: BRCA1, estrogen receptor (ER), p16, E-cadherin and retinoic acid receptor β genes (These genes will be discussed below).

1.4.1 Oncogenes

Most of the growth factors of the normal mammary gland share convergent signaling pathways which involve the action of nuclear proto-oncogenes (Roberts 1992, Silvennoinen et al. 1993, van der Burg et al. 1991). Nuclear oncogenes found to be up regulated in human breast cancer tissue include the EGF-R family (erbB, HER/neu) and c-myc gene.

1.4.1.1 EGF-R family

Enhanced expression of EGF-R on cancer cells has been associated with increased cellular proliferation and metastasis. One member of this family, HER2 gene (also known as neu and as c-erbB2), encodes for a tyrosine kinase receptor that shares homology with the other members of the EGF-R family (Hynes and Stern, 1994). HER2 gene has been found to be overexpressed in 25-30% of breast cancers and is predictive for a poor prognosis (Press et al., 1997). A human antibody (Herceptin) directed to the external domain of this receptor has shown to be a potent inhibitor of growth of human breast cancer cells that express high levels of HER2 (Carter et al., 1992). This antibody is currently used in the therapy of those breast cancers that over-express HER2 oncogene with positive preliminary results (Baselga et al., 1996; Baselga et al., 1998).

1.4.1.2 c-myc

The proto-oncogene c-myc encodes for a highly conserved nuclear phosphoprotein with domains that are common to many transcription factors (Marcu et al. 1992). c-Myc binds to the Max protein to form a heterodimer that binds specifically to the DNA to activate transcription of specific genes. Overexpression of c-myc by *in vitro* transfection has been demonstrated to transform cells.

Deregulation of expression of c-myc *in vivo* has been clearly linked to tumorigenesis (Morgenbesser and DePinho, 1994). In normal quiescent cells, expression of myc is low, and is induced by mitogenic stimuli. In neoplastic cells, where myc expression is deregulated, cells grow at faster rate (Karn et al. 1989). Overexpression of c-myc is thought to play a major role in breast cancer tumorigenesis because this gene is often found amplified and/or overexpressed (reviewed by Nass and Dickson 1997). Amplification of the c-myc gene is associated to high proliferative rate and poor prognosis in patients with breast cancer.

1.4.2 Tumor suppressor genes

Tumor suppressor genes normally suppress the growth potential of cells, regulating their progression in the cell cycle. Loss of expression of tumor suppressor genes plays an important role in progression of breast cancer. Tumor suppressor genes whose functions are deregulated in breast cancer are listed in **Table 1.1**, and summarized below.

1.4.2.1 p53 gene

Genetic alterations at the p53 locus on chromosome 17p have been found in a large variety of human tumors, including breast cancer (Harris and Hollstein, 1993). Point mutations in p53 gene are commonly observed in families with Li-Fraumeni syndrome that is characterized by a high incidence of breast cancer or sarcoma. The non-mutated p53 gene product is a DNA binding protein that triggers cellular

responses to DNA damage. The p53 protein induces cell cycle arrest to permit the damaged DNA to be repaired or to induce apoptosis if DNA damage is too severe for repair. Mutations in p53 are associated to genetic instability due to loss of its function resulting in a gradual accumulation of chromosomal alterations (Cullotta and Koshland, 1993).

1.4.2.2 BRCA

The two main breast cancer susceptibility genes, BRCA1 and BRCA2, have been extensively studied in patients with this disease. Mutations in BRCA1 are responsible for nearly half of inherited breast cancers and for about 80% of inherited ovarian cancers (Ford et al. 1994). BRCA2 gene mutations appeared to confer a relatively lower risk of ovarian cancer than BRCA1 and they seemed to be present more frequently in cases of male breast cancer (Wooster et al. 1994). Some studies have found a connection between BRCA1, as well as BRCA2, and the DNA repair machinery for the chromosome breaks that lead to genetic instability. This instability is linked to the loss of BRCA1 and BRCA2 expression resulting in a block in the mechanisms that trigger apoptosis (Chen et al., 1999; Venkitaraman, 1999).

Up to 80 distinct mutations in BRCA1 have been characterized in high risk families (Shattuck-Eidens et al. 1995). Mutations of BRCA1 in sporadic breast cancers have been seldom described, but lack expression of this gene has been observed. Rice et al. (1998) described silencing of BRCA1 gene by aberrant DNA methylation in these cancers.

1.4.2.3 p16 gene

The p16 gene encodes for a protein (CDKN2A) that is an inhibitor of cyclin dependent kinases CDK4 and CDK6. It binds to and inactivates these kinases in late G1 phase and suppresses cell division by blocking the phosphorylation of the retinoblastoma protein (Serrano et al. 1993). The p16 tumor suppressor gene maps on chromosome 9p21, a site that undergoes frequent LOH in breast cancer (Geraltis and

Wilson, 1996). In many types of cancer p16 deletion has been demonstrated to occur at a high frequency (Nobori et al., 1994; Kamb et al., 1994). p16 was also demonstrated to be inactivated by aberrant methylation in 33% of breast cancer cell lines and 31% of primary breast tumors (Herman et al., 1995). Its reactivation following exposure to the hypomethylating agent, 5-aza-2'-deoxycytidine (5-AZA-CdR) was reported (Otterson et al., 1995; Costello et al. 1996).

1.4.2.4 E-cadherin gene

The E-cadherin gene encodes for a transmembrane glycoprotein that is involved in cell to cell adhesion and thought to play an important role in metastasis suppression. The E-cadherin gene has been cloned and it is located on chromosome 16q22.1 (Berx et al., 1995), a region which has been reported to show LOH in about 30% of breast tumors (Sato et al., 1990). Expression of E-cadherin was reported to be reduced or absent in poorly differentiated breast tumors (Oka et al., 1993).

Transfection of E-cadherin gene into MDA-MB-231 breast carcinoma cells was shown to reduce their metastatic potential in nude mice (Mbalaviele et al., 1996). E-cadherin was demonstrated to be silenced by promoter hypermethylation in breast cancer by Graff et al. (1995).

1.4.2.5 Maspin gene

The maspin gene is a member of the serpin protease inhibitors family that shows tumor suppressor-like activity by inhibiting motility, invasion and metastasis of breast cancer cells (Zou et al., 1994; Seftor et al., 1998). Loss of maspin expression is a frequent event in breast cancer and it is usually associated to increased invasiveness and metastasis. Nevertheless, detection of chromosomal deletions or mutations in this gene has not been observed (Zou et al., 1994). Recently, Domann et al. (2000) have shown that the promoter region of the maspin gene is aberrantly methylated and not expressed in several in human breast cancer cell lines.

1.4.2.6 Estrogen Receptor (ER)

Growth of breast cancer cells expressing the ER gene can be regulated by estrogens (Fisher et al. 1993). Estrogen can bind to its receptor producing a conformational change that permits binding to the estrogen responsive elements (EREs) to modulate the transcription of specific genes. Approximately two-thirds of breast cancers express the ER gene at diagnosis. ER positive breast cancers respond to therapy with anti-estrogens such as Tamoxifen. Response of ER negative breast cancers to hormonal therapy is rare (Johnston et al. 1995).

The ER gene is located on chromosome 6q. There is no evidence of homozygous deletions or other significant alterations of the region surrounding the ER gene that would explain the loss of ER expression in ER negative breast cancers (Lapidus et al., 1998). Ottaviano et al. (1994) showed extensive methylation of the ER promoter region in ER negative breast cancers. Reactivation of ER expression in human breast cancer cell lines by treatment with the demethylating agent 5-AZA-CdR (Ferguson et al. 1995) lead to the hypothesis that some hormone unresponsive breast cancers may become sensitive to anti-estrogens after treatment with 5-Aza-CdR.

1.4.2.7 Mammary-derived Growth Inhibitor (MDGI)

The mammary-derived growth inhibitor (MDGI) is a member of a family of lipophilic intracellular proteins. The human MDGI gene maps on chromosome 1p35, a locus that was identified as a common site of LOH in primary human breast cancer (Bieche et al, 1993). The MDGI protein was shown to inhibit *in vitro* growth of breast carcinoma cell lines (Huynh et al. 1995). Transfection of MDGI into breast cancer cell lines reduces their clonogenicity, proliferative rate and tumorigenicity in nude mice. All these results suggested that MDGI act as a tumor suppressor gene. Mutations in this gene have not been detected (Phelan et al. 1996), but hypermethylation of its promoter region was shown and reactivation of its expression by 5-Aza-CdR was observed in some breast carcinoma cell lines (Huynh et al. 1996).

1.4.2.8 Retinoic Acid Receptor β (RARβ)

Retinoic acid receptors (RARs) act as nuclear transcription factors which can bind retinoic acid response elements (RAREs) on DNA to activate transcription of genes involved in differentiation of epithelial cells. The expression of RARβ is restricted to epithelial cells and it is induced by RA. Normal breast cells express RARβ (Swisshelm et al., 1994; Widschwendter et al., 1997). However, most breast cancer cell lines (Swisshelm et al., 1994: Seewaldt et al., 1995) and primary breast tumors do not express this gene (Widschwendter et al., Xu et al., 1997)

Senescing normal mammary epithelial cells show upregulated expression of RARβ (Swisshelm et al., 1994), which raises the possibility that RARβ may be involved in regulation of cell proliferation. RARβ gene is located on chromosome 3p24, a site that shows frequent LOH in breast cancer (Deng et al. 1996). Evidence that RARβ is a tumor suppressor gene also includes the reports that transfection of RARβ cDNA into some tumor cells induced terminal differentiation (Caliaro et al. 1994) and reduced their tumorigenicity in nude mice (Houle et al, 1993). This gene is described in more details in Chapter 4.

1.4.2.9 Glutathione S-Transferase (GST)

GSTs are a family of enzymes involved in the detoxification of genotoxic compounds and alkylating agents. Drug resistance to alkylating agents in tumors may be due to in some cases to increased expression of GST (Batist et al., 1986). The class of GSTP1 has been reported to be silenced by aberrant methylation of its promoter in 44% of breast cancer cell lines and in 30% of the primary breast tumors evaluated (Esteller et al., 1998).

1.4.2.10 Hypermethylated in Cancer (HIC)

HIC, a candidate tumor suppressor gene, is located on chromosome 17p13.3, a locus that undergoes frequent loss of heterozygosity in breast cancer. HIC encodes for a protein containing a zinc finger motif with an unknown function, but possibly involved in the regulation of transcription. Loss of expression due to extensive hypermethylation of the promoter region of HIC was observed in 67% of primary breast cancer specimens (Fujii et al. 1997, Ahuja et al. 1997).

1.4.2.11 Gelsolin

Gelsolin is a protein which binds to and regulates actin filament assembly and disassembly (Wang et al., 1997). The actin filament cytoskeleton is essential for normal cellular functions and is often deranged in tumor tissue. (Higley and Way, 1997). Gelsolin expression is markedly reduced in most human, murine and rat breast cancers (Asch et al., 1996). The gelsolin gene maps to human chromosome 9p33 and LOH has been described for this locus in human bladder cancers (Orlow et al., 1994), but not in breast cancers (Asch et al., 1996). The promoter region of the gelsolin gene is found within a CpG island. Altered methylation of gelsolin has been observed in human breast cancer cells, but not always correlated with down-regulation of its expression (Mielnicki et al., 1999). Epigenetic changes in appear to be involved in silencing of this gene in breast cancer.

1.4.2.12 Death Associated Protein Kinase (DAP-kinase) gene

DAP kinase, a protein that acts as a positive mediator of programmed cell death, was found to be downregulated in many types of cancer cell lines and primary tumors (Kissil et al. 1997). Many studies indicate that suppression of cell death can be one of the critical events in tumorigenesis. DAP-kinase gene maps to chromosome 9q34 (Feinstein et al., 1995), a locus that is frequently rearranged in human malignancies. Reactivation of expression of DAP-kinase in various cell lines after

exposure to 5-AZA-CdR, suggests that methylation can also be involved in the silencing of this gene (Kissil et al., 1997; Inbal et al., 1997).

1.4.2.13 14-3-3 σ gene

The 14-3-3 σ gene was originally identified as a gene inducible in response to DNA damage (Hermeking et al., 1997). The 14-3-3 σ gene product induces cell growth arrest in the G2 phase of the cell cycle which allows repair of damaged DNA (Chan et al., 1999). This gene has been found downregulated in 94% of breast tumors by Ferguson et al. (2000). Reactivation of expression by 5-AZA-CdR and very low frequency of mutations or LOH in this gene have supported the idea that hypermethylation could be a major cause of silencing of the 14-3-3 σ gene. Methylation analysis of the CpG island confirmed the presence of aberrant methylation in the majority of breast carcinoma cell lines and the primary tissues analyzed (Ferguson et al., 2000).

1.5 Therapy of breast cancer

Once breast cancer is diagnosed, the clinical stage of the disease needs to be determined. Carcinoma of the breast is staged according to the size and characteristics of the primary tumor, the involvement of regional lymph nodes and the presence of metastatic disease.

1.5.1 Surgery and Radiation therapy

Nearly all women with breast cancer will have some type of surgery. Lumpectomy is preferred today rather than total mastectomy in early breast cancers where the tumor mass is still confined. In almost all cases, 6 to 7 weeks of radiation therapy follows lumpectomy. Radiation can shrink a tumor before surgery, or destroy cancer cells left behind in the breast, chest wall, or armpit after surgery. This combination of lumpectomy and radiation is referred to as combination breast

conserving therapy and it is an option for most, but not all, women with breast cancer. After lumpectomy or mastectomy, additional surgery might be required to assess if surrounding lymph nodes contain malignant cells.

1.5.2 Adjuvant Therapy

Adjuvant therapy is administered to eradicate tumor cells that may have metastasized beyond the breast to other tissues. Examples of adjuvant treatments include chemotherapy and hormonal therapy. The goal of adjuvant therapy is to kill microscopic cells that were not eliminated by surgery or radiation therapy. Even in the early stages of the disease malignant cells can break away from the primary breast tumor, spread through the bloodstream and establish new tumors in other organs.

1.5.2.1 Chemotherapy

Chemotherapy is administered in cycles, with each period of treatment followed by an interval to allow the patient to recover from the major side effects of the drug treatment. Combination chemotherapy has proven more effective than a single drug since drug combinations reduce the frequency of drug resistance.

The most frequently used drug combinations for breast cancer are:

- Cyclophosphamide, methotrexate, and fluorouracil (CMF)
- Cyclophosphamide, doxorubicin (Adriamycin), and fluorouracil (CAF);
- Doxorubicin (Adriamycin) and cyclophosphamide (AC) with or without paclitaxel (Taxol);
- Doxorubicin (Adriamycin) followed by CMF.

The side effects of chemotherapy depend on the type of drugs used, the dose, and the length of treatment. The major side effect of chemotherapy is myelosuppression. Growth factors can accelerate bone marrow recovery after chemotherapy and can reduce the incidence of infections due to leukopenia.

Another approach that has been used to treat patients with advanced metastatic breast cancer is high dose chemotherapy plus hematopoietic stem cell

rescue. However, in a preliminary randomized trial when this experimental therapy was compared to conventional-dose chemotherapy, no significant differences were observed in the survival of these patients (Stadtmauer et al., 2000).

1.5.2.2 Hormone Therapy

One approach to block the growth-stimulatory effects of estrogen on mammary tissue is the use of tamoxifen, an antiestrogen. Tamoxifen acts by binding to the ER and thus blocking the interaction of estrogen with this receptor. Studies show that tamoxifen can reduce tumor progression in patients with breast cancer. However, a slight increase of incidence of endometrial cancer can occur in women taking tamoxifen. This effect can be avoided by using other antiestrogens, such as raloxifene, which does not show estrogen-like effect in endometrial tissue. Short-term side effects of antiestrogens may include thrombosis, hot flashes, mood swings, and cataracts.

1.5.3 New approaches in therapy of breast cancer

Investigation of genetic changes in breast cancer can lead to development of new approaches for early detection and therapy. Development of rapid and accurate methods for characterization of oncogenes and tumor suppressor genes status would be a very useful tool to design new therapies for breast cancer. For example, detection of the HER2 oncoprotein has been predictive of poor prognosis tumors. Based on these observations, the antibody to HER2, Herceptin, is being used in the therapy of breast cancers that overexpress this gene.

The observation that aberrant methylation of promoter regions of tumor suppressor genes is responsible for silencing of their expression in breast cancer provides a rationale to use inhibitors of DNA methylation as potential chemotherapeutic agents for this type of cancer. The analysis of the DNA methylation status of tumor suppressor genes could be used to screen tumor biopsies to select patients that will respond to therapy with DNA methylation inhibitors. The

ultimate goal would be to provide the rationale for cancer therapies determined by patients' genetic and epigenetic profiles.

Table 1.1 Genes silenced by DNA Methylation in breast cancer.

Gene	Function	Reference
p16	(TSG, cyclin kinase inhibitor)	Otterson et al. (1995)
Retinoic acid receptor β	(TSG, transcription factor)	Côté & Momparler(1997)
Mammary-derived growth inhibitor	(TSG, growth inhibitor)	Huynh et al. (1996)
BRCA-1	(DNA repair)	Dobrovic &Simpfendorfer(1997)
		Rice et al.(1998)
Estrogen receptor	(transcription factor)	Ferguson et al., (1995)
E-Cadherin	(invasion suppression)	Graff et al. (1995)
Maspin	(invasion suppressor, protease inhibitor)	Domann et al. (2000)
Glutathione S-transferase	(inactivates carcinogens)	Esteller et al. (1998)
Hypermethylated in Cancer	(unknown)	Ahuja et al. (1997)
Gelsolin	(inhibits metastasis?, actin-binding protein)	Mielnicki et al. (1999)
Death-Associated Protein kinase	(induces apoptosis)	Kissil et al. (1997)
14-3-3 σ	(allows DNA repair)	Ferguson et al. (2000)

TSG, tumor suppressor gene

CHAPTER 2: DNA METHYLATION

2.1 Introduction

Methylation of cytosine residues in DNA is an epigenetic change that has been shown to play an important role in control of gene expression. In mammalian cells, approximately 3 to 5% of cytosine residues in the genomic DNA are found as 5-methylcytosine (Ehrlich et al., 1992). Since the human genome contains about 6 x 10^9 base pairs, the estimated number of 5-methylcytosine molecules is about 10^8. Methylation of cytosines takes place immediately after DNA replication, and is catalyzed by DNA methyltransferase, using S-adenosyl-methionine as the methyl donor, as shown in **Fig. 2.1**.

2.2 Functions of DNA methylation

DNA methylation is involved in many cellular processes, such as embryonic development (Kafri et al., 1992), cell differentiation (Kochanek et al. 1990), genomic imprinting, inactivation of the X chromosome (Xi) (Riggs and Pfeifer, 1992), senescence (Cooney, 1993), and carcinogenesis. Inactivation of viral DNA that has integrated into the host mammalian genome by methylation illustrates its function as a protective mechanism against infective agents (Schaefer et al., 1997). Each of these areas are reviewed below with the major focus on the role of DNA methylation in carcinogenesis.

2.2.1 Differentiation

DNA methylation plays a key role in cellular differentiation. Methylation patterns are characteristic of each cell type and are highly conserved (Kochanek et al., 1990). During cellular differentiation, genes specific for a tissue undergo demethylation and subsequent activation to produce a specific phenotype. Several studies have shown that treatment of undifferentiated cells with hypomethylating agents, such as 5-AZA-CdR, can induce differentiation to a specific phenotype. An example of these phenomena is the work of Jones and Taylor (1980) who showed that

embryonic mouse fibroblasts could be converted into myoblasts, adipocytes or condrocytes when treated with this agent.

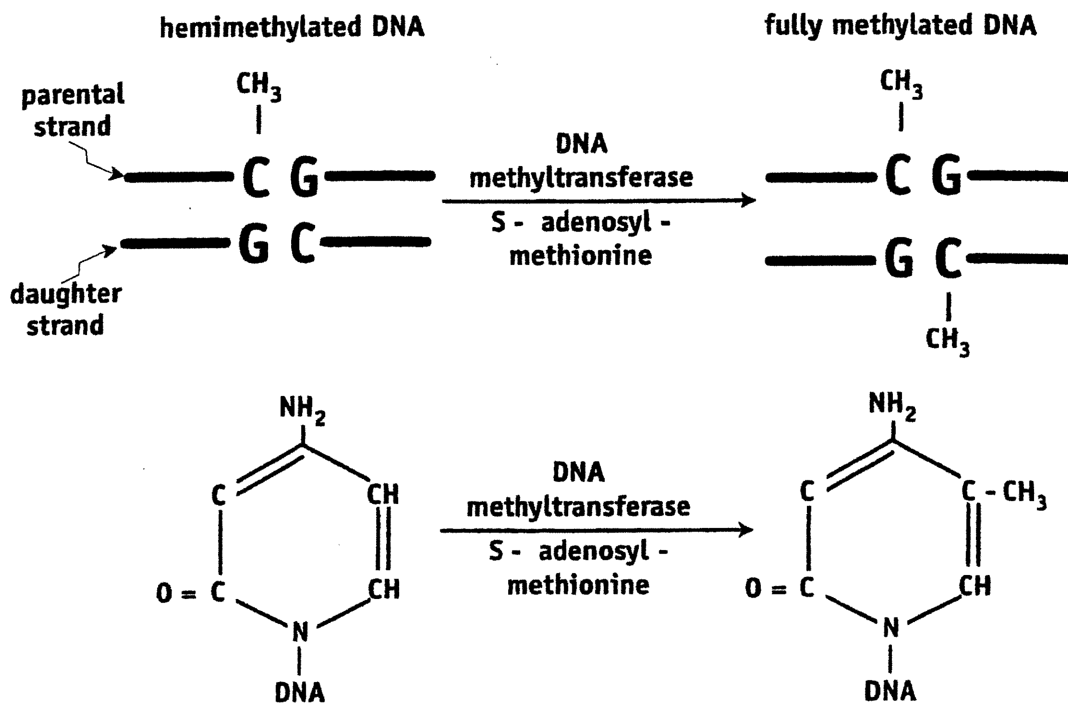

Fig. 2.1 Representation of the DNA methylation reaction by the DNA methyltransferase. After DNA replication, the daughter strand is methylated in order to maintain the methylation pattern characteristic of the cell (Top). S-adenosyl-methionine is the methyl donor.The structures of cytosine and 5-methylcytosine are shown (Bottom).

2.2.2 Embryonic development

DNA methylation is also involved in embryonic development (Kafri et al., 1992). After fertilization, during preimplantation development, genome-wide demethylation takes place, so that blastocysts are mostly non-methylated. Remethylation of the genome occurs rapidly after implantation resulting in a sequential activation of specific classes of genes that regulate embryonic development.

2.2.3 Genomic imprinting

Genomic imprinting is the phenomenon that determines the monoallelic gene expression from either the maternal or paternal chromosome. Only a small number of genes are imprinted. The existence of genomic imprinting imparts a heritable signal that can distinguish the parental origin of an active allele. The imprint must occur while the maternal and paternal chromosomes are still separated, either as separate gametes or at the zygote stage before karyogamy. After karyogamy it is not possible to mark parental specific chromosomes since any epigenetic change would be imparted to both alleles. Razin and Cedar (1994) showed that some parental-specific methylation can be inherited from gametes and is retained during preimplantation development, and may constitute imprints. An example of maternally imprinted gene is the insulin-like growth factor II (IGF-II) which encodes for a mitogenic protein that plays an important role in normal fetal growth and development.

An imprinted gene can be re-expressed after exposure to hypomethylating agents like 5-AZA-CdR. For example, 5-AZA-CdR was demonstrated to reactivate the hypoxanthine-guanine phosphoribosyltransferase gene on an inactive X-chromosome (Jones et al., 1982).

2.2.4 Inactivation of foreign DNA

Methylation can also function as a defense mechanism against foreign DNA. This would imply inactivation of foreign sequences that have integrated into the host genome by their methylation. In fact foreign DNA, such as virus sequences, or repetitive sequences like Alu are found to be highly methylated in DNA (Doerfler et al., 1995). After their integration in the genome, viral sequences are gradually methylated, which leads to silencing of their expression. This can be a problem in human gene therapy where retroviral gene transfer results in the integration of a therapeutic transgene in the host genome and its expression can be silenced by methylation (Challita and Kohn, 1994, Hoeben et al., 1991).

2.2.5 Carcinogenesis

Oncogenes and tumor suppressor genes play major roles in tumorigenesis. Oncogenes can result from any member of a given signal transduction pathway. Over-expression of oncogenes can contribute to signal-independent activation of the cascade, leading to the initiation or maintenance of the malignant phenotype, by allowing uncontrolled proliferation of the cells. Tumor suppressor genes have the normal function to suppress cell proliferation. Loss of function of these genes through mutation, deletion or methylation contributes to tumorigenesis.

Alteration of methylation patterns in cancer has been widely described (Baylin et al., 1998, Counts and Goodman 1995, Jones 1996). In cancer cells, genome-wide hypomethylation coexists with regional hypermethylation. Attempts to solve this methylation paradox have led to analysis of the role of DNA methyltransferase in the disruption of normal methylation patterns.

2.3 DNA methyltransferases (DMTases)

2.3.1. DMTase1

DMTase1 was the first methylating enzyme to be identified and cloned in mammalian cells (Bestor et al., 1988). This enzyme uses hemimethylated DNA as a template (Bouchard and Momparler, 1983). It is believed that DMTase1 acts as a maintenance DNA methyltransferase whose function is to preserve the methylation patterns of differentiated cells (Tucker and Bestor 1997). After DNA replication the newly formed daughter strand is not methylated. DMTase1 uses the parental DNA strand to identify the positions of 5-methylcytosine and to catalyze the methylation of the complementary cytosine on the daughter strand so as to maintain the methylation pattern (see **Fig. 2.1**). DMTase1 is a large enzyme (193.5 kDa) composed of a C-terminal catalytic domain and a large N-terminal regulatory domain (Chuang et al., 1997).

DMTase1 deficient mice show abnormal imprinting, reduced DNA methylation levels, and derepression of endogenous retrovirus (Li et al., 1993, Li et al., 1992, Walsh et al., 1998). The observation that DMTase1 -/- embryonic stem cells were viable and still showed the ability to de novo methylate DNA suggested the existence of other methyltransferases with this function (Lei et al., 1996).

2.3.2 DMTase 2, 3a, 3b

Recently, three more DNA methyltransferases have been cloned from mammalian cells: DMTase 2, DMTase 3a, and DMTase 3b. DMTase 2 appears to be responsible for the methylation of integrated viral sequences (Yoder and Bestor, 1998). Since DMTase 3a and 3b can methylate hemimethylated and unmethylated sequences with the same efficiencies, they are potential good candidates for the de novo methyltransferase (Xie et al., 1999).

2.4 DNA methylation and cancer

2.4.1 Increased DMTase activity in neoplastic cells

One event that may be involved in the DNA methylation imbalance, a characteristic of cancer cells, is an apparent increase in DMTase activity. Kautiainen and Jones (1986) found a multifold increase in levels of DNA methyltransferase activity in tumor cells as compared to the normal counterparts. These findings were confirmed by El-Deiry and coworkers (1991) who found that DMTase gene mRNA levels were significantly higher in neoplastic cells than in normal cells. In their study El-Deiry and coworkers also found that DMTase gene expression gradually increased in parallel with colon cancer progression (El-Deiry et al., 1991).

The significance of overexpression of DMTase1 in tumorigenesis is controversial. Since DMTase activity is correlated with DNA synthesis and cell cycle activity, some authors have considered the higher number of proliferating cells as a possible explanation for differences between neoplastic and normal cells (Lee et al., 1996). However, this is very unlikely to be the case, since some cancers have a proliferative index are only marginally higher than normal tissue, yet significant increase in DMTase activity is observed (El-Deiry et al., 1991; Issa et al., 1993). In a recent study, Robertson et al (1999) detected significant overexpression of DMTase3b, even when the proliferative status of the tumor was taken into account. This raises the question that the putative de novo methylase might be responsible of aberrant methylation characteristic of transformed cells.

2.4.2 DNA Hypomethylation and Cancer

Cancer cells can be also characterized by a general hypomethylation of DNA (Gama-Sosa et al., 1983; Feinberg et al., 1998), which coexists with hypermethylation of CpG rich regions. The resolution of this methylation paradox is essential for understanding of the role of methylation in carcinogenesis. Levels of DMTase are

usually increased in cancer cells (Issa et al., 1993; Ottaviano et al., 1994), but the implications of this increase on tumorigenesis are not clear.

What are the mechanisms by which CpG islands become preferentially hypermethylated? It has been widely shown that short repetitive sequences in eukaryotic DNA can form binding sites for DMTase (Tuker et al., 1997; Bestor, T.H. and Tycko, 1996; Yoder et al., 1997). Examples of these repetitive DNA sequences include satellite DNA, microsatellite DNA, Alu repeats, and imprinted genes containing repeats, etc. All these sequences tend to be targets for de novo methylation and transcriptional repression. Also, short inverted repeats are capable of forming foldback structures such as hairpins or loops containing single stranded DNA which can have a high affinity for binding of DMTase.

Based on these observations, Jill (1998) hypothesized that CpG palindrome asymmetry might provide a recognition site for DMTases for both maintenance and de novo methylation. In normal cells, DMTase1 recognizes the methyl-directed asymmetry as a signal for maintenance methylation at the replication fork. This would guarantee the maintenance of methylation patterns characteristic of different cell types.

In cancer cells, DNA damage might cause asymmetry due to abasic sites or mismatches caused by point mutations. These genetic lesions may create preferential binding sites for DMTase in tumor cells. In this situation, an increase of binding sites would deplete the number of DMTase molecules available for normal maintenance methylation. This would reduce the availability of the enzyme at the replication fork which can be one explanation for genome-wide hypomethylation typical of cancer cells. This hypothesis, even though very provocative, needs to be viewed in the light of the recent discovery of several DMTase which possess separate maintenance and/or de novo methylation activities (Robertson et al., 1999).

Recently, Bhattacharya et al. (1999) identified demethylase activity in mammalian cells. In the light of this finding, another possible explanation for genome-spread demethylation in cancer might be due to over-expression of demethylase activity.

2.4.3 5-Methylcytosine and Mutations

5-methylcytosine can undergo spontaneous deamination to form thymine at a rate much higher that the deamination of cytosine to uracil (Shen et al., 1994). If the deamination of 5-methylcytosine is unrepaired, it will result in a C to T transition mutation. Also the T:G mismatches are more difficult to recognize than the U:G mismatch because thymine is a natural base of DNA while uracil is not. These phenomena were used to explain the high incidence of CpG to TpG transition mutations observed in the p53 tumor suppressor gene (Rideout et al., 1990). In fact, in many cancers, about 25% of point mutations in p53 gene were at CpG sites. However, these phenomena may be more complex and may involve additional events (Schmutte and Jones, 1998). For example, the carcinogen, benzopyrene, was observed to preferentially form adducts at the methylated CpG sites of the p53 gene; these sites are hot spots for mutation, which might explain the high frequency of mutation at these sites in cancer (Denissenko et al., 1997). In addition, the accessibility of the CpG sites may be influenced by the chromatin structure of DNA. A more compact chromatin would make the DNA less accessible to carcinogen attack.

2.4.4 CpG islands

The big majority of 5-methylcytosines are found in the CpG dinucleotides (Bird, 1986). The distribution of CpG in the genome is not random and the majority of the genome is CpG poor (Cooper and Krawczak, 1989). Certain regions of the genome that possess a high frequency of CpG are known as CpG islands. CpG islands are associated with the promoter region of genes and for most housekeeping genes are usually unmethylated. About 60 to 70% of genes have CpG islands. It was estimated that there are about 45,000 CpG islands in the human genome (Costello et al., 2000).

What normally protects CpG islands from methylation? Several studies indicate that Sp1-binding motifs present in most CpG islands (Antequera and Bird,

1993), protect them from methylation (Brandeis et al 1994; Macleod et al 1994; Mummaneni et al. 1995).

In a study comparing CpG islands of different tumor suppressor genes, Graff et al (1997) observed that all the genes they studied had 2 Alu repeats located within 1kb 5' of the CpG island and another Alu repeat within 500 bp 3' of it. They also noticed that at both the 5' and 3' ends of the island, where the methylation density started to decrease, there were multiple Sp1 sites. The Alu sequences revealed to be methylated even in normal cells. The CpG island within Sp-1 sites were free of methylation in normal tissue, but not in tumors which do not express the gene. The Sp1 sites are located at both the 5' and 3' ends of the CpG island, in strategic positions to potentially protect the island from methylation spreading from the flanking regions. The presence of sharp boundaries that separate and protect unmethylated regions from the regions that are heavily methylated is intriguing in the view of their derangement during tumorigenesis.

2.5 Regulation of gene expression

Aberrant methylation of cytosine residues in CpG islands in promoter regions of tumor suppressor genes has been associated to their loss of expression (**Fig. 2.2**). Methylation of CpG sequences in the exons of genes appears to play a minimal role in the control of gene expression (Bender et al., 1999).

Two mechanisms have been described to explain how DNA methylation can inhibit transcription. The first mechanism is a direct inhibition due to the fact that 5-methycytosine residues protrude into the major groove of the DNA helix (Tate and Bird, 1993). The modified cytosine might interfere with the binding of the transcription factors to the promoter, preventing gene expression (Kass et al., 1997). It has been reported that transcription factors bind with less affinity to methylated target sequences (Iguchi-Ariga and Schaffner, 1989; Mancini et al., 1998).

The second mechanism implicates an indirect effect of 5-methylcytosines on repression of transcription that is mediated by proteins that recognize and bind methylated sequences. Recently, two families of such proteins are known: the family

of sequence specific-methylated DNA binding proteins, such as MDBP-1, MBDP-2, and methylated CpG binding proteins such as MeCP1 and MeCP2.

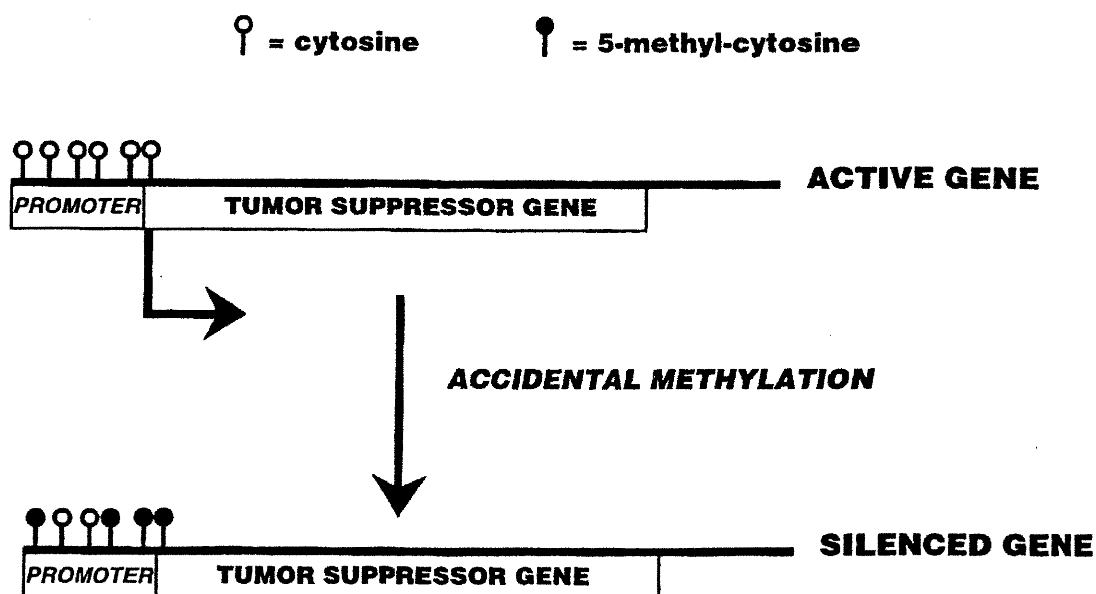

Fig. 2.2 Mechanism of silencing of tumor suppressor genes by accidental hypermethylation. Promoter regions of genes contain CpG sequences which can undergo accidental methylation during tumorigenesis resulting in gene silencing. The open lollipops represent cytosine residues; the closed ones represent 5-methylcytosines.

2.5.1 Aberrant DNA methylation of cancer-related genes.

The mammalian cell, if unchecked, has enormous growth potential. One cell with a doubling time of 24 hours can potentially form a mass of one kilogram (10^{12} cells) in only about 40 days. It is not surprising that cells contain many genes that can suppress this growth potential. The presence of two copies of these suppressor genes, one on the paternal and the other on the maternal chromosome, reduces the risk of unregulated growth due to the inactivation of a single gene copy. Knudsen (1971) in his analysis of the familial incidence of retinoblastoma (Rb) proposed that both alleles of the this tumor suppressor gene have to be inactivated to give rise to the malignant phenotype. This has been referred to as the "two hit" hypothesis of tumor suppressor gene inactivation.

Initially, point mutations and chromosomal deletions were considered to be the major events involved in the inactivation of tumor suppressor genes. The discovery that many tumor suppressor genes can be also inactivated by aberrant methylation of the CpG islands in their promoter region clearly indicates that epigenetic events also play a very important role in tumorigenesis (Jones and Laird, 1999). A model for the inactivation of both alleles of tumor suppressor genes by aberrant DNA methylation alone or in combination with mutations or deletions is shown in **Fig.2.3**. This model is supported by the reports that some cancer-related genes were found to be completely inactivated by biallelic methylation of CpG sequences (Batova et al., 1997; Veigl et al., 1998).

The molecular mechanisms by which the aberrant methylation of DNA takes place during tumorigenesis are still not clear. It is possible that the DMTase can make mistakes by methylating CpG islands in the nascent strand of DNA without a complementary methylated CpG in the parental strand. It is also possible that aberrant methylation may be due to the removal of CpG binding proteins that "protect" these sites from being methylated. Whatever the mechanism, the frequency of aberrant methylation is probably a rare event in mammalian cells.

Tumor-suppressor and other cancer-related genes that have been found to be hypermethylated in human cancer cell lines and in primary tumors are summarized in

Table 2.1. The genes that were activated in tumor cell lines by 5-AZA-CdR are also indicated. The different classes of genes that are silenced by DNA methylation include: tumor suppressor genes like p15 (Herman et al. 1996), p16 (Otterson et al 1994, Herman et al. 1995), RARβ (Côté and Momparler, 1997), retinoblastoma gene (Ohtani-Fujita et al. 1993); genes that suppress tumor invasion and metastasis like E-cadherin (Graff et al., 1995), DNA repair genes like hMLH1 (Kane et al., 1997), BRCA1 (Mancini et al., 1998) and MGMT (Qian and Brent, 1997); genes that suppress cell death such as DAP-kinase (Kissil et al., 1997), genes for hormone receptors, and genes that inhibit angiogenesis.

There are most likely many other cancer-related genes that are involved in tumorigenesis and are also silenced by methylation and still remain to be identified. For example, Costello et al. (2000), using restriction landmark genomic scanning, estimated an average of 600 CpG islands that were aberrantly methylated out of a total of 45,000 in 98 primary tumors studied as compared the normal corresponding tissue.

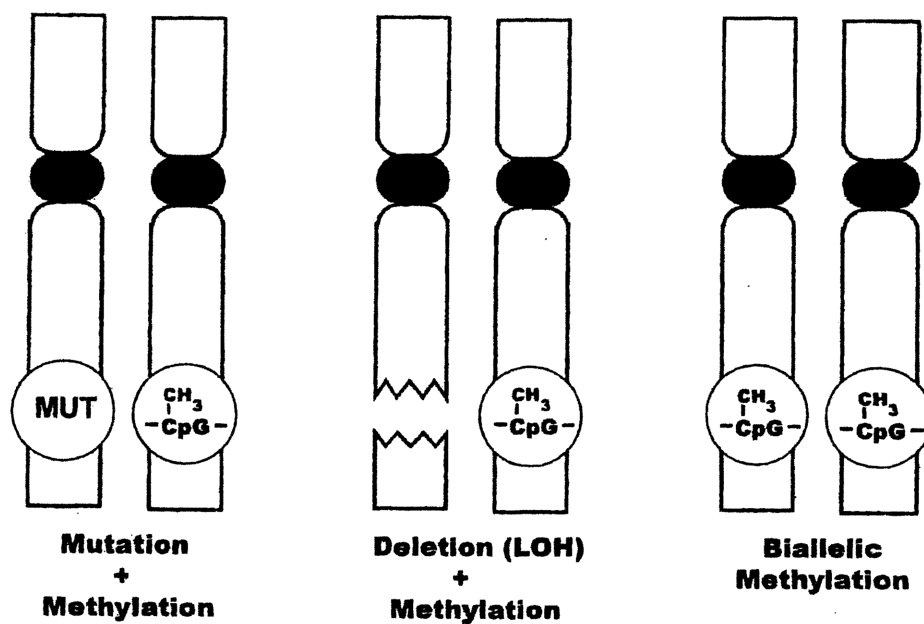

Fig. 2.3 Model for biallelic silencing of tumor suppressor genes by aberrant methylation with or without mutation or chromosomal deletion. Biallelic gene inactivation by mutation and/or deletion only are not shown. MUT, mutation; CH_3-CpG indicates methylation; broken line indicates deletion.

TABLE 2.1 Genes silenced by aberrant DNA Methylation and Activated by 5-Azadeoxycytidine (5-AZA-CdR) in human tumor cell lines

Gene	Activation 5-AZA-CdR	Reference
Tumor suppressor		
p15 INK4B (cyclin kinase inhibitor)	+	Herman et al. ,1996b
p16 INK4A (cyclin kinase inhibitor)	+	Otterson et al. ,1995; Merlo et al., 1995
p73 (p53 homology)	+	Corn et al. ,1999
ARF/INK4A (regulate level p53)	+	Robertson & Jones, 1998
Wilms tumor	+	Laux et al., 1997
von Hippel Lindau (VHL)	+	Herman et al. ,1994
retinoic acid receptor ß (RARß)	+	Côté & Momparler ,1997; Côté et al. ,1998
estrogen receptor	+	Ferguson et al., 1995
androgen receptor	+	Jarrard et al., 1998
mammary-derived growth inhibitor	+	Huynh et al. ,1996
Hypermethylated in cancer (HIC1)	nd	Ahuja et al.,1997
retinoblastoma (Rb)	nd	Ohtani-Fujita et al., 1993
14-3-3 σ	+	Ferguson et al. 2000
Inducer of apoptosis		
Death Associated Protein (DAP) kinase	+	Kissil et al., 1997
Invasion/metastasis Suppressor		
E-Cadherin	+	Graff et al. ,1995
Tissue inhibitor metalloproteinase-3 (TIMP-3)	+	Bachman et al., 1999
mts-1	+	Tulchinsky et al., 1995
CD-44	+	Verkaik et al., 1999
Maspin	+	Domann et al., 2000
Gelsolin	-	Mielnicki et al., 1999
DNA repair/detoxify carcinogens		
methylguanine methyltransferase	+	Qian & Brent ,1997
hMLH1 (mismatch DNA repair)	+	Herman et al. ,1998; Deng et al., 1999
glutathione S-transferase	nd	Esteller et al., 1998; Millar et al., 1999
BRCA-1	nd	Dobrovic & Simpfendorfer,1997; Mancini et al., 1998
Angiogenesis inhibitor		
thrombospondin-1 (TSP-1)	+	Li et al., 1999
TIMP-3	+	Bachman et al., 1999
Tumor antigen		
MAGE-1	+	Weber et al., 1994; Coral et al., 1999

nd, not done

2.6 Methods for the evaluation of DNA methylation

A variety of methods are used to evaluate the methylation status of genes: Southern blot analysis, bisulfite genomic DNA sequencing, restriction enzyme-PCR, methylation sensitive PCR (MSP) and the methylation-sensitive single nucleotide primer extension (MS-SNuPE). A report on the comparison of some of these methods has been published (Gonzalgo et al., 1997). A brief description of each of these methods is given below.

2.6.1 Southern blot

Southern blot is the most frequently used method for DNA methylation analysis. In this method the genomic DNA is cleaved with methylation-sensitive and insensitive endonucleases specific for the same sequence, such as Hpa II and Msp I. An analysis of the target sequence for potential methylated CpG sites should be performed to allow proper selection of the suitable restriction enzymes. The restriction fragments are then separated on an agarose gel, transferred to a membrane and hybridized with a probe specific for the target sequence. Autoradiography will reveal the presence of bands of the predicted size that will allow to assess the methylation status of the target sequence. The limitations of this technique are: a) the amount of DNA required for the Southern blot analysis (5-10 μg per sample) which becomes prohibitive if the tumor sample is small; b) possibility of incomplete enzymatic digestion can lead to results that are difficult to interpret.

2.6.2 Restriction enzyme PCR

Genomic DNA is cleaved with methylation-sensitive and -insensitive restriction enzymes, and the digested DNA is then amplified using primers flanking the target region. Using a methylation-sensitive endonuclease it should be possible to amplify DNA of a predicted size if the target sequence contains methylated CpG sites. With the use of a methylation-insensitive endonuclease prior to PCR no

amplification will be possible regardless of the methylation status of the target DNA sequence. The major limitation of this method is that the enzyme digestion must be complete. Another limitation is that if several CpG sites are present in the target region, some methylated and others not methylated, the results obtained will be inconclusive. This technique is nevertheless very helpful as a primary screening of DNA methylation in a target sequence (Kane et al., 1997).

2.6.3 Bisulfite DNA sequencing

The bisulfite method for sequencing DNA containing 5-methylcytosine has led to the development of new techniques to analyze the methylation status of genes (Grigg and Clark, 1994). Bisulfite genomic sequencing was first described by Frommer et al (1992). This technique provides an excellent tool for the detection of single 5-methylcytosine residues in genomic DNA. The method is based on the ability of sodium bisulfite to deaminate all the cytosines to uracil in single stranded DNA, while leaving the 5-methylcytosines unchanged (Hayatsu et al 1970). The reactions involved in the conversion of 5-methylcytosine to uracil are shown in **Fig 2.4**. The reaction involves a first step of sulphonation of the cytosine residue in position 6 with formation of cytosine sulphonate. This step is followed by hydrolytic deamination to uracil sulphonate. A desulphonation step will then lead to conversion to uracil. The modified DNA is then amplified by PCR, using a set of primers specific for the converted sequence; these primers will amplify a fragment in which all the uracil residues (converted cytosines) are amplified as thymine (T) and only the 5-methylcytosines remain as C. The amplified fragments can then be sequenced directly or after cloning of individual molecules. The cloning strategy is very useful for the analysis of the methylation differences between alleles (Clark et al., 1994, Clark et al 1995). It is very important to verify if the bisulfite conversion is complete, since it has been reported that C adjacent to methylated CpG sites can be resistant to bisulfite treatment (Harrison et al, 1998).

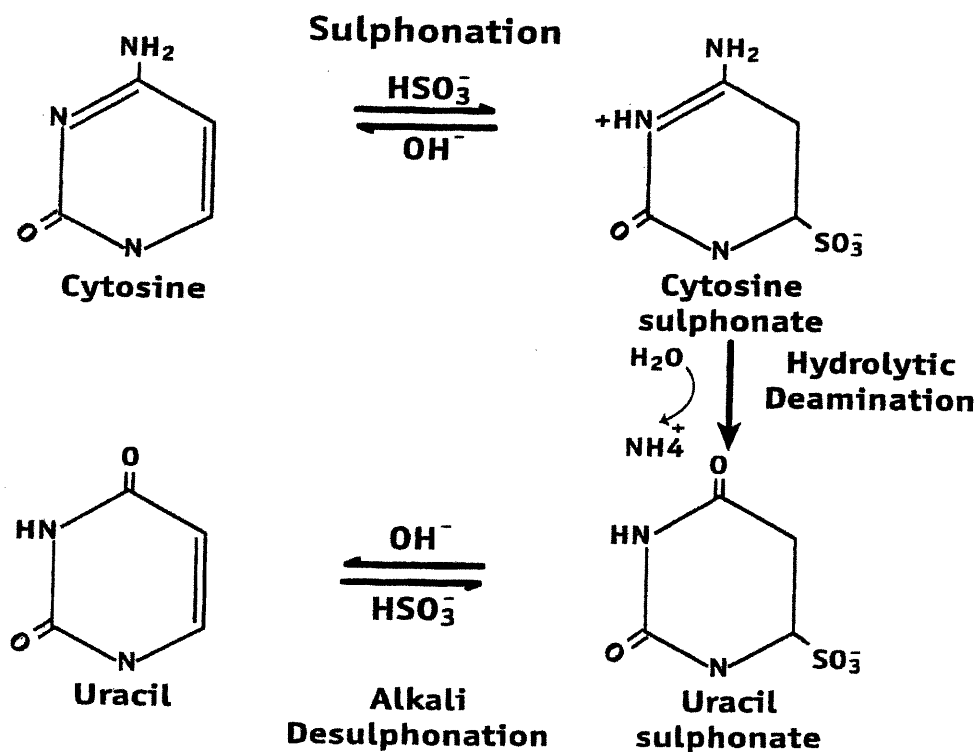

Fig. 2.4 Schematic representation of the sodium bisulfite modification of DNA leading to hydrolytic deamination of cytosine residues to uracil. In the presence of sodium bisulfite cytosine is sulphonated to form cytosine sulphonate. Hydrolitic deamination will lead to formation of uracil sulphonate. Removal of the HSO_3^- leads to formation of uracil.

2.6.4 Methylation Sensitive PCR (MSP)

MSP is a technique that was first described by Herman et al. (1996). It takes advantage of the sequence difference existing between methylated and unmethylated DNA after bisulfite treatment. Cytosines are deaminated to uracil, which replicates as thymine (T) during PCR, allowing the design of primers that will specifically anneal to sequences that contain either methylated (C) or modified (U) sites, as shown in detail in **Fig.2.5**. Following PCR, amplified DNA will be obtained with the pair of primers specific for unmethylated or methylated sequences (Herman et al., 1996). MSP is a rapid and qualitative method for the analysis of presence of methylation in a given region of DNA. Careful selection of primers is very important since it is possible to obtain false positives with both methylated and unmethylated primer pairs making it difficult to interpret the results. The method of selection of primers for the MSP analysis of RAR β is shown in Appendix II. The sequence of the region where the primers were designed is also shown in Appendix II. Incomplete bisulfite modification of genomic DNA will also give false positives for methylated C. Another approach that can be used is to determine if the PCR product is sensitive to digestion by restriction enzymes that will only digest the DNA if their recognition sequence was methylated prior to treatment with bisulfite (Li et al., 1999).

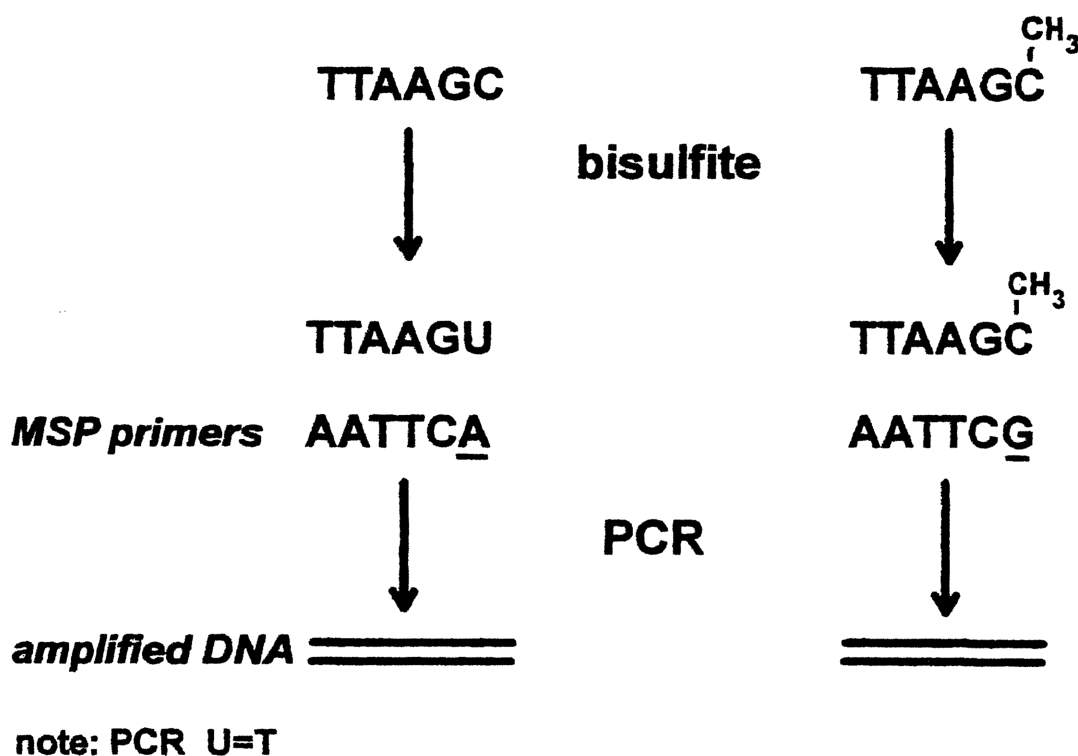

Fig 2.5 Schematic outline for the MSP method. The differences between the methylated and unmethylated DNA after sodium bisulfite modification are shown for a given sequence. Specific differences in the design of primers specific for the methylated (M) or unmethylated (U) DNA are also underlined.

2.6.5 Methylation-sensitive single nucleotide primer extension (MS-SNuPE)

The SNuPE assay was first described by Kuppuswamy et al. (1991) for the detection of mutations in abnormal alleles. Gonzalgo and Jones (1997) modified this method for the quantitation of methylation differences at specific CpG sites. An outline of the method is represented in **Fig.2.6**. After bisulfite treatment of DNA and amplification of the target sequence with primers specific for the converted DNA, the resulting amplified DNA can be used as a template for the MS-SNuPE reaction. The primers used for this single nucleotide extension reaction are designed so that the primer ends just one nucleotide before the incorporation site designated for methylation analysis. The purified amplified DNA is incubated with either radioactive dCTP or dTTP and DNA polymerase. If the target site is methylated, a C will be incorporated during nucleotide extension, if the site is unmethylated, a T will instead be incorporated. Quantitation of the relative C and T incorporated will allow the determination of the methylation status of the target site. As discussed above, primer design and complete modification of DNA are important in order to obtain good results with this assay.

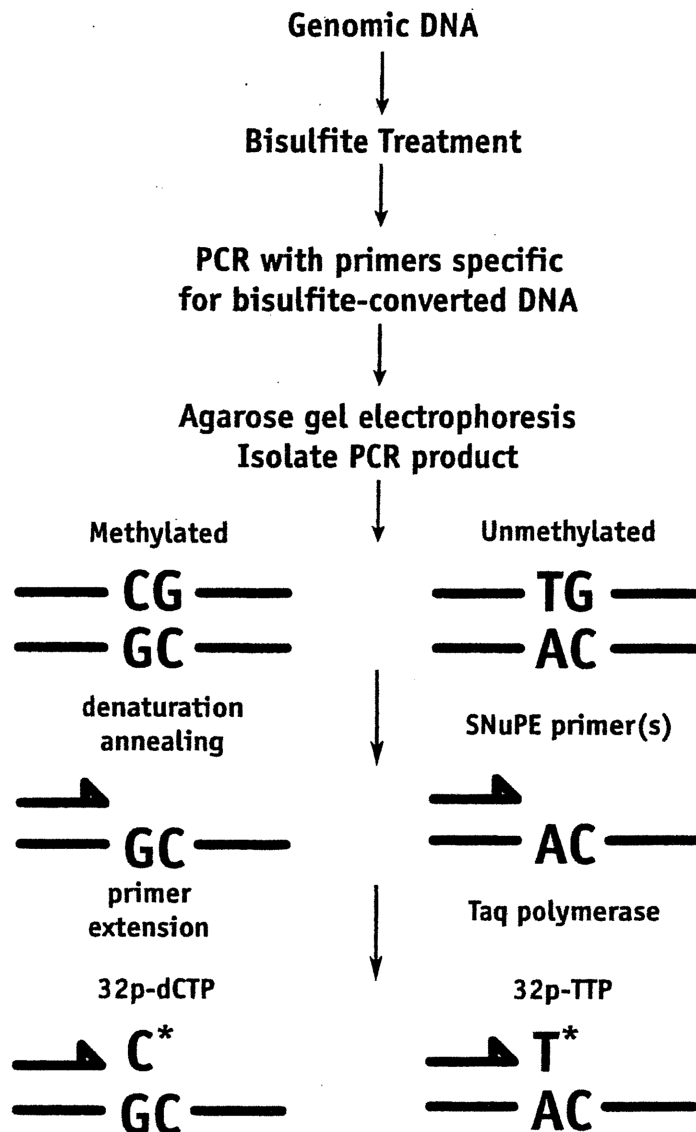

Fig. 2.6 Outline for the SNuPE method for the analysis of methylation status of genes at specific sites. Genomic DNA is treated with sodium bisulfite followed by PCR to amplify a target sequence. PCR products are separated and isolated from agarose gel. The DNA is incubated with a single [^{32}P]dNTP, primer, Taq and DNA polymerase. SNuPE primers are designed to anneal to DNA a single nucleotide prior to the target site. Radioactivity quantification allows quantitation of incorporation of specific [^{32}P]dNTPs, and quantitation of the methylation status of the target sequence.

CHAPTER 3: CHROMATIN STRUCTURE AND HISTONE ACETYLATION

3.1 Chromatin structure

3.1.1 Structure and organization of histones

In eukaryotic cells, DNA exists as a well-organized pattern of chromatin structures. This results in the compaction of DNA of about 10,000-fold. Chromatin is organized in structural units called nucleosomes (Van Holde, 1988). Each nucleosome core is composed of an octamer of proteins called histones and a short filament of DNA of about 146 bp wrapped around the octamer. The core histones are named H2A, H2B, H3 and H4. They are organized as an $(H3\text{-}H4)_2$ tetramer and two H2A and H2B on each side of the tetramer (**Fig 3.1**).

Histones have an extremely defined spatial position within the nucleosome, which makes interactions between histones and DNA highly specific. Each histone has a similar folding pattern for each peptide chain that is based on a long α-helix, flanked on both sides by shorter helices that interact with DNA. Finally, 15-30 residues at the amino-termini of all the histones are unstructured and commonly referred to as tails. Histone display an electrostatic interaction with the phosphodiester backbone of DNA with minor non-polar interaction with the deoxyribose moiety (Arents and Moudrianakis, 1995).

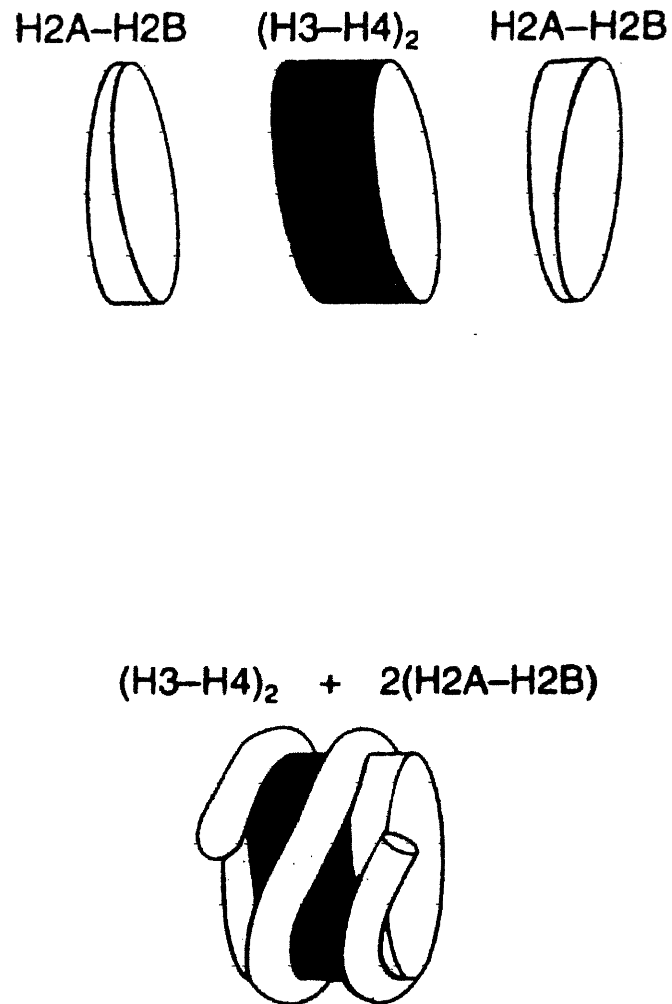

Fig. 3.1 Spatial organization of histones and DNA in the nucleosome. The core histones are arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers positioned on each face of the tetramer. A 146bp loop of DNA is wrapped around the core of histones.

3.1.2 Organization of nucleosomes

Adjacent nucleosomes are joined by linker DNA which binds histone H1. Variations in the length of linker DNA is important for the diversity of gene regulation (Spadafora et al., 1976). Despite this variation, a chain of nucleosomes can coil and fold in a regular fashion to form a chromatin fiber. The lack of sequence specificity of the histone-DNA interactions, evident from the capacity of the histone octamer to package virtually any DNA, gives rise to the question: what positions nucleosomes on the DNA?

Different factors are implicated in the correct positioning of nucleosomes: sequences that are more easily flexible tend to be incorporated into nucleosomes due to the lower energy necessary for their bending around the core histones. Although all the processes involved in the positioning of histones have not been elucidated yet, it seems clear that in regions close to gene promoters the positioning of the nucleosomes is sequence specific. Positioning might serve to prevent specific proteins binding to sites within the nucleosome or to potentiate binding to sites on linker DNA (Straka and Horz, 1991). A degree of sequence specificity is also seen in the binding of the linker histone H1 (Travers and Muyldermans, 1996). Histone H1 association with DNA leads to higher degrees of chromatin compaction.

3.2 Histones and gene expression

Chromatin was once thought to be a static and inactive structural element. It is now clear that histones are a dynamic component of the machinery responsible for the regulation of gene transcription. The structure of transcriptionally active chromatin differs from that of the compact DNA in its susceptibility to digestion to endonucleases and its salt solubility. DNaseI accessibility and salt solubility of chromatin fragments are standard methods used to identify transcriptionally active domains of chromosomes (Rocha et al., 1984).

Packaging of a gene within nucleosomes can block their *in vitro* transcription by eukaryotic RNA polymerase (Knezetic and Luse, 1986; Lorch et al., 1987). It is

most likely that nucleosomes exert these same inhibitory effects on transcription in the cell (Han and Grunstein, 1998). What exactly happens *in vivo* to chromatin?

3.2.1 Chromatin conformation changes

In vitro studies on the compact chromatin fibers show that decrease in ionic strength leads to a more relaxed, beads on a string conformation which is accessible to DNA binding proteins. Restoring the initial ionic strength can reverse this conformational transition.

In the cell chromatin conformation changes are due to both ATP-dependent chromatin remodeling and/or to histone post-translational modifications.

3.2.2 ATP-dependent Chromatin Remodeling Complexes

Biochemical studies in eukaryotic cells have revealed the existence of a class of multisubunit complexes that remodel nucleosomal structure in an ATP-dependent manner. The most characterized chromatin-remodeling complex is that of yeast SWI/SNF. It is composed of 11 subunits with a molecular weight of 2 MDa and contains an ATPase catalytic domain (reviewed by Tyler and Kadonaga, 1999).

How do remodeling complexes move the nucleosome along the DNA? The complexes use the energy derived ATP to promote nucleosome mobilization, but how the histone-DNA bonds are changed is not clear. Perhaps the energy derived from ATP breaks the histone-DNA contact within the nucleosome, but no clear evidence of a direct action of the remodeling factors is yet available. An interesting feature of nucleosome remodeling factors is that displacement of the nucleosomes might favor transcriptional activation by making specific regions of the genome more accessible to transcription factors.

3.2.3 Histone post-translational modifications

Post-translational modifications of histones present in nucleosomes include acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation (van-Holde, 1988). All these modifications take place on the N-terminal tails which protrude from the nucleosome core and are protease sensitive. Histone modifications may alter chromatin structure by influencing histone-histone and histone-DNA interactions (Wolffe and Hayes, 1999). On the basis of multiple histone changes associated to different cellular events, the importance of N-terminal modifications is becoming more evident. The modified histone may interact with other proteins or protein complexes leading to different downstream events. (Strahl and Allis, 2000).

Phosphorylation of histones H1 and H3 has long been implicated in chromosome condensation that occurs during mitosis. New evidence also suggests that histone H3 phosphorylation of lysine at position 10 can be correlated with the induction of genes such as c-jun, c-fos and c-myc which play an important role in the initiation of cellular proliferation (Mahadevan et al., 1991; Chadee et al., 1999).

The role of methylation of lysine and arginine residues in histones H3 and H4 is a poorly understood. This post-translational modification seems to target specific lysines at positions 4, 9, and 27 in histone H3 (van Holde, 1988; Strahl et al., 1999). Recent observations support the idea that histone methylation may contribute to transcriptional activation. For example, CARM1, a nuclear receptor and transcriptional coactivator possesses histone H3 specific methyltransferase activity. This protein functions through association to a transcriptional coactivator containing histone acetylase activity. These observations suggest that large multiprotein complexes containing different histone modifying activities work in concert to regulated gene transcription (Strahl and Allis, 2000)

The most important post-translational modification of histones is acetylation. The spacing between acetylatable lysines is strikingly regular at the amino-termini of histones (Strahl and Allis, 2000). For example, lysines at positions 9,14,18,and 23 are acetylated in histone H3, whereas the lysines at positions 5,8,12, and 16 are acetylated in histone H4 as shown in **Fig. 3.2**. The enzymes responsible for histone

acetylation are called histone acetyltransferases (HATs). The cytoplasmic HATs responsible for histone acetylation during nucleosome assembly are named B type HATs. A second class of HATs referred to as type A is located in the nucleus and is involved in the regulation of gene expression as discussed below.

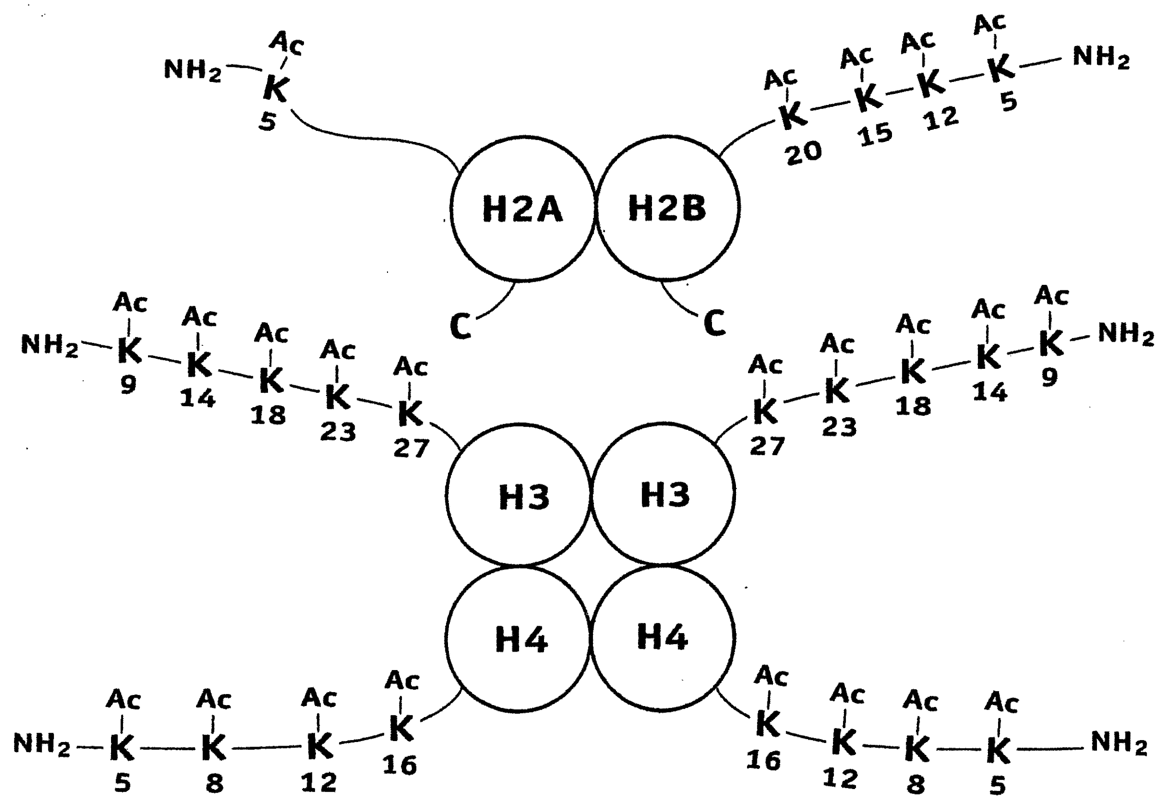

Fig. 3.2 Amino acid positions of lysines that undergo acetylation on core histones H2A, H2B, H3 and H4.

3.3 Histone acetylation and transcriptional regulation

Since the cloning of the first HAT (Brownell et al., 1996), a multitude of transcriptional coactivator complexes have been shown to possess HAT activity (Struhl and Moqtaderi, 1998). These observations provided the evidence for a regulatory role of the nucleosome in transcriptional regulation (Bjorklund et al., 1999). Highly acetylated chromatin is generally associated to actively transcribed DNA, whereas poorly acetylated chromatin is associated with repression of transcription.

How does acetylation of chromatin regulate gene expression? A commonly held view is that acetylation of specific lysine residues in the histone N-terminal tails weakens histone-DNA interaction, by reducing the positive charge on histones, and reducing its affinity for negatively-charged phosphates of DNA (Allfrey et al., 1964). Lysine deacetylation on the histone tails would restore the positive charge, then favoring histone-DNA interaction leading to more compact structure. This phenomenon is actually very complex. Many other factors contribute to transcriptional regulation.

In vertebrates, many transcription factors and modulators have HAT activity. For example, the p300/CBP complex, a transcription factor that possesses intrinsic HAT activity, is able to bind transcription activators (ex. pCAF) which also have HAT activity. It is possible that pCAF and p300/CBP interact in order to acetylate chromatin in the region of certain promoters.

What brings chromatin acetylating proteins in proximity of gene promoters or genes that are to be transcribed? Many transcription factors, like TFIID, are multiprotein complexes that bind to the RNA polymerase holoenzyme. They comprise the TATA binding protein (TBP) and many associated factors known as TAFs (Transcription present Associated Factors)(Struhl and Moqtaderi, 1998). A subset of the TAFs in TFIID are also components of histone acetylase complexes distinct from TFIID (Grant et al., 1998; Ogryzko et al., 1998). The presence of TBP assures the correct positioning of the RNA polymerase II complex with respect to the site for initiation of transcription. The molecular role of TAFs is not well understood.

The observation that some of the TAFs in TFIID can form a substructure similar to the histone octamer (Xie et al., 1996) might suggest that the histone-like TAFs octamer can displace chromosomal histones, facilitating the access of the transcription machinery to the DNA.

The relative levels of histone acetylation are determined by the activities of both HATs and histone deacetylases (HDACs). These enzymes can modify chromatin structure in regions ranging from a single nucleosome to entire chromosomes (mammalian X chromosome inactivation). There are two known classes of HDACs, based on their homology with the yeast HDACs Rpd3 and Hda1 (Grozinger et al., 1999).

Since both HATs and HDACs do not have preference for specific DNA sequences, how can they influence transcription of specific genes? One possible explanation is that they associate to coactivators and/or corepressors that are targeted to specific nuclear receptors resulting in a complex that binds sequence-specific transcriptional regulators.

Nuclear coactivators can be grouped into two general classes: members of the SWI/SNF family, and members of the histone acetyltransferase (HAT) family. Two well-characterized HDAC-associated corepressor families include a major family of nuclear receptor corepressor (NCoR), and silencing mediator of retinoid and thyroid receptors (SMRT) proteins; and a second family including the transcription intermediary factor-1 (TIF-1).

3.3.a Nuclear coactivators

Both classes of coactivators facilitate transcription by either indirectly releasing the tight chromatin structure, or possibly by protein-protein interactions. Targeting of the coactivators to their specific nuclear receptors is mediated by the receptor binding motifs LXXLL, where L is a leucine and N any amino acid. These motifs, their spacing and their flanking sequences are all important for determining coactivator specificity, and nuclear receptor transcriptional activation (McInerney et al., 1998).

One of the first nuclear receptor coactivator to be cloned was the steroid receptor coactivator-1 (SRC-1), also known as p160 (Kamei et al., 1996). SRC-1 enhances ligand-dependent transcriptional activation (AF-2) by many nuclear receptors including progestin receptor (PR), estrogen receptor (ER), thyroid hormone receptor (TR), and retinoid X receptor (RXR). To date three members of the SRC family have been isolated: SRC-1/NCoA-1, TIF2/GRIP1/NcoA-2, and p/CIP/ACTR/RAC3/AIB1/TRAM-1 (refs).

AIB-1 is a member of the SRC family of nuclear coactivators, also known as SRC-3. AIB-1 is highly expressed in the mammary gland, uterus, testis, pituitary gland and muscle, suggesting a physiological role in the endocrine response of these tissues (Suen et al., 1998). It interacts in a ligand-dependent manner with the estrogen receptor resulting in increased levels of ER-dependent transcription. AIB-1 is amplified and overexpressed in a number of ovarian cancers and ER+ breast cancer (Anzik et al., 1997), implying a role of this coactivator in development of these cancers (Bautista et al., 1998).

A potential mechanism of transcriptional activation by members of the SRC family is acetylation of histones. SRC-1 contains a moderate intrinsic HAT activity (Spencer et al., 1997). SRC-1 has been shown to recruit other HATs including p300, CBP (c-AMP response element (CREB) response element), and pCAF (p300/CBP associated factor). p300/CBP and pCAF can enhance transcriptional activation independently and in synergy with SRC-1. A very interesting observation was that ER transcriptional activation by AIB-1 was enhanced by its phosphorylation by mitogen-activated protein kinase (MAPK). Activation of AIB-1 increased its ability to recruit p300/CBP and its associated HAT activity, providing a mechanism by which MAPK signaling is coupled to the regulation of ER and possibly other nuclear receptors (de Mora et al., 2000).

Ligand binding to the nuclear receptors leads to release of the corepressor complex and recruitment of an SRC coactivator to the target gene promoter. SRC binds to the AF-2 domain of the nuclear receptors though its LXXLL motifs, and recruits a wide variety of additional coactivators, including p300/CBP, pCAF. Once this complex is formed, the HAT activities of its components serves to remodel the

chromatin structure, facilitating the access of other coactivators, transcription factors and the basal transcription machinery to the target gene to promote its transcription (Leo and Chen, 2000).

3.3.b Nuclear corepressor

Nuclear corepressors can facilitate transcriptional repression by nuclear receptors in the absence of ligand or in the presence of antagonists. They act by recruiting multiprotein complexes containing HDACs. Two classes of nuclear receptors-associated corepressors have been identified: the major including NCoR and SMRT, the second family including TIF-1 proteins. The association of nuclear receptors to the corepressors takes place at a region (the CoR box) in the hinge between the DBD and the LBD (Horlein et al., 1995). Both classes of corepressors act by recruiting HDAC and stabilizing the chromatin structure in the vicinity of the receptor. NCoR and SMRT interact with the ligand-free TR or with RAR as a RAR-RXR heterodimer or also with antagonist- (e.g.: Tamoxifen) bound ER (Lavinski et al., 1998). NCoR and SMRT are both part of a complex that contains HDAC and Sin3. The main function of Sin3 seems to serve as an intermediary between the receptor and HDACs (Wong and Privalsky, 1998). These complexes contain domains that bind additional transcription associated proteins such as Mad, MeCP2, Ikaros etc., which facilitate targeting of the complex. The interaction of MeCP2 with Sin3 and HDAC is described in some detail in the next paragraph.

3.4 DNA Methylation meets Acetylation

Recent investigations have shown that DNA methylation interacts with histone acetylation to regulate the expression of specific genes. This interaction involves proteins that bind methylated DNA. An example is MeCP2 which is an abundant chromosomal protein that binds to methylated CpG dinucleotides in DNA. It requires a single methylated CpG site for preferential binding to DNA (Nan et al., 1997). One interesting finding is that MeCP2 coprecipitates with histone deacetylase

activity (Nan et al., 1998; Jones et al., 1998), providing direct evidence of a link between methylation and chromatin structure. MeCP2 is associated with the human Rett Syndrome which is characterized by mental retardation in women very young in age. The incidence of this syndrome is 1 in 10,000, and it is caused by mutations in region q28 of the X chromosome, where MeCP2 gene is located (Bienvenu et al., 2000).

As discussed above, posttranslational modifications of histones can modulate gene expression (Davie and Hendzel, 1994). Methylated inactive genes contain under-acetylated histones whereas unmethylated active genes preferentially associate with highly acetylated histones. How do histone acetylation and DNA methylation interact? The mechanism for this interaction follows the path where MeCP2 recognizes the methylated sequence and binds them (Bestor, 1998; Eden et al., 1998). MeCP2 exists in complexes in association with HDACs and transcriptional corepressors, like Sin3, which act together resulting in the final suppression of transcription and condensation of heterochromatin, as shown in the model in **Fig. 3.3**.

Trichostatin A (TSA), a specific histone deacetylase inhibitor (HDI), can activate some genes that are methylated, but not all methylated genes (Jones et al., 1998; Cameron et al., 1999), indicating that the interaction between the methylated DNA, MeCP2 and histone deacetylase is complex and involves different factors and possibly different mechanisms. An interesting observation is that partial demethylation of some genes with the DNA methylation inhibitor, 5-AZA-CdR interacts synergistically with TSA to activate methylated genes that were previously insensitive to this histone deacetylase inhibitor alone (Cameron et al., 1999). These data suggest that removal of methyl groups from cytosines is enough to release the tighter conformation of chromatin around methylated DNA. In fact individual chromosomes assembled on methylated DNA appear to interact together more stably than on unmethylated templates (Keshet et al., 1986).

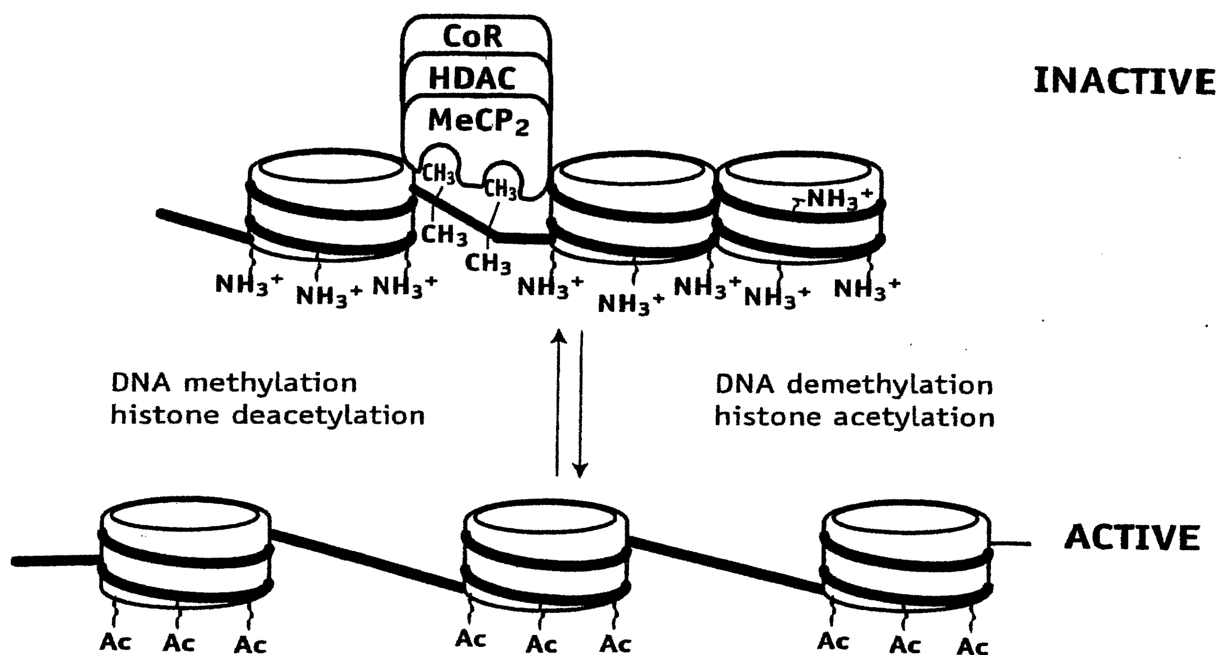

Fig. 3.3 Model for interaction between DNA methylation and histone deacetylation on silencing of gene expression. The methylated DNA binding protein (MeCP2) binds to 5-methylcytosines in the promoter region of target genes. This protein complexes with histone deacetylases (HDACs) and transcriptional corepressors (CoR) resulting in gene silencing and compaction of chromatin.

3.5 Histone deacetylase inhibitors

The role of acetylated histones has been elucidated by using inhibitors of histone deacetylation such as sodium butyrate and TSA. TSA was first isolated from *Streptomyces hygroscopicus* as an antifungal antibiotic (Tsuji et al., 1976). However, only 10 years later its antiproliferating and differentiating effects on leukemia cells were reported (Yoshida et al., 1987). TSA is a specific inhibitor of HDAC which leads to hyperacetylation of histones (Yoshida et al., 1990). The molecular structures TSA and lysine are shown in **Fig. 3.4**. It is interesting to note the similarity in structure of TSA and lysine, a key aminoacid in histones. This explains the ability of TSA to act as an inhibitor of histone deacetylases. HDAC inhibitors induce G1 cell cycle arrest and morphological changes in various cell lines (Fallon and Cox 1979; Nakajima et al., 1998). Inhibition of HDACs by TSA can also lead to induction of differentiation and apoptosis or alteration of transcription (Yoshida et al., 1995). The molecular mechanisms leading to cell cycle arrest remain to be elucidated.

In order to understand the effects of TSA on gene expression, Kim et al (1999) screened for cell cycle regulators whose expression is affected by TSA. They found that p21WAF1 was greatly upregulated in various cell lines following TSA treatment. p21 gene encodes for a protein which acts as a cyclin dependent kinase inhibitor. p21 protein is able to halt cells the cell cycle G1 phase in response to DNA damage (Sang et al., 1995). Another cyclin dependent kinase inhibitor, p16 INK4a, was also upregulated by TSA. All the other cyclins and CdKs were not significantly affected by treatment with TSA. These results seem to suggest that histone acetylation and deacetylation plays an important role in p21WAF1 expression and that overexpression of this gene is responsible of the cell cycle arrest that follows TSA treatment. Treatment with TSA can also affects expression of other genes involved in cell migration, adhesion and cytoskeletal structure. Induction of these genes may explain the morphological changes described after treatment with TSA (Kim et al., 1999).

The potent activity of TSA and other HDACs in inducing cell cycle arrest and apoptosis, suggests their usefulness in cancer chemotherapy. TSA did not prove to be

effective in *in vivo* models, maybe due to its instability or rapid metabolic inactivation. Some of the new HDACs that are being tested today, such as depsipeptide, show interesting antitumor activity on experimental tumor models (Nakajima et al., 1998).

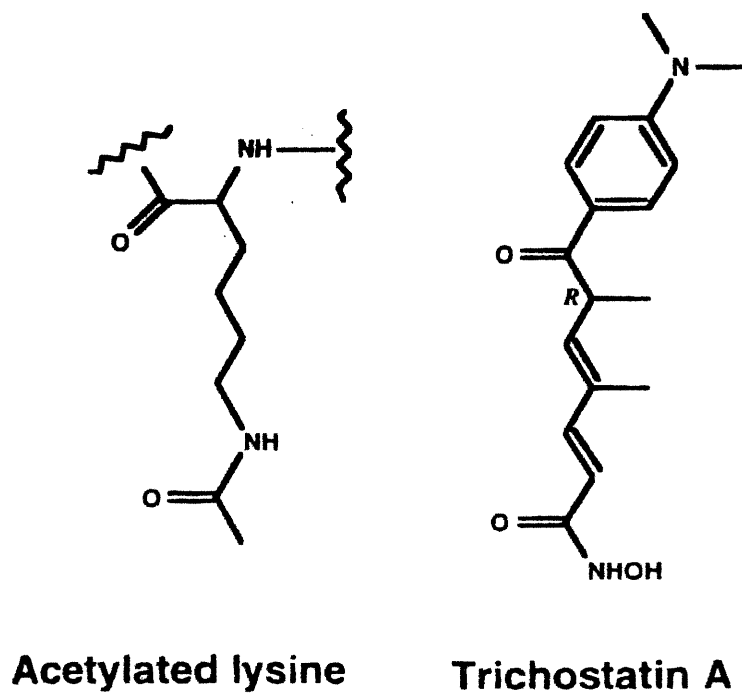

Fig. 3.4 Structure of acetylated lysine and of Trichostatin A, a specific inhibitor of histone deacetylase. Note the structural similarities of the two molecules.

CHAPTER 4: RETINOIC ACID RECEPTOR β

4.1 Retinoic Acid Receptors

Nuclear retinoic acid receptors (RARs) belong to the steroid/thyroid hormone superfamily of transcription factors. The interaction of these receptors with their ligand, retinoic acid, results in gene activation or suppression producing the biological effects characteristic of retinoids. Two families of nuclear retinoic acid receptors have been described: the retinoic acid receptors (RARs) (Chambon et al, 1991) and the retinoid X receptors (RXRs) (Leid et al., 1992, Mangelsdorf et al., 1992).

Upon activation by a ligand, RARs form heterodimers with RXRs (RAR-RXR), and bind to specific DNA sequences called RA response elements (RAREs) in the promoter regions of target genes, to activate their transcription. Nuclear receptors are also able to bind coactivators through the AF-2 domain, which enhance the transcriptional activation via mechanisms that include recruitment of other coactivators and histone acetylases. For example, RAR(α)-RXR is able to bind p300/PCAF complex as a coactivator, enhancing the transcriptional activation of developmentally regulated genes, such as those involved in hematopoiesis (Lenny et al., 1997). In the absence of ligand, the RAR(α)-RXR heterodimers repress basal transcription by binding to NCoR corepressor. NCoR is able to bind Sin-3 and to recruit HDACs leading to a compact chromatin structure, suppressing transcription of target genes (Grignani et al., 1998, Xu et al., 1999). In acute promyelocytic leukemia, RAR-α gene is fused to the PML gene, following chromosomal translocations. As a fusion protein, RAR-α is not responsive to physiological levels of RA, so it becomes a constitutive transcriptional repressor, which blocks normal differentiation and leads to leukemia.

Both RAR and RXR families comprise 3 subtypes of receptors: α, β, and γ. Each of these subtypes exhibits specific patterns of expression during embryonal development and different distribution in adult tissues (Mangelsdorf et al., 1994). In this chapter we will focus on the function and importance of the RARs in tumorigenesis.

4.2 Genomic organization of the human RAR

As mentioned above three major subtypes of RARs exist: α, β, and γ. These retinoic acid receptors share a structure which is similar to the structure of the other members of the nuclear receptor superfamily, which is composed of six distinct domains called A/B-C-D-E/F (See Fig. 4.1).

The N-terminal A domain is highly variable and is characteristic for each class of receptors RAR. The B region is moderately conserved for the three RAR types. The A/B region contains the ligand independent transactivation domain (AF-1), whose function is to modulate the activation of target genes in specific cell-types (Tora et al., 1988 a,b, Dahlman-Wright et al., 1995). The AF-1 domain makes functional, direct interactions with the basal transcriptional machinery (Ford et al., 1997)

The C region corresponds to the DNA binding domain (DBD). The DBD has the most conserved amino acid sequence among the members of the steroid receptor superfamily. It contains the zinc fingers motifs. These motifs contain protein structures in which an atom of zinc binds 4 cysteines forming a finger-like tridimensional conformation that favors interactions with DNA. The zinc fingers interact with the RARE in the promoter region of the target genes. The tridimensional structure of human RARβ DBD has been determined by nuclear magnetic resonance (NMR), and it shows structure similarities with the glucocorticoid receptor (GR) and other members of the nuclear receptor superfamily (Knegtel et al., 1993).

The function of the D (Hinge) region and F region is poorly understood. These two regions, together with A region, are the least conserved among the different receptors. The central part of the region D is RAR type specific and may be linked to some still unknown important function. The amino-terminal part of region D contains a high number of basic amino acids and may function as a nuclear localization signal (Ylikomi et al., 1992).

The C-terminal E region encodes for ligand binding domain (LBD) which provides a class-specific ligand binding site and includes a homodimerization domain

(Forman and Samuels, 1990). It is highly conserved among different RARs showing 76-90% of sequence homology. The C-terminal part of this region also contains a ligand inducible activation function called AF-2 (Allenby et al., 1993, Nagpal et al., 1993).

4.2.1 RAR subtypes

Three subtypes of RARs have been identified in mammalian cells: RARα (Giguere et al., 1987; Petkovich et al 1987), RARβ (de The et al 1987, Brand et al., 1988) and RARγ (Krust et al., 1989; Zelent et al., 1989). In the adult organism RARα seems to be expressed almost ubiquitously, whereas RARβ and RARγ show higher tissue-specificity (Krust et al 1989). RARβ is mostly expressed in epithelial tissues (Dollé et al., 1990); RARγ is predominantly expressed in skin and cartilage (Kastner et al., 1990). These receptors are encoded by different genes: RARα is located on chromosome 17q21 (Mattei et al, 1988a), RARβ gene maps on chromosome 3p24 (Mattei et al., 1988b) and RARγ gene maps on chromosome 12q13 (Ishikawa et al., 1990).

Each receptor subtype consists of different isoforms, which share identical B to F regions, but differ in their A region. These isoforms are generated by the same RAR gene by differential promoter usage and alternative splicing as depicted in **Fig. 4.1**. There are 2 isoforms for RARα (α1 and α2), 4 isoforms for RARβ (β1-β4), and 2 isoforms for RARγ (γ1 and γ2).

The RARα gene is involved in the development of acute promyelocytic leukemia (APL). In fact, in a study on 30 APL patients, all patients showed a characteristic translocation that leads to the fusion of the RARα gene on chromosome 17 to the PML gene on chromosome 15, which codes for a putative transcription factor (Miller et al., 1992).

The 4 different isoforms of the RARβ gene are generated by usage of 2 different promoters, P1 and P2, and alternative splicing. P1 is used for expression of β1 and β3 isoforms. They differ in their A region in that the β3isoform presents 27

extra amino acids encoded by E2, besides the ones shared with β1, encoded by E1. Transcription of isoforms β2and β4 is controlled by P2 which is 20kb downstream from P1. The β4 isoform is a truncated form of β2 with the 5 -UTR and A region almost entirely deleted (Nagpal et al., 1992) (See **Fig.4.1**).

Isoforms β1 and β3 are expressed mostly in fetal and adult brain where they are involved in the development of the central nervous system (Zelent et al., 1991). The β2 isoform is found in the embryo stem cells following treatment with retinoic acid and may be involved in the initial steps of the embryonic development. This β2 isoform is also found in the epithelium of the lungs, breast, intestine, liver, heart and kidneys. It seems that RARβ2 plays a role in the differentiation, apoptosis and homeostasis of epithelial tissues. The β4 could also play a role in the homeostasis of epithelial tissues (Berard et al., 1994).

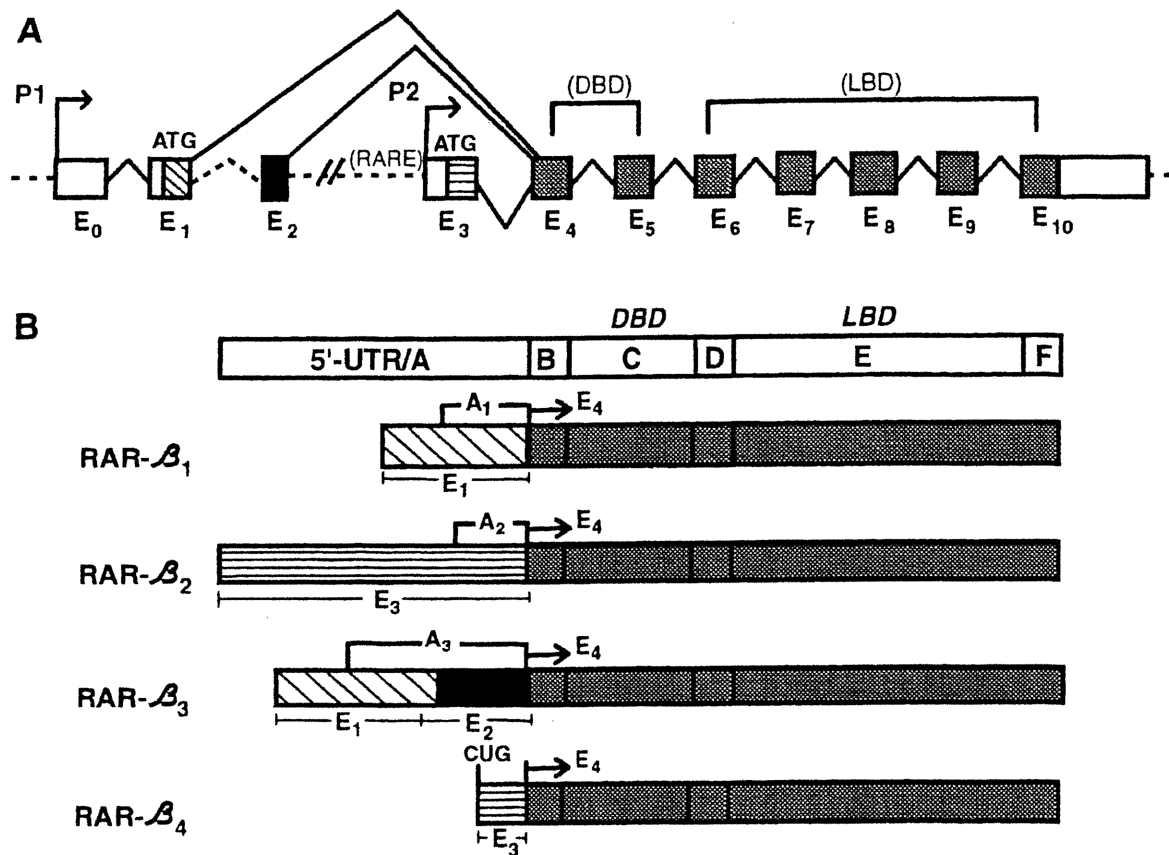

Fig. 4.1 Genomic organization of retinoic acid receptor β (A) and its isoforms (B). The coding region of the gene includes 10 exons (E_1-E_{10}). Two promoters (P1 and P2) are used to initiate the synthesis of the different isoforms of the RAR receptors. Alternative splicing and the use of the two promoters lead to the formation of the 4 isoforms of the RAR (β_1-β_4). The retinoic acid responsive elements (RARE) are found upstream from the P2. DBD, DNA binding domain. LBD, ligand binding domain. The 5'-UTRs are specific for each isoform.

4.3 RARβ role in tumorigenesis

RAR β plays a very important role in tumorigenesis of the lung, the head and neck squamous cells, the colon and the breast. Houle et al. (1993) were the first to propose the tumor suppressive activity of RARβ. Their initial interest in RARβ2 as a putative tumor suppressor gene was based on their observation of frequent LOH at 3p21 in lung cancer, the chromosomal location of this gene. These investigators observed a lack of expression of RARβ in several tumor types, including lung cancer. In their study on expression of RARβ in lung cancer cells, Houle et al. (1993) found that the gene was not expressed in most lung tumor cells even in the presence of normal copies of the gene. Transfection of RARβ cDNA in tumor cells not expressing this gene decreased their growth rate and tumorigenicity in nude mice.

In order to determine if inactivation of RARβ2 can predispose to development of lung cancer, Berard et al. (1996) studied the effects of an antisense to RARβ2 in transgenic mice. After 14-18 months of antisense treatment the transgenic mice developed lung tumors, while the control mice did not. The increase in tumor formation was due to decreased expression of RARβ2.

More than 90% of the malignancies that occur in the head and neck are squamous cell carcinomas (SSCs). Several studies have demonstrated that some head and neck SSC cell lines (Crowe et al., 1991; Lotan et al., 1995) and oral premalignant and malignant lesions (Hu et al., 1991; Zou et al., 1994) express low or no RARβ. Normal counterparts of the oral mucosa express RARβ mRNA, suggesting that RARβ may be important for the normal differentiation of epithelial cells, and that its suppression may play a key role in their transformation (Lotan et al., 1995). Transfection of RARβ in head and neck SCC cells that did not express this gene led to their normal differentiation to stratified epithelium in culture (Crowe, 1998), or to suppression of aberrant keratinization (Wan et al. 1999). Xu and coworkers (1994) examined biopsies from 31 HNSCC, and they found that RARβ expression was decreased in 35% of samples analyzed. Moreover, treatment of SSC patients with 13-*cis*-RA for 3 months resulted in an increased expression of RARβ, and a

coincident positive clinical response. All these data suggest that the decreased expression of RARβ may be associated with HNSCC development.

Down-regulation of RARβ expression in colon cancer has been well documented and studied in our laboratory by Côté et al. (1995). These investigators observed that the DLD1 colon carcinoma cell line was resistant to RA. A synergy between 5-AZA-CdR and RA was observed with respect to their anti-neoplastic activity against these tumor cells. Northern blot analysis showed that 5-AZA-CdR activated expression of RARβ. RARβ silencing was shown to be due to DNA hypermethylation by Southern blot analysis (Côté and Momparler, 1997). These authors also used the bisulfite DNA sequencing technique to identify the methylated cytosines in a 297 bp fragment of the promoter region of RARβ gene (Côté and Momparler, 1998).

Decreased expression of RARβ has been described in many breast cancer cell lines. Swisshelm et al. (1994) found that most of breast tumor cells they studied showed down-regulated RARβ. Transfection of RARβ in MDA-MB-231 and MCF-7 breast carcinoma cell lines which do not express this gene, restored sensitivity of the cells to RA which led to marked growth inhibition and induction of apoptosis (Seewaldt et al., 1995). Loss of RARβ expression has also been described in 13 out of 14 breast tumors by Widschwendter et al. (1997), and in about 50% of breast tumor biopsies if compared to normal breast tissue by Xu et al. (1997). In a recent report, Widschwendter et al. (2000) reported silencing of the RARβ2 gene in breast carcinoma cell lines by aberrant methylation in the promoter region and the first exon of this gene. Treatment of these cells with 5-AZA-CdR and RA was able to restore RARβ2 expression. These authors also reported methylation in the 5' region of the RARβ2 gene in 6 of 16 breast tumor biopsies analyzed. RARβ2 expression was found to progressively decrease in more advanced disease.

All these reports suggest that RARβ is a general regulator of cellular proliferation of epithelial tissues and its selective inactivation allows cells to bypass the growth control mechanisms. RARβ gene is located at chromosome 3p24, a site that shows frequent LOH in breast cancer (Deng et al., 1996). These observations

support the hypothesis that it acts as a tumor suppressor gene in breast cancer. Additional evidence that RARβ is a tumor suppressor gene includes the reports that transfection of RARβ cDNA into tumor cells induces terminal differentiation (Caliaro et al., 1994).

CHAPTER 5: 5-AZA-2'-DEOXYCYTIDINE

5.1 Introduction

5-Aza-2'-deoxycytidine (5-AZA-CdR, Decitabine) is an analog of 2'-deoxycytidine, in which the carbon in position 5 of the cytosine ring has been replaced by a nitrogen. The chemical structure of 5-AZA-CdR and its comparison to 2'-deoxycytidine is shown in **Fig. 5.1**. This nucleoside analog was first synthesized by Sorm and Pliml in 1964, and first demonstrated to be an active antileukemic agent in animal models by Sorm and Vesely (1968).

5.2 Metabolism

5-AZA-CdR enters the cell either by both transportation system for nucleosides and by passive diffusion (Plagemann et al., 1978). As a nucleoside analog, 5-AZA-CdR is incorporated readily into the DNA. A schematic representation of 5-AZA-CdR metabolism is shown in **Fig. 5.2.** In order to become active inhibitor, this analog has to be phosphorylated to its triphosphate form, 5-AZA-dCTP. The enzyme responsible for its phosphorylation to 5-AZA-dCMP is the deoxycytidine kinase. In the cells, 5-AZA-dCMP is rapidly converted to 5-AZA-dCDP and 5-AZA-dCTP by other kinases. The limiting step in this chain of reactions is the formation of 5-AZA-dCMP; leukemic cells deficient in the deoxycytidine kinase gene are completely resistant to the antitumor activity of 5-AZA-CdR (Momparler et al., 1982). The incorporation of 5-AZA-dCTP into the DNA is catalyzed by DNA polymerase and produces a potent inhibition of DNA methylation (Bouchard and Momparler 1983). 5-AZA-dCTP is incorporated in the DNA with an affinity for DNA polymerase comparable to that of the native substrate, dCTP.

The 2 enzymes responsible for 5-AZA-CdR degradation are deoxycytidine-5'-monophosphate deaminase (dCMP deaminase) and cytidine deaminase (CR deaminase). The dCMP deaminase catalyses the deamination of 5-AZA-dCMP to 5-AZA-dUMP (5-AZA-2'-deoxyuridine-5'-monophosphate). The second enzyme, CR deaminase, catalyzes the conversion of 5-AZA-CdR to 5-AZA-UdR (5-AZA-2'-uridine), an inactive metabolite (Chabot et al., 1983; Eliopoulos et al., 1998). The

presence of high levels of CR deaminase in human liver is responsible for the short half life (~15-20 min) of 5-AZA-CdR in man (Momparler et al., 1997).

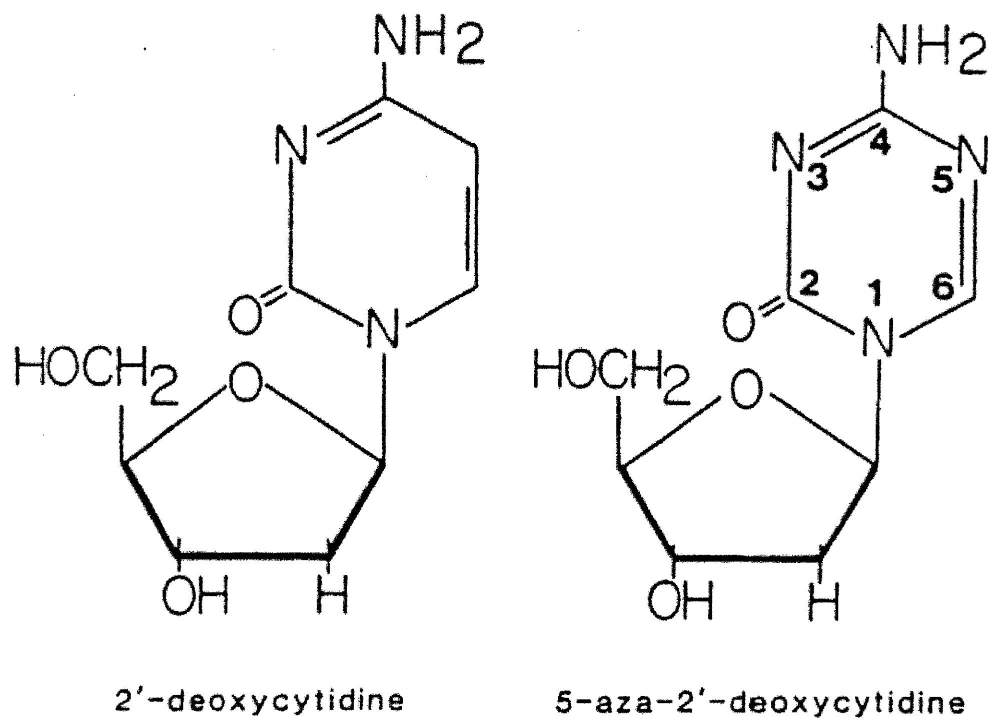

Fig 5.1 Molecular structure of 2'-deoxycytidine and 5-aza-2'-deoxycytidine (5-AZA-CdR). The carbon in position 5 of the pyrimidine ring is replaced by an atom of nitrogen in 5-AZA-CdR. Once incorporated in the DNA, the position 5 of the 5-AZA-cytosine cannot be methylated.

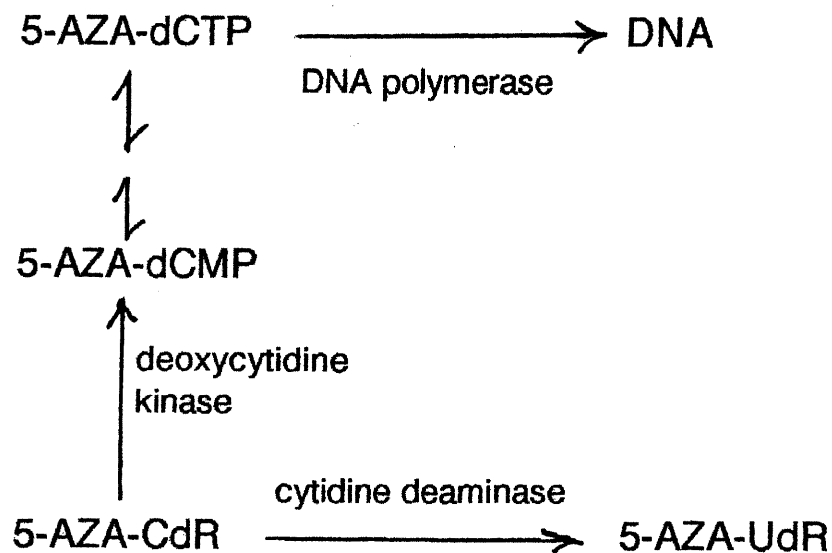

Fig. 5.2 Metabolism of 5-AZA-CdR. As a nucleoside analog, 5-AZA-CdR is incorporated in the DNA by a DNA polymerase. The molecule needs to be phosphorylated to its triphosphate form: deoxycytidine kinase is the enzyme responsible for the phosphorylation to 5-AZA-dCMP, the rate limiting step. 5-AZA-dCMP is phosphorylated to 5-AZA-dCTP by other kinases. Cytidine deaminase is responsible of the deamination of 5-AZA-CdR to the inactive metabolite, 5-AZA-UdR.

5.3 Mechanism of action

5-AZA-CdR is an S-phase specific agent and it does not affect the cells that are in other phases of the cell cycle (Momparler et al., 1984b). This is due to the fact that to exert its antitumor action 5-AZA-CdR needs to be incorporated into the DNA.

Incorporation of 5-AZA-dCTP into DNA produces an inhibition of methylation of cytosine to 5-methylcytosine, a reaction that is catalyzed by the enzyme DMTase (Bouchard and Momparler 1983; Creusot et al., 1982). Once incorporated in the DNA, 5-AZA-dCMP forms covalent adducts with DMTase (Wu and Santi, 1985, Jütterman et al, 1994). This results in depletion of DMTase from the cells resulting in demethylation of genomic DNA following treatment with this cytosine analog (see **Fig. 5.3**) (Creusot et al., 1982, Michalowsky and Jones, 1987). 5-Aza-dCMP in DNA is not a substrate for DMTase because of the presence of a nitrogen (N) on the position 5 of the cytosine ring that can not be methylated.

The target of the antineoplastic action of 5-Aza-CdR is to reactivate tumor suppressor genes and other cancer-related genes that have been accidentally silenced by aberrant methylation (see **Fig. 5.4**). Many cancer-related genes have been reported to be silenced in different tumor cell lines and in primary tumors (see **Table 2.1**). Most of these genes have been shown to be re-expressed in tumor cell lines following *in vitro* treatment with the demethylating agent, 5-AZA-CdR.

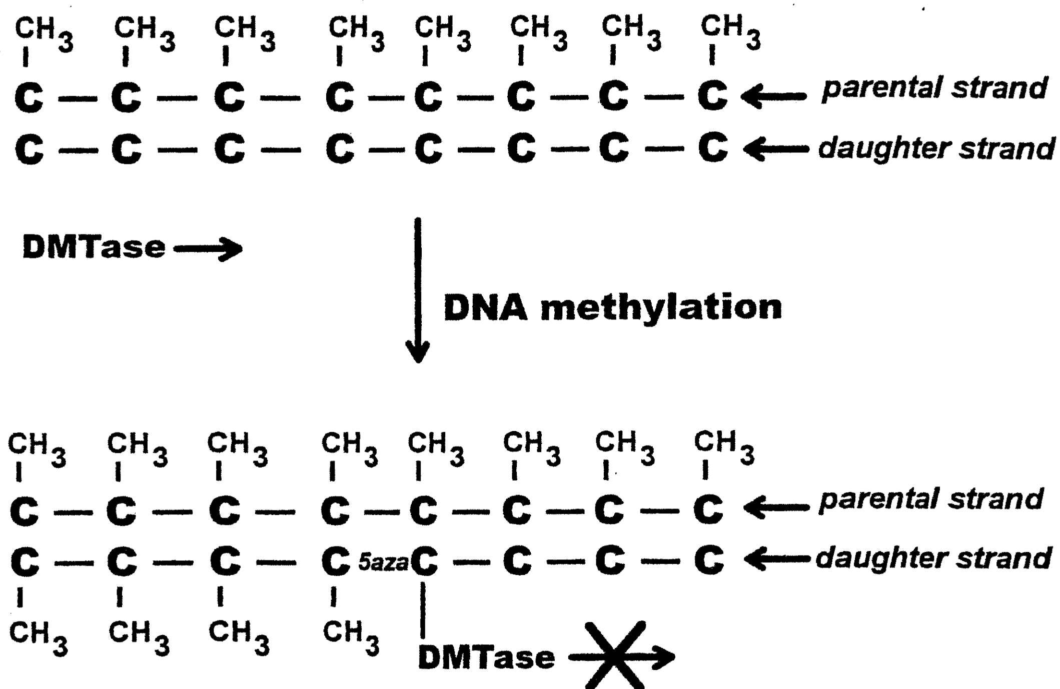

Fig. 5.3 Mechanism of action of 5-AZA-CdR. DNA methyltransferase (DMTase) maintains the methylation patterns in genomic DNA by converting the complementary cytosines to 5-methylaytosines in the new daughter strand using parental DNA strand as the template. The presence of 5-azacytosine at the methylation sites in the daughter strand inactivates DMTase by formation of a covalent adduct with this enzyme. Depletion of DMTase results in hypomethylation of the newly synthesized DNA.

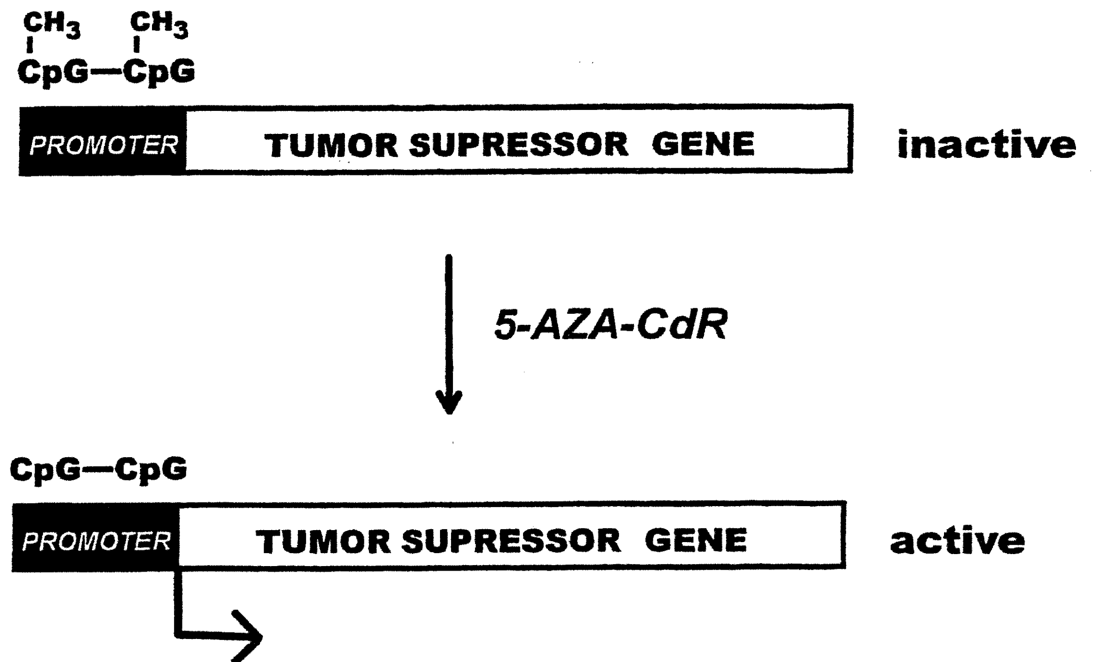

Fig. 5.4 Reactivation of tumor suppressor genes in tumor cells by treatment with 5-AZA-CdR. Demethylation of the CpG islands in the promoter region of tumor suppressor genes leads to reactivation of their expression.

5.4 Toxicity

The main toxicity of 5-Aza-CdR is myelosuppression. In patients with leukemia Rivard et al. (1981) and Momparler et al. (1986) observed a gradual decrease in the white blood cell count with a nadir at about day 14 to 21 and recovery at about day 28 following therapy with 5-AZA-CdR. The terminal differentiation of the hematopoietic progenitor cells induced by this analog is a slow process that requires many days. Treatment with growth factors such as G-CSF or GM-CSF after 5-AZA-CdR treatment may reduce the duration of granulocytopenia produced by the chemotherapy (Crawford et al., 1991).

Another approach to overcome bone marrow toxicity following 5-AZA-CdR is the use of gene therapy. In our laboratory we have used the cytidine deaminase gene cloned into a retroviral vector to transduce cells. The transduced cells showed drug resistance to 5-AZA-CdR and other cytosine analogs (Eliopoulos et al, 1998, Momparler et al 1996b). The future approach will be to make normal bone marrow cells resistant to 5-AZA-CdR by transduction with the cytidine deaminase gene and to reinfuse these cells back into the patients to confer chemoprotection to 5-AZA-CdR (Momparler et al., 2000).

5.5 Resistance to 5-AZA-CdR

The major mechanisms of resistance to 5-AZA-CdR are linked to the activity of the enzymes involved in the metabolism of this drug (See **Fig.5.2**). Deficiency in deoxycytidine kinase, the enzyme that catalyzes the phosphorylation of 5-AZA-CdR to 5-AZA-dCMP, will make cells completely resistant to cytosine analogs (Momparler et al., 1982). Also, increase in cytidine deaminase, the enzyme that deaminates 5-Aza-dCR to an inactive metabolite can make cells resistant these cytosine analogs. An increased pool of cellular dCTP can also induce drug resistance by either feedback inhibition deoxycytidine kinase to reduce its activation by phosphorylation, or by competing with 5-AZA-dCTP for DNA polymerase to reduce its incorporation into DNA (reviewed by Momparler et al 1997).

5.6 Cancer therapy with inhibitors of DNA methylation

The large number of target genes related to tumorigenesis that are silenced by aberrant DNA methylation (**Table 2.1**) suggest that inhibitors of this process may have interesting potential in cancer therapy. The potential target genes for therapy with 5-AZA-CdR include tumor suppressor genes, metastasis inhibitors genes, angiogenesis inhibitor genes, DNA repair genes and tumor-associated antigen genes. In preclinical and preliminary clinical studies 5-AZA-CdR shows promising activity as an anticancer agent.

5-AZA-CdR is a potent and specific inhibitor of DMTase. A model for the activation by 5-AZA-CdR of tumor suppressor genes that have been silenced by aberrant methylation is displayed in **Fig. 5.4**. The inhibition of DNA methylation as a target for cancer therapy is supported by the interesting observation that antisense to DMTase shows *in vitro* antitumor activity and some potential to reverse the malignant phenotype (MacLeod and Szyf, 1995). The antisense oligonucleotide to DMTase also inhibits tumor growth in an animal model (Ramchandani et al., 1997).

Interest in the chemotherapeutic potential of inhibitors of DNA methylation has been retarded by the reports that analogs of 5-azacytosine are carcinogenic (Carr et al, 1984) and can activate oncogenes by hypomethylation (Counts & Goodman, 1994). There has been some confusion in this field since results obtained with the riboside analog, 5-azacytidine, have been assumed to also apply to 5-AZA-CdR. There are large differences in the pharmacology of these two analogs. Most of 5-azacytidine is incorporated into RNA, interfering with its function (Momparler et al., 1976) and only a small fraction of this analog is incorporated into DNA producing hypomethylation. Due to this dual action of 5-azacytidine, it cannot be assumed that all its pharmacological action is due to its effects only on DNA methylation, making data obtained with this analog difficult to analyze. An example of this is the report that 5-azacytidine was carcinogenic in rats, whereas 5-AZA-CdR was not carcinogenic (Carr et al., 1988). In fact, in the mouse model 5-AZA-CdR was demonstrated to suppress intestinal neoplasia (Laird et al., 1995). It has been reported that 5-AZA-CdR can produce mutations in a bacterial gene in a mouse model

(Jackson-Grusby et al., 1997). This model is difficult to interpret due to the lack of knowledge on DNA repair of a bacterial gene present in a murine genome. Mutagenesis assays on 5-AZA-CdR in mammalian cells in tissue culture indicate that 5-AZA-CdR is negligibly or not mutagenic (Landolph and Jones, 1982; Momparler et al., 1984c).

5-AZA-CdR is a S phase specific agent with a short *in vivo* half-life and pharmacological activity that is very dose-schedule dependent (Momparler, 1986). Clinical investigations on 5-AZA-CdR were stimulated by the observations that this analog was a much more potent antileukemic agent in the mouse model that cytosine arabinoside (Momparler et al., 1984b). Also, in support of clinical trials on leukemia with this analog were the observations that it could induce *in vitro* differentiation of human leukemic cells (Pinto et al., 1984; Momparler et al., 1985). In the mouse model the antileukemic activity of 5-AZA-CdR correlates with its inhibition of DNA methylation (Wilson et al., 1983). The first clinical trials on 5-AZA-CdR showed that this analog could induce complete remissions in patients with acute leukemia in relapse (Momparler et al., 1986). The dose-schedule of 5-AZA-CdR used in this trial produced a significant inhibition of DNA methylation in the leukemic blasts (Momparler et al., 1984a). Currently, 5-AZA-CdR shows interesting clinical activity in patients with chronic myeloid leukemia in blast crisis (Kantarjian et al., 1997) and in patients with myelodysplastic syndrome, a preleukemic disease (Zagonel et al., 1993).

Several pilot clinical trials on the antitumor activity of 5-AZA-CdR have been performed. Preclinical studies on human tumor xenografts in the mouse model showed that this analog is a potent antitumor agent (Braakhuis et al., 1988). 5-AZA-CdR also shows interesting *in vitro* antitumor activity against breast cancer (Bovenzi et al., 1999). In preliminary studies on patients with head and neck cancer and patients with prostate cancer, 5-AZA-CdR showed minimal to moderate antitumor activity (van Groeningen et al., 1986; Thibault et al., 1998) due to the suboptimal dose-schedule used. Interesting antitumor activity was observed with 5-AZA-CdR using a more intense dose-schedule in patients with stage IV non-small cell lung cancer, including one patient that survived more than 6 years; this response was

remarkable due to the short life expectancy of patients with this disease (Momparler et al., 1997). The clinical antitumor activity of 5-AZA-CdR was complex to evaluate due to its delayed antineoplastic action, a characteristic of an agent that induces terminal differentiation. In fact, after treatment some tumor progression was observed for some patients followed by growth arrest. It is interesting to note that a similar phenomenon occurred in some leukemic patients after treatment with 5-AZA-CdR (Momparler et al., 1986).

More clinical studies have to be performed to fully evaluate the antitumor activity of 5-AZA-CdR. The development of tests that can detect the methylation of tumor suppressor genes in fragments of genomic DNA present in the serum (Esteller et al., 1999) and the tests to quantitate the extent of inhibition of DNA methylation (Gonzalgo and Jones, 1997; Lo et al., 1999; Bovenzi and Momparler, 2000) will permit the monitoring of these events before and during therapy with 5-AZA-CdR and help clarify its antineoplastic activity.

Hematopoietic toxicity is the major side effect produced by 5-AZA-CdR and limits the dose intensity (Momparler et al., 1986; 1997). It may be possible to overcome this problem by using gene therapy as discussed above to insert the drug resistance gene, cytidine deaminase, into hematopoietic cells to protect them from the toxicity produced by 5-AZA-CdR (Momparler et al., 1996). Elevated expression of cytidine deaminase in cells confers drug resistance to 5-AZA-CdR (Eliopoulos et al., 1998).

Future clinical investigations on 5-AZA-CdR therapy in patients will clarify the chemotherapeutic potential of this interesting cytosine analog against cancer.

OBJECTIVES OF PRESENT WORK

1. To evaluate the *in vitro* antineoplastic activity of the inhibitor of DNA methylation, 5-AZA-CdR on human MDA-MB-231 breast carcinoma cells.

2. To evaluate the *in vitro* of the inhibitor of histone deacetylation, trichostatin A (TSA) on human MDA-MB-231 breast carcinoma cells.

3. To evaluate the *in vitro* antineoplastic activity of 5-AZA-CdR and TSA in combination on human MDA-MB-231 breast carcinoma cells.

4. To evaluate the methylation status of the promoter region of the tumor suppressor gene, RARβ, in MDA-MB-231 cell line and breast tumor biopsies using the methylation specific PCR reaction (MSP).

5. To evaluate the methylation status of the promoter region of RARβ in MDA-MB-231 cells using an improved and simplified methylation sensitive single nucleotide primer extension (Ms-SNuPE) assay.

6. To quantitate the inhibition of DNA methylation following exposure of MDA-MB-231 cells to 5-AZA-CdR using the modified Ms-SNuPE assay.

7. To evaluate the activation of expression of RARβ gene and the estrogen receptor gene by 5-AZA-CdR and TSA, alone and in combination, on human MDA-MB-231 breast carcinoma cells using the reverse transcriptase PCR (RT-PCR) reaction.

PART 2: PRESENTATION OF MANUSCRIPTS

CHAPTER 6: DNA METHYLATION OF RETINOIC ACID RECEPTOR β IN BREAST CANCER AND POSSIBLE THERAPEUTIC ROLE OF 5-AZA-2'-DEOXYCYTIDINE.

Preface

We have investigated the activity of 5-AZA-CdR on the clonogenic and growth potential of the MDA-MB-231 breast carcinoma cells. We found that this analog acts as a potent inhibitor of clonogenicity and growth on these cells *in vitro* at concentrations that have shown clinical activity against leukemia and lung cancer. These preliminary data support the rationale for a pilot clinical trial on 5-AZA-CdR in patients with breast cancer.

Thirteen breast cancer biopsies were screened for the methylation status of the tumor suppressor gene, retinoic acid receptor β (RARβ), using the MSP technique. We found that 30% (4 of 13) of the biopsies presented methylation of the promoter region of the gene analyzed. Two biopsies that showed strong signal for methylation were cloned and sequenced for identification of methylation sites in a 393bp region of the RARβ gene. Sequencing analysis revealed that the positions of 5-methylcytosines in the breast cancer biopsies are identical to those found in the DLD1 colon carcinoma cells by Côté et al. (1998). These results suggest that important sites of methylation appear to be conserved in different tumor types. The RARβ gene can be considered as an additional target for treatment of breast cancer with demethylating agents such as 5-AZA-CdR.

DNA methylation of retinoic acid receptor ß in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine

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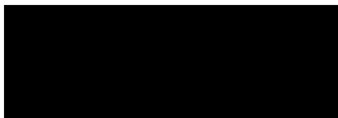

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Abstract

The retinoic acid receptor β (RARβ), a putative tumor suppressor gene, has been reported to be poorly expressed in breast cancer. In this report using the methylation specific PCR reaction we observed DNA methylation in the promoter region of RARß in several primary breast tumors. DNA sequence analysis showed that the positions of 5-methylcytosine in the RARß promoter region was almost identical to that reported previously by our laboratory for human DLD-1 colon carcinoma cells (*Anti-Cancer Drugs 1998; 9: 743*). Several other cancer-related genes have been also reported to be silenced by DNA methylation, including the p16 tumor suppressor gene, E-cadherin, an invasion suppressor gene and the estrogen receptor gene in breast cancer cell lines. Since breast cancer cells have several potential target genes for the DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-AZA-CdR), we investigated the *in vitro* antineoplastic activity of this analog on the human breast cancer cell line MDA-MB-231. We report that 5-AZA-CdR is a potent growth inhibitor and a potent cytotoxic agent against the breast carcinoma cells. These results suggest that 5-AZA-CdR may be an interesting agent to investigate in patients with breast cancer resistant to conventional chemotherapy.

Introduction

About 30% of women diagnosed with breast cancer ultimately die from this disease.[1] Postmenopausal women with breast tumors that are estrogen receptor positive respond initially to therapy with antiestrogens.[2] However, many patients eventually will become resistant to hormonal therapy. When metastatic breast tumors arise, these patients may respond initially to cytotoxic drug therapy, but eventually tumor progression occurs and their survival is limited. It is urgent to consider new approaches for the therapy of this disease. Recent results suggest that tumor suppressor genes may be considered as interesting targets for the therapy of breast cancer.

Lack of expression of the retinoic acid receptor beta (RARβ), a putative tumor suppressor gene, has been reported for breast cancers[3, 4] and other types of cancer. [5-8] RARβ gene is located at chromosome 3p24, a site that shows frequent loss of heterozygosity in breast cancer.[9] Additional evidence that RARβ is a tumor suppressor gene includes the reports that transfection of RARβ cDNA into some tumor cells induced terminal differentiation[8] and reduced their tumorigenicity in nude mice.[10]

It is possible that the loss of expression of RARβ in breast cancer is due to aberrant methylation of CpG islands in its promoter region. We reported previously that RARβ is methylated and not expressed in DLD-1 human colon carcinoma cells.[11] Recently, we identified the specific sites of 5-methylcytosine in the promoter region of RARβ in these tumor cells.[12] In this study, using the methylation specific PCR (MSP) assay,[13] we detected methylation of RARβ in several breast tumor biopsies. In addition, using DNA sequence analysis we observed similar positions of 5-methylcytosine in the promoter region of RARβ in two primary human breast tumors as reported previously for the DLD-1 colon tumor cells.

It is interesting to note that a large number of cancer-related genes have been found to be methylated in breast cancer, including BRCA1,[14] the invasion suppressor gene, E-cadherin,[15] the estrogen receptor gene,[16] the tumor suppressor genes, p16,[17] MDGI (mammary-derived-growth- inhibitor),[18] and HIC-1 (hypermethylated in

cancer).[19] In most of these cases, gene methylation results in the silencing of their expression. 5-aza-2'-deoxycytidine (5-AZA-CdR), a potent inhibitor of DNA methylation,[20] has been reported to activate the *in vitro* expression of most of these genes. We reported previously that 5-AZA-CdR activates the expression of RARβ in DLD-1 colon carcinoma cells.[12] This analog may be a novel agent to investigate against breast cancer considering the many interesting target genes for its chemotherapeutic action. In clinical studies 5-AZA-CdR has shown activity in patients with leukemia[21, 22, 23] and promising activity in patients with advanced lung cancer.[24] In this study we observed that 5-AZA-CdR produced a potent growth inhibition and a profound reduction in the clonogenicity of MDA-MB-231 human breast carcinoma cells, supporting the hypothesis that this analog has some potential in the therapy of breast cancer.

Materials and Methods

Cell line and tumor samples

Human MDA-MB-231 breast cancer cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium (Life Technologies, Burlington, Ontario) containing 10% heat inactivated fetal calf serum (Wisent, St-Bruno, Quebec) and 1 mM sodium pyruvate in a 5% CO2 incubator at 37°C. Genomic DNA was obtained from a tumor bank of breast tumors and purified by proteinase K digestion and phenol-chloroform extraction.

Bisulfite treatment of genomic DNA

Bisulfite treatment of single stranded genomic DNA deaminates all the cytosines into uracil, with no change in 5-methylcytosine. This reaction was carried out as described by Frommer *et al.*,[25] and Herman *et al.*,[13] with minor modifications. Briefly, 2 μg of genomic DNA was digested with 10U of MboII (Life Technologies). The DNA was

denaturated with freshly prepared 0.6 M NaOH for 20 min at 45°C. Freshly prepared bisulfite (Sigma, Oakville, Ontario), pH 5, and hydroquinone (Sigma) were added to each sample to a final concentration of 3 M and 5 mM, respectively, and incubated in the dark at 55°C for 16-20h. The bisulfite-converted DNA was desalted using the Prep-A-Gene DNA purification kit (BioRad Laboratories, Mississauga, Ontario), according to the manufacturer's instructions. The DNA was desulfonated by addition of fresh 0.3 M NaOH by incubation at 40°C for 15 min. The sample was then neutralized with 2 M ammonium acetate (pH 7) and precipitated with ethanol. The DNA was resuspended in 10 mM Tris, 1 mM EDTA, pH 7.5.

DNA cloning and sequencing

Bisulfite-converted DNA specific primers were used to amplify a 395 bp fragment of RARβ promoter region (from position 738 to position 1131) prior to cloning in the pCR2.1 TOPO vector (InVitrogen, Carlsbad, CA): sense primer 5'-GGA GTT GGT GAT GTT AGA TTA GTT G-3' (position 738-762) and antisense primer 5'-TCC AAA TAA TCA TTT ACC ATT TTC C-3' (position 1107-1131). PCR amplifications were performed in 25 μl reaction mixture containing 1 to 25 ng of bisulfite treated DNA, 2.5 mM dNTPs, 0.2 μM primers, 1 mM $MgCl_2$, 8% glycerol, PCR buffer and 1.25 U Taq Polymerase (Pharmacia, Baie d'Urfé, Quebec), in a thermal minicycler (MJ Research, Watertown, MA) under the following conditions: 94°C for 2 minutes; 94°C for 30 sec, 50°C for 30 sec, 72°C for 1min for 45 cycles; and 72°C for 5 min. The 395 bp amplified fragment was cloned into pCR2.1-TOPO vector (InVitrogen) according to the manufacturer's instructions. Plasmid DNA was purified using the Qiagen Plasmid Mini kit, and sequenced using the AutoRead 1000 sequencing kit and ALF automatic sequencer (Pharmacia).

Methylation Specific PCR (MSP) assay

MSP is a technique that takes advantage of DNA sequence differences existing between methylated and unmethylated DNA after bisulfite treatment.[13] Primer pairs for the RARβ promoter region (RARβ-M and RARβ-U) were described by Côté *et al.*.[12] These primers were used to amplify a 146-bp fragment of the RAR-β promoter region. MSP amplification was performed as described previously.

Growth inhibition and clonogenic assay

For growth inhibition evaluation, MDA-MB-231 cells were plated at 100,000 cells per well of a 6-well dish in 2 ml of media. 5-AZA-CdR (Decitabine) was obtained from Pharmachemie BV, Haarlem, The Netherlands, dissolved in 0.45% NaCl containing 10 mM sodium phosphate (pH 6.8) and stored at -70°C. The cells were treated with different concentrations of 5-AZA-CdR ranging from 1 to 1000 ng/ml. Cells were counted at 48, 72 and 96 hr after the beginning of drug treatment. The growth inhibition values are expressed as the cell count of the drug-treated cells relative to the cell count of the untreated control cells.

For the clonogenic assay, MDA-MB-231 breast carcinoma cells were plated at 100 cells per well on a 6-well dish containing 2 ml of media and treated with 5-AZA-CdR as described above for the growth assay. The cells were incubated with 5-AZA-CdR at 37°C for 24-96 hr. The colonies were counted on day 10-14 after drug treatment. The loss of clonogenicity is expressed as the number of colonies formed by drug-treated cells relative to the number of colonies formed by untreated control cells. Due to the chemical instability of 5-AZA-CdR, for both the growth inhibition and the clonogenic assay, fresh drug was added to the medium every 24 hr.

Results

Methylation Specific PCR (MSP)

After DNA isolation from breast tumor biopsies and corresponding normal tissue and bisulfite treatment, we assessed the methylation status of the RAR ß promoter region by MSP. As shown in Fig. 6.1, we detected significantly positive signs of methylation of RARβ in 4 of 13 (30%) breast tumor biopsies (T2, T5, T7, and T8). Using the unmethylation-specific primers we detected strong bands of unmethylation in 10 of 13 (77%) of the tumor biopsies. Two tumor biopsies (T11 and T12) showed very weak bands by MSP, presumably due to unsuitable DNA for PCR analysis. For the tumors that showed significant methylation, an amplified DNA band was also produced by the unmethylation-specific primers for tumor samples T2 and T8. It is likely that these tumor samples contained significant amounts of normal tissue. In normal corresponding tissue we detected significant sign of methylation of RARβ in sample N8, but only very weak bands in samples N7 and N9.

DNA sequence analysis

In Table 6.1 are shown the positions of 5-methylcytosine in the promoter region of RARβ of human DLD-1 colon carcinoma cells and two human breast tumor samples (T2 and T8). In the promoter region from position 738 to 1131, there were 17 sites of 5-methylcytosine present in the DLD-1 cells. Tumor biopsy T2 had the identical sites of 5-methylcytosine as DLD-1 cells. For tumor biopsy T8, all the positions of 5-methylcytosine were the same as the DLD-1 cells, except at positions 784, 798 and 810 at the 5' end and positions 1082 and 1095 at the 3' end of the sequenced DNA fragment.

Growth inhibition and clonogenic assay

The effects of 5-AZA-CdR on the growth of MDA-MB-231 human breast carcinoma cells are shown in Fig. 6.2. A 48 hr treatment of 5-AZA-CdR at a concentration of 10 and 100 ng/ml produced 30 and 47% inhibition of growth, respectively. At this time point the lower concentration of 1 ng/ml produced only 14% growth inhibition. The growth inhibitory effects of 5-AZA-CdR increased with exposure time. At 72 hr 5-AZA-CdR at 10 ng/ml produced 48.2% growth inhibition whereas 100 ng/ml produced 68.5% inhibition.

To evaluate the effects of this analog on the colony formation of the MDA-MB-231 breast carcinoma cells, we performed a clonogenic assay using different concentrations of 5-AZA-CdR at different exposure times (Fig. 6.3). 5-AZA-CdR at 10 and 100 ng/ml for 48hr exposure produced 30.8 and 97.6 % loss of clonogenicity, respectively. These concentrations of 5-AZA-CdR for a 24 hr exposure produced 23 and 91.3% loss of clonogenicity, respectively (data not shown). The loss of clonogenicity produced by 5-AZA-CdR increased with the duration of exposure. For example, for the 96 hr exposure 10 ng/ml of this analog produced a 73.5% loss of clonogenicity whereas for 100 ng/ml the decrease in clonogenicity was >99%.

Discussion

Tumorigenesis arises via the accumulation of specific genetic and epigenetic alterations that give tumor cells growth advantages over the normal cells. Aberrant methylation of CpG islands in the promoter regions of genes involved in the regulation of cell growth has been described in both tumor cell lines[26] and primary tumors.[27] Hypermethylation of promoter regions of these growth regulatory genes results in silencing of their expression.[11, 28] Interestingly, many cancer-related genes have been found to be silenced by *de novo* methylation in breast cancer.[14-19]

We evaluated the methylation status of RARβ gene in tumor biopsies from 13 patients with breast cancer. Low expression of RARβ has been reported to occur frequently in breast cancer.[3,4] We reported previously that this putative tumor suppressor gene is silenced by methylation in DLD-1 human colon carcinoma cells.[29] Using DNA sequence analysis we identified the sites of methylation of the RARβ promoter region.[12] In this report using MSP analysis of breast cancer biopsies from patients, we observed that in 4 of 13 samples analyzed (30%) showed methylation of the RARβ gene (Fig.6.1). With unmethylation specific primers (RARβ-U) we detected positive signals in most tumor samples which may have been due to their contamination with normal tissue. Normal tissue, adjacent to the breast tumor, was also screened for the methylation status of RARβ. For the normal sample N8 we observed a strong positive signal for DNA methylation. One possible explanation for this result is that the MSP reaction detected the presence of malignant cells in the normal tissue or that some normal cells contained a methylated RARβ which may represent an early neoplastic change that could eventually lead to their malignant transformation.

The sequencing of a 395 bp fragment of bisulfite-treated DNA from the RARβ promoter region of two human breast cancer biopsies (T2 and T8) revealed that the positions of 5-methylcytosine were similar to those in DLD-1 human colon carcinoma cell line (Table 6.1). In particular, in patient T2 all the 5-methylcytosine positions correspond precisely to those detected in DLD-1 cells. A similar pattern was

observed for patient T8, except for the cytosines at the 5' and 3' ends of the DNA fragment. In the latter case, it is possible that these unmethylated cytosines may not play an important role in the control of the expression of RARβ. It is intriguing to note that these results indicate that primary breast tumors can contain a methylation pattern of RARβ that is almost identical to that of the DLD-1 colon tumor cell line, suggesting the importance of this epigenetic change in different types of neoplastic cells.

Many genes involved in tumor progression have been shown to be silenced by DNA methylation and their expression activated by 5-AZA-CdR in breast cancer. RARβ is another target gene for this analog. These findings suggest that 5-AZA-CdR may be an interesting agent to investigate for the treatment of breast cancer. In order to explore this possibility we have studied the *in vitro* antineoplastic activity of 5-AZA-CdR on MDA-MB-231 human breast carcinoma cell line. Growth inhibition and clonogenic assays show that this analog is a potent inhibitor of cell growth and clonogenic potential in this breast carcinoma cell line. We observed that the growth inhibitory action and the loss of clonogenicity produced by 5-AZA-CdR increased with exposure time and drug concentration. 5-AZA-CdR shows clinical activity against leukemia[21] and advanced lung cancer.[24] Pharmacokinetic analysis shows that the plasma concentration of this analog that produced clinical responses[24] is in the range of the concentration that showed effective *in vitro* antineoplastic activity (Figure 3). Based on these findings, a pilot clinical study in patients with breast cancer resistant to conventional therapy has been initiated with 5-AZA-CdR.

Acknowledgment

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Table 6.1 Comparison of the positions of 5-methylcytosine in the promoter region of RARβ in DLD-1 colon carcinoma cells and human breast tumor biopsies.

Position of 5-methylcytosine[a]	DLD-1 cells	breast tumor #2	breast tumor #8
784	+	+	-
798	+	+	-
810	+	+	-
927	+	+	+
951	+	+	+
957	+	+	+
959	+	+	+
963	+	+	+
968	+	+	+
990	+	+	+
993	+	+	+
1005	+	+	+
1010	+	+	+
1016	+	+	+
1077	+	+	+
1082	+	+	-
1095	+	+	-

[a]The positions of cytosine as designated by the DNA sequence of the $RAR\beta_2$ promoter region (GenBank accession no. X56849).+ , 5-methylcytosine present; - , 5-methylcytosine absent.

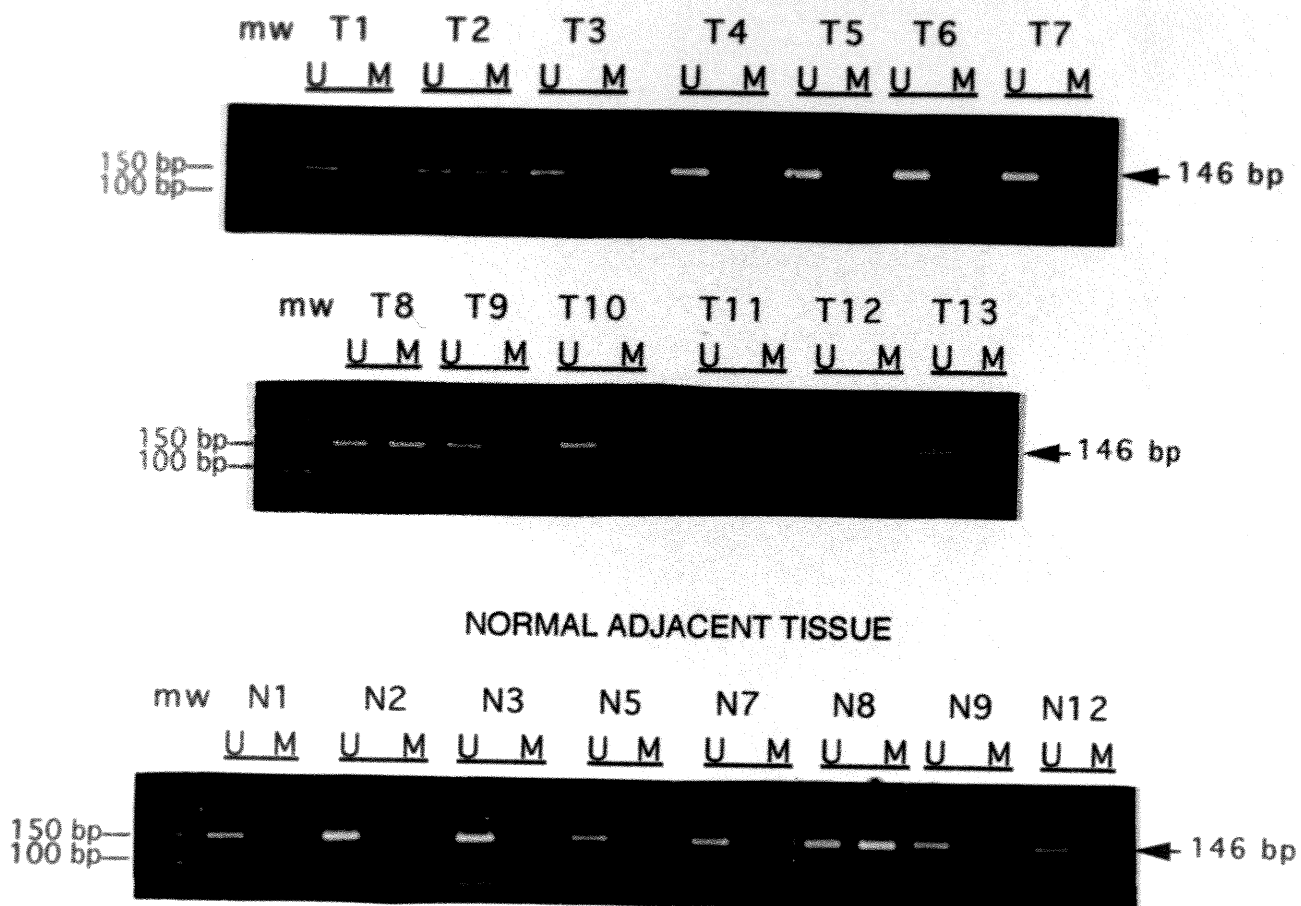

Figure 6.1. Methylation-specific PCR (MSP) analysis of the promoter region of RARβ gene in breast cancer biopsies (T) and normal adjacent breast tissue (N). Genomic DNA was isolated from breast tumors and normal adjacent tissue from different patients. After treatment with bisulfite the DNA was used in the MSP assay with methylation-specific primers (M) and unmethylation-specific primers (U) to amplify a 146 bp DNA fragment.

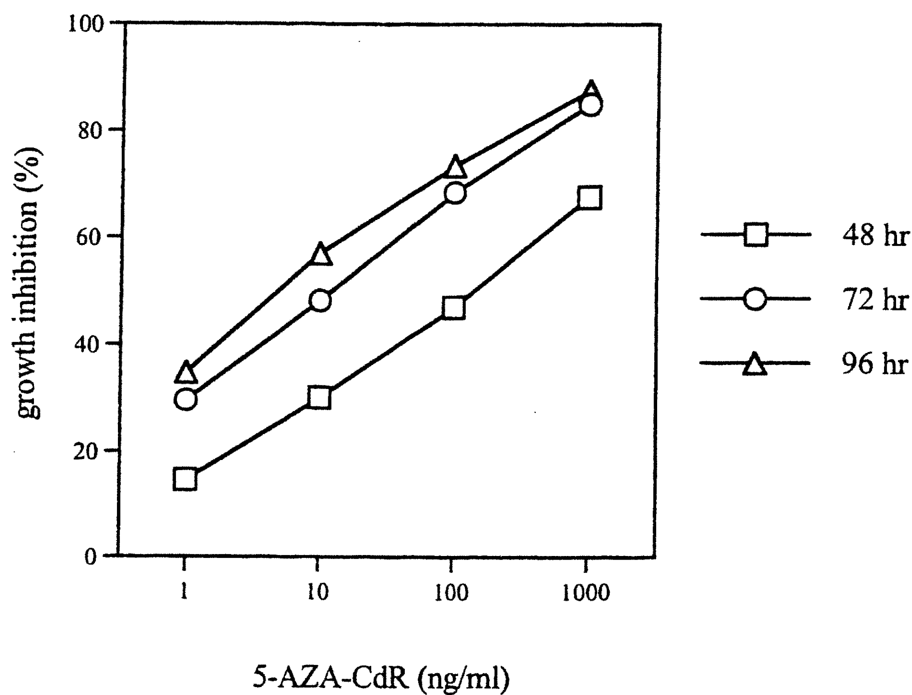

Figure 6.2. Effect of different concentrations of 5-AZA-CdR on the inhibition of growth of human MDA-MB-231 breast carcinoma cells. Cell counts were performed after 48, 72 and 96 hr of drug exposure. Mean values with SE ± 15% are shown, n = 6.

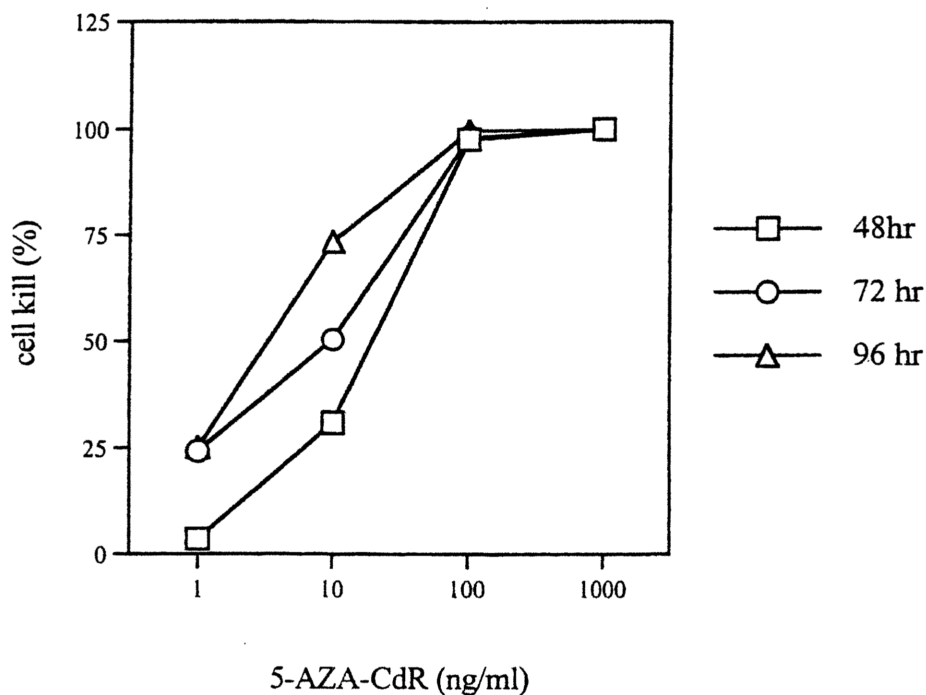

Figure 6.3. Effect of different concentrations of 5-AZA-CdR on colony formation by human MDA-MB-231 breast carcinoma cells. The cells were exposed to the indicated concentrations of 5-AZA-CdR for 48, 72 or 96 hr. The colonies were counted 10-14 days later. Cell kill (%) is defined as the number of colonies formed by drug-treated cells divided by the number of colonies formed by control cells. Mean values with SE ± 15% are shown, n = 6.

CHAPTER 7: QUANTITATION OF THE INHIBITION OF DNA METHYLATION OF RETINOIC ACID RECEPTOR β GENE BY 5-AZA-2'-DEOXYCYTIDINE IN TUMOR CELLS USING SINGLE NUCLEOTIDE PRIMER EXTENSION ASSAY.

Preface

In order to further increase our understanding of the role of DNA methylation in cancer it is important to develop methods that can quantitate the level of methylation of specific genes in tumors. We modified the methylation-sensitive single nucleotide primer extension (Ms-SNuPE), described by Gonzalgo and Jones (1997). This method allows the quantitative analysis of the methylation status of specific sites in a target region.

Bisulfite converted DNA from MDA-MB-231 breast carcinoma cells and DLD1 colon adenocarcinoma cells was used to amplify a 404bp fragment of the promoter region of the RARβ gene by semi-nested PCR. The amplified DNA was used for the SNuPE reaction using specific primers which permit the incorporation of only one molecule of [^{32}P]dCTP if the target site is methylated or of [^{32}P]dTTP if the site is not methylated. The method by Gonzalgo and Jones used polyacrylamide gel electrophoresis and quantification of radioactivity by phosphorimage analysis. Our modifications simplified this method by binding of the labeled DNA to DEAE discs for direct counting of radioactivity. The method has proven to be specific, linear and gives reproducible results. We demonstrated that our method could quantitate the inhibition of methylation produced by 5-AZA-CdR of the RARβ gene in tumor cells.

Quantitation of the Inhibition of DNA methylation of Retinoic Acid Receptor β Gene by 5-Aza-2'-deoxycytidine in Tumor Cells Using Single Nucleotide Primer Extension Assay

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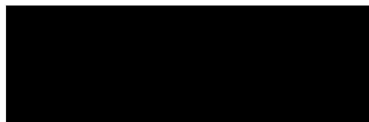

Running Title: QUANTITATION OF INHIBITION OF DNA METHYLATION

ABSTRACT

The expression of several cancer-related genes has been reported to be silenced by DNA methylation of their promoter region. 5-Aza-2'-deoxycytidine (5-AZA-CdR), a potent and specific inhibitor of DNA methylation, can reactivate the *in vitro* expression of these genes. In future clinical trials in tumor therapy with 5-AZA-CdR a method to quantitate its inhibition of methylation of specific tumor suppressor genes would provide important data for the analysis of the therapeutic efficacy of this analogue. We have modified the methylation sensitive-single nucleotide primer extension assay reported by Gonzalgo and Jones (*Nucleic Acid Res.* **25**, 2529, 1997). Genomic DNA was treated with bisulfite and a fragment of the promoter region of human retinoic acid receptor β (RARβ) gene, a tumor suppressor gene, was amplified using semi-nested PCR. Using two different primers we quantitated the inhibition of methylation produced by 5-AZA-CdR at two specific CpG sites in the RARβ promoter in a human colon and a breast carcinoma cell line. The results obtained with the modified assay show a precise and reproducible quantitation of inhibition of DNA methylation produced by 5-AZA-CdR in tumor cells.

Key Words: DNA methylation, tumor suppressor genes, retinoic acid receptor β, 5-aza-2'-deoxycytidine.

INTRODUCTION

DNA methylation is growing in interest as an epigenetic mechanism that can silence the expression of many cancer-related genes (1). The presence of 5-methylcytosine in CpG islands in the 5' promoter region of certain genes presumably interferes with the binding of transcription factors and/or promotes the interaction with methylation-dependent DNA binding proteins (MDBP) to repress transcription. During tumorigenesis *de novo* methylation of CpG islands occurs in different gene types, including tumor suppressor genes (2-5), genes involved in DNA repair (6), tumor metastasis (7, 8) and angiogenesis (9). *In vitro* treatment of human tumor cells with the demethylating agent, 5-AZA-CdR, has been shown to reactivate the expression of many of these cancer-related genes. 5-AZA-CdR shows clinical activity in the treatment of hematological malignancies (10-12) and promising activity in tumor therapy (13). In the evaluation of the clinical response to this analogue in cancer therapy, a method to quantitate its inhibition of DNA methylation in tumor biopsies and leukemic blasts would be very useful in the design of its optimal dose-schedule.

Kuppuswamy et al. (14) were the first to describe the single nucleotide primer extension assay to detect mutations in abnormal alleles. Gonzalgo and Jones (15) extended this assay to assess methylation differences at specific CpG sites and designated it as the Methylation Sensitive Single Nucleotide Primer Extension (MS-SNuPE). One major advantage of the MS-SNuPE method is that it requires only small amounts of genomic DNA. In this method, genomic DNA was treated with bisulfite which converts non-methylated cytosine to uracil while leaving 5-methylcytosine unchanged. The desired sequence was amplified by PCR using specific primers for the modified DNA. The amplified DNA was then used as a template for the single nucleotide extension reaction using a specific primer which permits the incorporation of a single molecule of [^{32}P]dCTP if the target site is methylated or a single molecule of [^{32}P]dTTP if it is not methylated. The product of this reaction was loaded onto a denaturing polyacrylamide gel and radioactivity quantitated by phosphorimage

analysis. This method gave very good quantitation of the methylation status of the p16 tumor suppressor gene (15, 16).

Our laboratory has been investigating the methylation status of the human retinoic acid receptor β (RARβ), a tumor suppressor gene (17,18). We have observed that the promoter region of RARβ is frequently methylated in human colon and breast carcinoma cell lines and primary tumors (19). In the DLD-1 human colon carcinoma cells in which RARβ expression is suppressed by methylation, we demonstrated that 5-AZA-CdR could reactivate its expression (20).

In order to quantitate the inhibition of DNA methylation in these cells with this analogue we modified the Gonzalgo and Jones (15) method by omitting the electrophoresis and phosphorimage analysis and performing direct measurements of radioactivity on DEAE paper circles. In addition, to increase the sensitivity of the method we used semi-nested PCR to eliminate the possible contamination of the DNA template with amplified unmodified DNA. We found that the modified method can quantitate with precision the inhibition of DNA methylation by 5-AZA-CdR of the RARβ in human tumor cells.

MATERIALS AND METHODS

Tissue Culture and Drug Treatment. The human DLD-1 colon adenocarcinoma cell line and the human MDA-MB-231 breast carcinoma cell line were obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were maintained in culture in RPMI 1640 medium (Canadian Life Technologies, Burlington, Ontario) with 10% of fetal calf serum from Wisent (St.Bruno, Quebec) in a 5% CO_2 incubator at 37°C. 5-AZA-CdR (Decitabine) was obtained from Pharmachemie, (Haarlem, The Netherlands). For the methylation analysis, the cells were treated with 1, 10 or 100 ng/ml (as indicated) of 5-AZA-CdR for 72 hr and the genomic DNA was extracted at 96 hr from the beginning of drug treatment. Due to its chemical instability, fresh 5-AZA-CdR was added to the medium every 24 hr. Genomic DNA was extracted using the DNA Extraction kit (Stratagene, La Jolla, CA) following the manufacture's instructions.

Bisulfite treatment of genomic DNA. The genomic DNA obtained from the tumor cells was treated with bisulfite in order to convert all the unmethylated cytosines to uracil, leaving the methylated cytosines unconverted, as described previously (19).

PCR amplifications and primers. The bisulfite-treated genomic DNA was amplified using semi-nested PCR. The primers: sense 5'-TTA AG(C/T) TTT GTG AGA ATT TTG-3' at position #717-736 and the antisense: 5'-CCT ATA ATT AAT CCA AAT AAT C-3' at position #1120-1141 amplified a 426 bp fragment of the promoter region of RARβ (GenBank acc. no. X56849). Using the 426 bp DNA fragment as template a semi-nested PCR was performed with a new sense primer: 5'-GAG TTG GTG ATG TTA GAT TAG-3' at position #738-758 and the same antisense primer used above to amplify a 404 bp fragment. PCR amplifications were performed in 50μl reaction mixture containing 5-10 ng of bisulfite-treated DNA or 1,000-fold dilution of the first PCR product for the semi-nested PCR. The reaction mixture contained 10μM dNTPs, 2.5 μM primers, 1x Qiagen buffer containing 1.5 mM $MgCl_2$ and 1.2 units of Qiagen HotStar polymerase. The reaction was carried on

in a thermal minicycler (MJ Research, Watertown, MA) under the following conditions: 94°C for 15 min as suggested by the manufacturer followed by 94°C for 1 min, 50°C for 30 sec, 72°C for 1 min for 38 cycles; and 72°C for 3 min. The PCR products were isolated on a 2% agarose gel and purified using the QIAquick Gel Extraction Kit by Qiagen (Qiagen, Mississauga, Ontario).

Single Nucleotide Primer Extension Reaction: The Single Nucleotide Primer Extension assay was performed as described by Gonzalgo and Jones (15) with some modifications. The 404 bp amplified fragment of RARβ promoter region was used for the quantitation of methylation at the CpG sites at positions #951 (primer A) and #990 (primer B). The sequence for nucleotide extension primer A was: 5'-GTT GTT TGA GGA TTG GGA TGT-3' located at position #930-950; and for nucleotide extension primer B was: 5'-AGT AGG GTT TGT TTG GGT AT-3' located at positions #970-989. The reaction (25 μl) contained 20 ng of 404 bp amplified DNA, 1x buffer (GIBCO/BRL), 1 mM $MgCl_2$, 1 μM primers A or B, 0.1-1.0 μCi of either [α^{32}P]dCTP or [α^{32}P]dTTP, and 1 unit of Platinum Taq Polymerase (GIBCO/BRL). The conditions for primer extension were: 94°C for 5 min, 94°C for 1 min, 50°C for 2 min and 72°C for 1 min. The reaction was stopped by addition of 1 μl of 0.5 M EDTA to each tube. The complete reaction mixtures were applied onto 2.3 cm diameter Whatman DE81 circles (Fisher Scientific, Montreal) and placed at room temperature for 10 min. Individual circles were placed into each well of a Costar 6-multiwell dish (3.5 cm diameter/well) and then washed 5 times with 4 ml of 3X SSC (0.45 M NaCl; 0.045 M sodium citrate; pH 7.0) and 3 times with 4 ml H_2O. The circles were in the presence of each wash solution for 10 min with continuous shaking at 100 rpm using Junior Orbit Shaker (Lab-Line, Melrose Park, Ill). The circles were placed in 5 ml of EcoLite scintillation liquid (ICN) and the radioactivity measured in Beckman LS 6000IC scintillation counter.

Standard Curve: After bisulfite treatment and semi-nested PCR, the standard curve for DNA containing different amounts of 5-methylcytosine in the promoter region of RARβ was prepared by mixing normal human genomic White Blood Cell

(WBC) DNA (Roche Diagnostics, Laval, Quebec) containing unmethylated RARβ with genomic DNA from DLD-1 human colon carcinoma cells in which the promoter region RARβ gene was fully methylated (20). The 404 bp amplified DNAs were mixed at different ratios to obtain a mixture of 100, 75, 50, 25 and 0% methylated DNA. The cpm of each sample obtained with the Single Nucleotide Primer Extension assay were determined for each radioactive substrate. The C/T ratio was calculated by dividing the [^{32}P]dCTP cpm by the [^{32}P]dTTP cpm. The C/T ratio was plotted on a semilogarithmic scale against a linear scale of 5-methylcytosine (5-MC) content in the standard samples.

RESULTS

In order to establish a standard curve we mixed human DLD-1 DNA containing 100% 5-MC RARβ promoter DNA with normal human WBC DNA containing 0% 5-MC RARβ promoter DNA to obtain a mixture of 100, 75, 50, 25 and 0% 5-MC DNA. The starting DNAs were previously treated with bisulfite, the 404 bp DNA fragments of the RARβ promoter were synthesized by semi-nested PCR, and the DNAs purified. Semi-nested PCR was needed in order to achieve specific amplification of the target sequence from bisulfite-treated DNA. This gave us the template specificity needed for the nucleotide extension reaction. Single PCR amplification of bisulfite-treated DNA produced inconsistent results with the nucleotide extension reaction due to non-specific amplification of unconverted DNA (data not shown).

The Single Nucleotide Primer Extension was performed using these purified DNAs as templates with primer A or B and dCTP-[^{32}P] or dTTP-[^{32}P] as substrates. The radioactivity incorporated for each of these nucleotides was used to calculate the C/T ratio for each template (Table 7.1). The C/T ratio for the 50% 5-MC, which should theoretically be 1, differs from this expected value most likely due to the different specific activities of the two radioactive substrates. In addition, their rates of incorporation by the Taq DNA polymerase may differ as reported for the dideoxynucleotides (21). For the same reason, the incorporation of dCTP for the 100% 5-MC is higher than the incorporation of dTTP for the 0% 5-MC. In Fig. 7.1 is shown the plot of the C/T values on a log scale versus the 5-MC content using the data from Table 7.1. The correlation coefficient (r) between C/T values and the 5-MC content was excellent for both primers A and B giving values of >0.96. The 75, 50 and 25% 5-MC DNAs show a linear decrease of incorporation of C over T that parallels the decreasing content of 5-MC. The C/T ratio was calculated from the mean of three experiments. This figure was used to estimate the reduction of % 5-MC content in the RARβ promoter after treatment with 5-AZA-CdR in two human tumor cell lines.

For DLD-1 tumor cells containing 100% 5-MC, the incorporation of C was significantly higher than the incorporation of T for both primers A and B and gave

C/T values greater than 10. On the other hand for the WBC containing 0% 5-MC, the incorporation of T was much greater than C for both primers and gave C/T values less than 0.08 (Table 7.1).

Primer A which detects methylation at position #951 of the RARβ promoter appeared to be more specific than primer B which detects methylation at position #990. Primer B showed higher incorporation of dTTP (>2,000 cpm) for the 100% 5-MC sample as compared to <400 cpm for primer A. For the 0% 5-MC sample, primer B also showed higher incorporation of dCTP (>1,500 cpm) as compared to primer A (<400 cpm).

We quantitated the inhibition of DNA methylation of RARβ produced by 5-AZA-CdR in human DLD-1 colon carcinoma cells and human MDA-MB-231 breast carcinoma cells. After treatment of these tumor cells with 100 ng/ml for 72 hr, genomic DNA was isolated at 96 hr and prepared for the single nucleotide primer extension assay as described above. The inhibition of methylation after 5-AZA-CdR was estimated from the standard curves in Fig.7.1. Treatment of DLD-1 tumor cells with 100 ng/ml 5-AZA-CdR produced 50.0 ± 2.0 and 50.6 ± 2.9 % methylation inhibition for primers A and B, respectively (Table 7.2). A similar treatment with 5-AZA-CdR of the MDA-MB-231 tumor cells produced a 38.7 ± 4.7 and 35.7 ± 5.3% inhibition of methylation for primers A and B, respectively. The results obtained with lower concentrations of this analogue were more variable. For the DLD-1 tumor cells 10 ng/ml of 5-AZA-CdR showed 30.1 ± 4.0 and 18.7 ± 0.9% inhibition of methylation for primers A and B, respectively. For the MDA-MB-231 tumor cells 5-AZA-CdR at 1 ng/ml produced 13.7 ± 3.6 and <1% inhibition with primers A and B, respectively.

DISCUSSION

Silencing of the expression of cancer-related genes, especially tumor suppressor genes, by methylation of CpG islands in their promoter regions appears to play an important role in tumorigenesis (1). The bisulfite method for sequencing DNA containing 5-methylcytosine (22) has led to the development of new approaches to analyze the methylation status of genes, including methylation-specific PCR (MSP) (23) and SNuPE (14). One advantage of the SNuPE method is that it can quantitate the differences in methylation status of genes. The activation of tumor suppressor genes and other cancer-related genes by the hypomethylating agent, 5-AZA-CdR (3, 24-26), has created noteworthy interest in its clinical potential in cancer therapy. Clinical trials on 5-AZA-CdR in patients with cancer would be more informative if a method to quantitate its inhibition of DNA methylation in tumor biopsies could be used.

In this study we have modified the method of Gonzalgo and Jones (14) to quantitate the inhibition of methylation of the human RARβ gene in tumor cells after treatment with 5-AZA-CdR. The advantages of our revised method are the omission of the polyacrylamide gel electrophoresis, its simplicity and rapidity. These modifications did not compromise the specificity of our method. The semi-nested PCR gives us the specificity we needed to run the assay without electrophoresis. In order to determine if our method could also be used for other target genes, we performed the nucleotide extension assay on the p16 tumor suppressor gene. We performed semi-nested PCR for this gene and used the extension primers of Gonzalgo and Jones (1997). Our preliminary results produced standard curves with correlation coefficients for the C/T values versus the %5-MC content of >0.98 for their primers 1 and 2 and of >0.95 for their primer 3 (data not shown).

Our modified method gave excellent reproducibility and precision for the tumor cells treated with 100 ng/ml of 5-AZA-CdR. For example, with primer A this drug treatment produced 50.0 ± 2.0 % inhibition of methylation as compared to 50.6 ± 2.9 % inhibition with primer B in the DLD-1 colon tumor cells (Table 7.2). With the MDA-MB-231 breast tumor cells this 5-AZA-CdR treatment produced 38.7 ± 4.7 % inhibition of methylation for primer A as compared to 35.7 ± 5.3 % inhibition for

primer B (Table 7.3). The differences in the absolute counts reported for the incorporation of radioactive nucleotides in some experiments was due to the different amounts of μCi of ^{32}P used in the assay. The use of C/T ratio corrects for these differences in the total amount of radioactivity used in the reaction.

The reproducibility and precision of our method is also illustrated by the excellent correlation of the C/T values with the % 5-MC as shown by the correlation coefficients of 0.967 for primer A and 0.986 for primer B (Fig. 7.1). The lower concentrations of 5-AZA-CdR (1 and 10 ng/ml) showed results that were more variable. This was most likely not due to our nucleotide extension assay, but resulted from the low drug concentrations that were in the range that produced a weak and variable response in our *in vitro* clonogenic assays on tumor cells (19).

Primer A appeared to be more specific than primer B since the former primer gave a relative incorporation of dCTP much greater than dTTP for the 100% 5-MC template (Table 7.1). In addition, with the 0% 5-MC template the relative incorporation of dTTP was much greater than dCTP for primer A as compared to primer B. This difference in primer specificity may be due to improper annealing of a small fraction of primer B to the template. It is interesting to note that the DNA melting temperature of primer A is higher than primer B.

In our initial experiments we used direct PCR to amplify bisulfite-treated DNA with a single set of primers and obtained variable results with our single nucleotide extension assay. This was probably due to amplification of variable amount of unmodified DNA (27). In order to overcome this problem we used semi-nested PCR which is an excellent approach to eliminate the presence of any contaminating unmodified DNA. We also selected primers for semi-nested PCR that contained cytosines that were converted to uracils (thymine) by the bisulfite treatment.

It has been reported that after bisulfite treatment, DNA containing 5-MC may amplify at a different rate that DNA containing no 5-MC (28). In order to check for this bias, we suggest that standard RARβ DNA containing 50% 5-MC be amplified by PCR and used in the SNuPE assay. Any large difference from the theoretical 50% 5-MC should be apparent. Using this type of assay we did not detect significant bias

(<10%) from the theoretical value for the amplification of the bisulfite-converted DNA for RARβ (data not shown).

Our modified method can be used to rapidly analyze many samples due to the facility of working with DEAE discs as compared to polyacrylamide electrophoresis and phosphorimage analysis. It should be possible to also use tritium labeled nucleotides with this method. We have used tritium labeled substrates for enzyme assays on DEAE discs (29).

In conclusion, we have demonstrated that our modification of the single nucleotide extension assay can be a useful method to quantitate with precision the changes in 5-methylcytosine content in DNA produced by inhibitors of DNA methylation.

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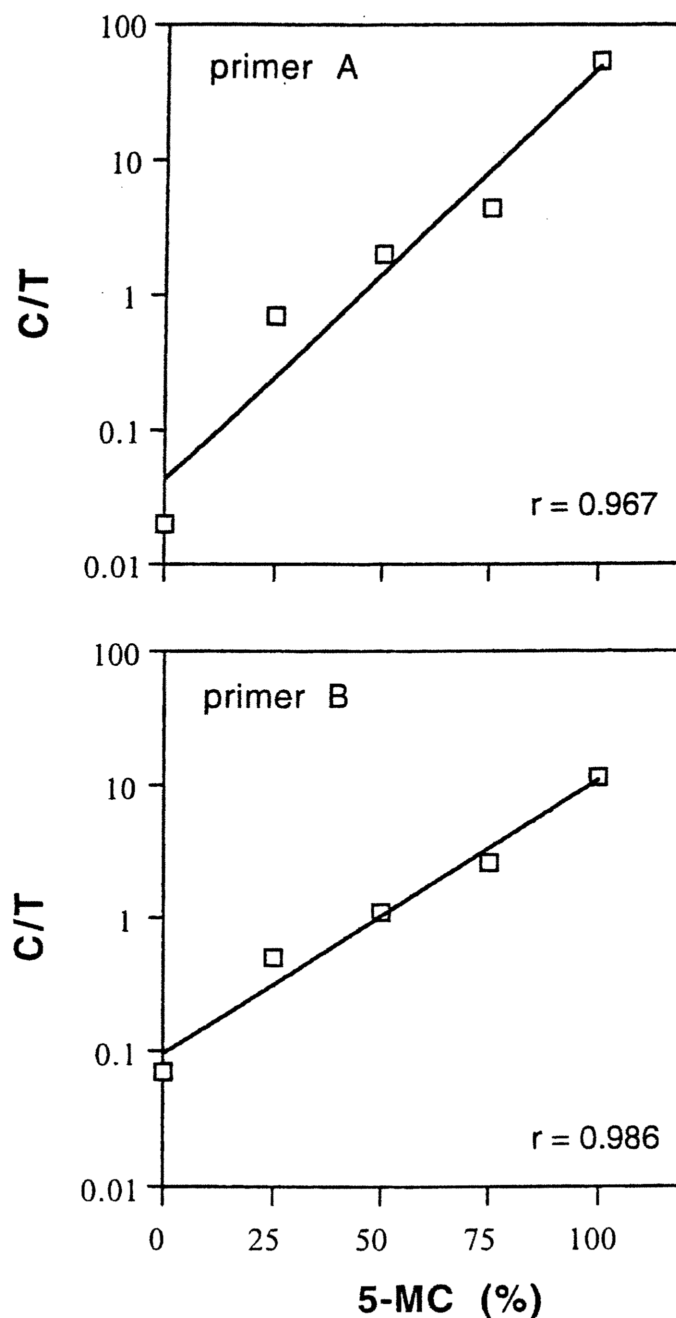

FIG. 7.1 Standard curve for the correlation of % of 5-methylcytosine (5-MC) in the RARβ promoter DNA with the C/T value for primer A and primer B using the single nucleotide primer extension assay. Experimental details are given in the legend to Table 1 and in Materials and Methods.

TABLE 7.1

Incorporation of [^{32}P]-dCTP or [^{32}P]-dTTP into Promoter Region of Human RARβ Gene Containing Different Amounts of 5-Methylcytosine Using Nucleotide Primer Extension Reaction

Primer	5-MC (%)	Experiment 1 [^{32}P]-dCTP (cpm)	Experiment 1 [^{32}P]-dTTP (cpm)	Experiment 2 [^{32}P]-dCTP (cpm)	Experiment 2 [^{32}P]-dTTP (cpm)	Experiment 3 [^{32}P]-dCTP (cpm)	Experiment 3 [^{32}P]-dTTP (cpm)	C/T
A	100	22,271	383	14,288	296	92,983	1,631	54 ± 3
A	75	11,630	3,417	13,812	2,444	73,504	17,707	4.4 ± 0.6
A	50	10,922	5,892	9,602	3,781	53,579	33,440	2.0 ± 0.2
A	25	5,951	8,331	6,392	7,489	25,588	48,361	0.7 ± 0.09
A	0	324	10,266	176	9,301	1,021	58,095	0.02 ± 0.01
B	100	30,936	2,083	34,178	3,629	70,885	7,030	11.4 ± 1.7
B	75	23,488	7,914	31,619	13,210	52,377	21,827	2.6 ± 0.2
B	50	17,023	11,814	19,801	19,271	38,368	38,523	1.1± 0.1
B	25	9,000	15,275	12,397	23,357	21,245	48,948	0.5 ± 0.05
B	0	1,737	18,016	2,163	30,576	4,463	58,455	0.07 ± 0.01

Genomic DNA from human DLD-1 tumor cells containing fully methylated CpG sites (100%) of the promoter region of RARß was prepared. This methylated DNA and non-methylated RARß DNA from human WBC were treated with bisulfite and used to amplify 404 bp of RARß promoter region by semi-nested PCR. DNA containing different % of 5-methylcytosine (5-MC) were prepared by mixing methylated with non-methylated RARß DNA. Primers A and B detect 5-MC at positions #951 and #990, respectively, of the RARß promoter region (acc. no. X56849). C/T values are mean ± S.E.; n = 3. Differences in the absolute cpm between experiments was due to the different amounts of total radioactivity used in the assay.

TABLE 7.2

Nucleotide Primer Extension Analysis of Incorporation of [^{32}P]-dCTP or [^{32}P]-dTTP into Promoter Region of RARβ Gene in DLD-1 Human Colon Carcinoma Cells before and after Treatment with 5-AZA-CdR

Primer	5-AZA-CdR (ng/ml)	Experiment 1		Experiment 2		Experiment 3			Inhibition DNA methylation (%)
		[^{32}P]-dCTP (cpm)	[^{32}P]-dTTP (cpm)	[^{32}P]-dCTP (cpm)	[^{32}P]-dTTP (cpm)	[^{32}P]-dCTP (cpm)	[^{32}P]-dTTP (cpm)	C/T	
A	0	21,806	410	19,082	294	1,642	27	59.5 ± 3.4	-
A	10	16,631	2,723	12,866	2,482	1,269	225	5.6 ± 0.3	30.1 ± 4.0
A	100	9,771	6,116	8,374	6,470	689	470	1.4 ± 0.01	50.0 ± 2.0
B	0	55,513	4,806	69,258	5,250	40,326	3,358	12.2 ± 0.5	-
B	10	44,784	10,304	52,647	12,137	30,495	6,131	4.5 ± 0.2	18.7 ± 0.9
B	100	25,630	22,981	30,844	28,312	19,513	15,073	1.1 ± 0.06	50.6 ± 2.9

DLD-1 tumor cells were treated at the indicated concentrations of 5-AZA-CdR for 72 hr and genomic DNA isolated at 96 hr. The DNA was treated with bisulfite and semi-nested PCR used to amplify a 404 bp fragment of RARß promoter region. The nucleotide primer extension assay was performed as described in Table 1. Inhibition of DNA methylation was estimated from the standard curves in Fig. 1. C/T and inhibition of DNA methylation values are mean ± S.E.; n = 3. Differences in the absolute cpm between experiments was due to the different amounts of total radioactivity used in the assay.

TABLE 7.3

Nucleotide Primer Extension Analysis of Incorporation of [^{32}P]-dCTP or [^{32}P]-dTTP into Promoter Region of RARβ Gene in MDA-MB-231 Human Breast Carcinoma Cells before and after Treatment with 5-AZA-CdR

Primer	5-AZA-CdR (ng/ml)	Experiment 1 [^{32}P]-dCTP (cpm)	Experiment 1 [^{32}P]-dTTP (cpm)	Experiment 2 [^{32}P]-dCTP (cpm)	Experiment 2 [^{32}P]-dTTP (cpm)	Experiment 3 [^{32}P]-dCTP (cpm)	Experiment 3 [^{32}P]-dTTP (cpm)	C/T	Inhibition DNA methylation (%)
A	0	60,333	12,404	68,125	8,603	35,932	4,802	6.7 ± 0.9	-
A	1	62,182	25,963	81,967	27,840	38,263	7,897	3.3 ± 0.7	13.7 ± 3.6
A	100	37,838	51,466	50,619	56,870	24,217	14,459	1.1 ± 0.3	38.7 ± 4.7
B	0	22,795	1,278	33,180	2,064	40,512	1,764	18.9 ± 2.1	-
B	1	31,403	1,305	40,377	1,128	43,783	860	37.0 ± 7	<1
B	100	18,011	9,162	32,033	15,358	34,408	11,710	2.2 ± 0.3	35.7 ± 5.3

MDA-MB-231cells were treated at the indicated concentrations of 5-AZA-CdR for 72 hr and genomic DNA isolated at 96 hr. The DNA was treated with bisulfite and semi-nested PCR used to amplify a 404 bp fragment of RARβ promoter region. The nucleotide primer extension assay was performed as described in Table 1. Inhibition of DNA methylation was estimated from the standard curves in Fig. 1. C/T and inhibition of DNA methylation values are mean ± S.E.; n = 3. Differences in the absolute cpm between experiments was due to the different amounts of total radioactivity used in the assay.

CHAPTER 8: ANTINEOPLASTIC ACTION AND EXPRESSION OF RETINOIC ACID RECEPTOR β GENE IN BREAST CARCINOMA CELLS TREATED WITH 5-AZA-2'-DEOXYCYTIDINE AND AN HISTONE DEACETYLASE INHIBITOR[1]

Preface

Based on the observations by Cameron et al (1999) of a synergistic interaction of 5-AZA-CdR and TSA, a specific histone deacetylase inhibitor, on reactivation of some tumor suppressor genes, we investigated the antineoplastic potential of these two agents on MDA-MB-231 breast carcinoma cells. Using a clonogenic assay, we found that both agents alone show antitumor activity on these cells. The combination of the two drugs resulted in synergistic interaction that produced a complete loss of clonogenicity of the breast carcinoma cells.

In order to understand the molecular mechanisms underlying this interaction, we investigated the effects of 5-AZA-CdR, TSA and their combination on the expression of two tumor suppressor genes, RARβ and estrogen receptor (ER). These two genes have been reported to be silenced by aberrant methylation in primary breast tumors and in the MDA-MB-231 breast carcinoma cell line. We found that 5-AZA-CdR reactivated expression of both RARβ and ER, but TSA only reactivates RARβ expression. These results suggest the presence of different levels of gene expression regulation by interaction of epigenetic mechanisms that involve DNA methylation and histone deacetylation.

Antineoplastic Action and Expression of Retinoic Acid Receptor β Gene in Breast Carcinoma Cells Treated with 5-Aza-2'-deoxycytidine and an Histone Deacetylase Inhibitor[1]

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[3]The abbreviation used are: HDAC, histone deacetylase; 5-AZA-CdR, 5-aza-2'-deoxycytidine; TSA, trichostatin A; RARβ, retinoic acid receptor ß; ER, estrogen receptor.

ABSTRACT

During tumorigenesis it has been reported that several cancer-related genes can be silenced by aberrant methylation. In many cases these silenced genes can be reactivated by exposure to the DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-AZA-CdR). Histone acetylation also plays a role in the control of expression of some genes. Deacetylation of histones leads to a compact chromatin structure that is transcriptionally inactive. In a recent report, Cameron et al. (Nature Genet. 21: 103,1999) observed synergy between 5-AZA-CdR and the histone deacetylase inhibitor, Trichostatin A (TSA) for the activation of tumor suppressor genes. We have studied the antineoplastic activity of 5-AZA-CdR and TSA combination on MDA-MB-231 breast carcinoma cells. We have found that both drugs alone showed significant antineoplastic activity. The combination of the two drugs was synergistic with respect to its cell kill. MDA-MB-231 cells have been reported to lack expression of the tumor suppressor gene, retinoic acid receptor β (RARβ) and of the estrogen receptor gene (ER). 5-AZA-CdR alone activated the expression of both RARβ and ER. TSA alone only activated RARβ, but not ER. These agents in combination appeared to produce a greater activation of both genes than either agent alone. Different epigenetic mechanisms appear to control the expression of these two genes. One hypothesis is that ER is locked in the silenced state predominantly by methylation. On the other hand, neither methylation nor histone deacetylation alone can completely silence the expression of RARβ. This interesting interaction provides some rationale for the use of inhibitors of DNA methylation and histone deacetylation in combination for the chemotherapy of breast cancer.

INTRODUCTION

It has been widely shown that an epigenetic mechanism that involves the methylation of CpG islands in their promoter region can be responsible for the silencing of many cancer-related genes (1,2). Another epigenetic mechanism that can regulate gene expression is the acetylation of chromosomal histones (3). Highly acetylated regions of chromatin contain transcriptionally active regions while hypoacetylated chromatin is transcriptionally silent (4). Methylated DNA is often found associated to deacetylated histones (5,6). These observations led to the hypothesis that these two phenomena could be linked and act in concert on regulation of gene expression. Jones et al. (7) and Nan et al. (8) found that a methyl-CpG binding protein, MeCP2, coprecipitates with HDAC[3] activity. MeCP2 is found in multiprotein complexes that can include HDACs and transcriptional corepressors (9). The MeCP2 complex recognizes and binds the methylated CpG dinucleotides leading to gene silencing. Their interaction was clarified by Cameron et al. (10) who observed a synergy between the DNA methylation inhibitor, 5-AZA-CdR, and the HDAC inhibitor, TSA, with respect to the reactivation of tumor suppressor genes.

Based on the observation of this synergistic activation of tumor suppressor genes, we proposed the hypothesis that HDAC inhibitors, such as TSA, should enhance the antineoplastic action of 5-AZA-CdR on tumor cells. 5-AZA-CdR is an experimental anticancer drug that has shown promising preclinical (11,12) and clinical activity (13,14). HDAC inhibitors also show promising preclinical antineoplastic activity (15-17). Both classes of inhibitors can induce differentiation in neoplastic cells (17-19) suggesting that the combination may have enhanced antitumor activity.

In this study we investigated the *in vitro* antineoplastic activity of 5-AZA-CdR and TSA on MDA-MB-231 breast carcinoma cells. In addition, we studied the effects of these agents on the tumor suppressor gene, retinoic acid receptor β (RARβ) and the estrogen receptor (ER), two genes that play important roles in breast cancer progression. The MDA-MB-231 cells lack expression of RARβ (20), and of ER gene (21). Lack of expression of the RARβ has been reported for several primary tumors, including breast cancers (20,22). We reported previously that RARβ is silenced by

methylation in DLD-1 colon carcinoma cells and it can be reactivated by exposure to 5-AZA-CdR (23). Loss of ER expression occurs frequently in advanced breast cancer that becomes unresponsive to hormonal therapy (24). Hypermethylation of CpG islands in the 5'-region of the ER gene has been found in different tumor types, including breast cancer and its expression was demonstrated to be reactivated after treatment 5-AZA-CdR (25).

In this report, using a clonogenic assay, we demonstrated that the drug combination was synergistic with respect to its antineoplastic activity on the MDA-MB-231 breast carcinoma cells. Our results also showed that both RARβ and ER genes were reactivated by 5-AZA-CdR alone, whereas only RARβ was significantly reactivated by treatment with TSA alone. In addition, the combination appeared to produce greater gene activation than either agent alone. Furthermore, we showed that TSA did not further decrease promoter demethylation produced by 5-AZA-CdR. A preliminary report of this work was presented (26).

MATERIALS AND METHODS

Cell line. The human MDA-MB-231 breast carcinoma cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium (Life Technologies, Burlington, Ontario) containing 10% heat-inactivated fetal calf serum (Wisent, St. Bruno, Quebec) and kept in a 5% CO_2 incubator at 37°C.

Clonogenic assay. MDA-MB-231 cells were plated at 100 cells/well of a six-well dish in 2 ml of medium. 5-AZA-CdR was obtained from Pharmachemie (Haarlem, The Netherlands), dissolved in 0.45% NaCl containing 10mM sodium phosphate (pH 6.8), and stored at -70°C. TSA was obtained from Wako BioProducts, Richmond, Va, dissolved absolute ethanol and stored at -20°C. For establishing a dose response curve, cells were treated with 5-AZA-CdR or TSA at concentrations ranging from 5 to 100 ng/ml, for 48 hours. Due to its chemical instability, 5-AZA-CdR was added to the medium every 24 hours. To assess the effect of combination of the two drugs on MDA-MB-231 cells, we treated these cells with 5-AZA-CdR 25

ng/ml and TSA 50 ng/ml, sequentially for 48 hours. Colonies were counted at day 10.

Reverse Transcriptase PCR (RT-PCR) Analysis. In order to study the reactivation of RARβ and ER genes in MDA-MB-231 cells, we treated the cells with 5-AZA 50 ng/ml (250nM) and TSA 181ng/ml (600nM), alone or in combination as a sequential exposure for 48 hours. Cells were harvested 15-20 hours after removal of the drugs. Total RNA was extracted from 1x106 cells using the RNeasy mini kit (Qiagen) the remaining cells were pelleted and frozen for subsequent DNA extraction. For cDNA synthesis, 500 ng of total RNA were used in 20 μl of reaction mixture using the RT OmniScript by Qiagen. The reaction mixture also contained 1x RT buffer (Qiagen), dNTPs 0.5mM each, random hexamers (1μM), RNase inhibitor (0.5 μM) ± RT (4 units). The reaction was performed at 37°C for 1h, followed by 5 min at 93°C to inactivate the enzyme. The PCR amplification was performed using HotStar Taq Polymerase (Qiagen), using specific primers spanning different exons for RARβ and ER. The primers of RARβ (accession no. Nm_000965) were: sense primer 5'-AGA GTT TGA TGG AGT TGG GTG GAC-3' (position 229-252) and antisense primer 5'-GAC GAG TTC CTC AGA GCT GGT G-3' (position 495-516). For the ER gene (accession no. NM_000125) the primers were: sense primer 5'-CAC CCT GAA GTC TCT GGA AG-3' (position 1752-1771) and antisense 5'-GGC TAA AGT GGT GCA TGA TG-3' (position 2200-2219). Microglobulin housekeeping gene was amplified as an internal control. The PCR profile consisted of 95°C for 15 min to allow the antibody to detach from the Taq polymerase and activate it; 94°C for 1 min, 59-61°C, 72°C for 1m for 5 cycles, and then the annealing temperatures were lowered to 57-59°C for 30 more cycles for a total of 35 cycles. Less cycles were used for the internal control gene, microglobulin. The reactions were performed in a programmable minicycler. For each gene we made sure that the DNA amplification did not reach a plateau. The PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining.

Methylation sensitive-single nucleotide primer extension (MS-SNuPE) Assay. Following drug treatment of MDA-MB-231 cells as described for RT-PCR, cell pellets were thawed and genomic DNA was extracted using the DNeasy mini kit

(Qiagen). Genomic DNA was treated with bisulfite as previously described (11). Seminested PCR was performed to amplify a 404 bp fragment of the promoter region of RARβ gene. This amplified DNA was used to perform the MS-SNuPE assay as previously described (27). Briefly, 20 ng of the 404 bp amplified DNA were used in 25 μl of reaction containing 1x buffer (Gibco/BRL), 1mM $MgCl_2$, 1 μM primers A or B, 0.1-1 μCi of either [α^{32}P]dCTP or [α^{32}P]dTTP, and 1 unit of platinum Taq polymerase (Gibco/BRL). The conditions for primer extension were as described previously (27). The reaction mixtures were applied onto DEAE circles and the circles washed 5 times with 3xSSC and then rinsed with water. The radioactivity was measured in a Beckman LS 6000IC scintillation counter.

RESULTS

The dose-response curve of 5-AZA-CdR and TSA shows that 5-AZA-CdR at higher concentrations is a more potent antitumor agent than TSA against MDA-MB-231 breast carcinoma cells (Fig.8.1). At a concentration of 100 ng/ml, 5-AZA-CdR produced 89.2% cell kill, while TSA at the same concentration produced a cell kill of only 64.7%. We were interested in a possible additive effect on cell kill when MDA-MB-231 cells were exposed to both 5-AZA-CdR and TSA. Cells were exposed sequentially for 48 hours to 5-AZA-CdR 25ng/ml and then to TSA 50 ng/ml for 48 additional hours (Fig.8.2). For the combination, we chose the concentrations of drugs that gave a cell kill of approximately 50%. We observed an additive effect of the two agents on MDA-MB-231 cell kill using the Valeriote and Lin (28) analysis. This result is particularly interesting since the combination of 5-AZA-CdR and TSA reaches values of cell kill of 100%, which may have relevance in the clinical treatment of patients with breast cancer.

In order to study the effect of 5-AZA-CdR and TSA on the reactivation of cancer-related genes that have been silenced by epigenetic mechanisms, we designed primers for two tumor suppressor genes, RARβ and ER, reported to be silenced in MDA-MB-231 cells (20,21). Treatment of the cells with 5-AZA-CdR 50 ng/ml reactivated both genes, as expected, by demethylation of DNA and subsequent reactivation of these genes. (Fig. 8.3). Our results also show that RARβ, but not ER, is reactivated by TSA alone. We also wanted to determine if the effect of increased reactivation of RARβ by TSA in combination with 5-AZA-CdR was not due an enhancement of promoter hypomethylation produced by this nucleoside analog. We performed a MS-SNuPE assay to quantitate the methylation status of two specific CpG sites in the promoter region of RARβ after drug treatment (Table 8.1). Our results show that TSA did not alter the methylation status of the target sites. In addition, exposure of cells to TSA after previous treatment with 5-AZA-CdR had no further effect on DNA methylation.

DISCUSSION

5-AZA-CdR has been shown to be an interesting antineoplastic agent that shows clinical activity both in leukemia and lung cancer (13,14). 5-AZA-CdR is a potent demethylating agent. Demethylation of cancer-related genes that have been silenced by aberrant methylation by 5-AZA-CdR can lead to gene activation and differentiation (25). This mechanism of action of 5-AZA-CdR makes it a very promising agent to use in cancer chemotherapy. The use of this analog in combination with other agents that induce differentiation may lead to an even more effective therapy for breast cancer.

HDAC inhibitors are a new class of antineoplastic agents which increase histone acetylation leading to a more "open" chromatin structure that is accessible to transcription factors (29). This action by HDAC inhibitors can lead to re-expression of some genes and induction of differentiation. HDAC inhibitors show interesting antineoplastic activity against various tumor cell lines (30).

Interaction between DNA methylation and histone acetylation in the control of gene expression is currently under intensive investigation. In a recent report Cameron et al. (10) showed that 5-AZA-CdR and TSA in combination produced a synergistic reactivation of tumor suppressor genes. These results suggested to us that the combination of inhibitors of DNA methylation and histone deacetylation might show enhanced antitumor activity.

Our results as evaluated by clonogenic assay showed that the human breast cancer cell line MDA-MB-231 was sensitive to the antineoplastic action of either 5-AZA-CdR or TSA (Fig. 8.1). Interestingly, sequential treatment of these cells led to a synergistic interaction between the two agents in which the combination produced 100% cell kill (Fig. 8.2).

In order to understand the mechanism of this interaction we studied the effect of 5-AZA-CdR and TSA on the activation of two genes silenced by aberrant methylation in MDA-MB-231 cells. It has been reported that this cell line and other breast cancers lack or show reduced expression of RARβ (20,22). In our laboratory we showed previously that RARβ gene was silenced by DNA methylation and reactivated after treatment with 5-AZA-CdR in human DLD1 colon carcinoma cells

(23). In this study we demonstrated that in MDA-MB-231 breast cancer cells RARβ can be reactivated by treatment of either 5-AZA-CdR or TSA alone (Fig. 8.3). This finding confirms the report by Sirchia et al. (31). The reactivation of RARβ by the combination appears to be greater than either agent alone which is in agreement with the reports by Cameron et al. (10) for several cancer-related genes and Chiurazzi et al. (32) for the FMR1 gene. The hypothesis to explain these results is that RARβ gene silencing is due to both DNA methylation and histone deacetylation. It appears that DNA methylation alone is not sufficient to lock this gene in the silenced state without concomitant histone modifications.

The second gene that we investigated was the ER gene which was reported to be silenced by hypermethylation in the MDA-MB-231 breast cancer cell line (25). In contrast to the RARβ gene, we observed that expression of ER gene could not be induced by TSA alone (Fig. 8.3). However, after exposure of MDA-MB-231 cells to both 5-AZA-CdR and TSA in combination significant gene activation took place. 5-AZA-CdR alone only produced a weak activation of ER. These results suggest that methylation plays the dominant role in the silencing of ER and that histone deacetylation plays a complementary role in this process. It appears that DNA methylation is essential to maintain the silenced state, but when some demethylation occurs, histone deacetylation partially maintains the gene in the silenced state.

In order to determine if TSA could indirectly affect the extent of demethylation produced by 5-AZA-CdR we performed quantitative methylation analysis of 2 specific sites in RARβ gene by MS-SNuPE assay. This assay showed that the increased re-expression of this gene after treatment of cells with 5-AZA-CdR and TSA is not due to further decrease in methylation of these sites after TSA treatment. These results suggest that increased expression of some genes can take place even in the presence of partial methylation of their promoter regions and that two different epigenetic mechanisms play a role for the maintenance of the silenced state for these two genes.

In conclusion, the observations of a synergistic interaction of 5-AZA-CdR and TSA on the loss of colony formation and in the reactivation of two cancer-related genes suggest that the combination of inhibitors of DNA methylation and HDAC may

have interesting potential in the therapy of breast cancer, and possibly other cancers. The rationale of this therapy is to activate the key genes that induce irreversible terminal differentiation so as the tumor cells lose completely their proliferative and metastatic potential. It will be very interesting to test this form of differentiation therapy in future clinical trials in patients with advanced breast cancer.

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Table 8.1 Effect of treatment of MDA-MB-231cells with 5-AZA-CdR ± TSA on the methylation status of RAR-ß using the SNuPE assay.

Primer	Additions	Concentration	Relative inhibition DNA Methylation (%)
A	5-AZA	50 ng/ml	36.0 ± 3.0
A	TSA	600 nM	<1
A	5-AZA + TSA	50 ng/ml + 600 nM	30.8 ± 2.2
B	5-AZA	50 ng/ml	28.3 ± 2.0
B	TSA	600 nM	<1
B	5-AZA + TSA	50 ng/ml + 600 nM	33.2 ± 2.2

The cells were exposed sequentially for 48 hr to the drugs. DNA was isolated at 15-20 hr after treatment and treated with bisulfite. Seminested PCR was performed to amplify gene fragments from the promoter region of RARβ which were used for SNuPE assay with primers A and B as described under "Methods". The values represent the mean ± SE for 3 different experiments.

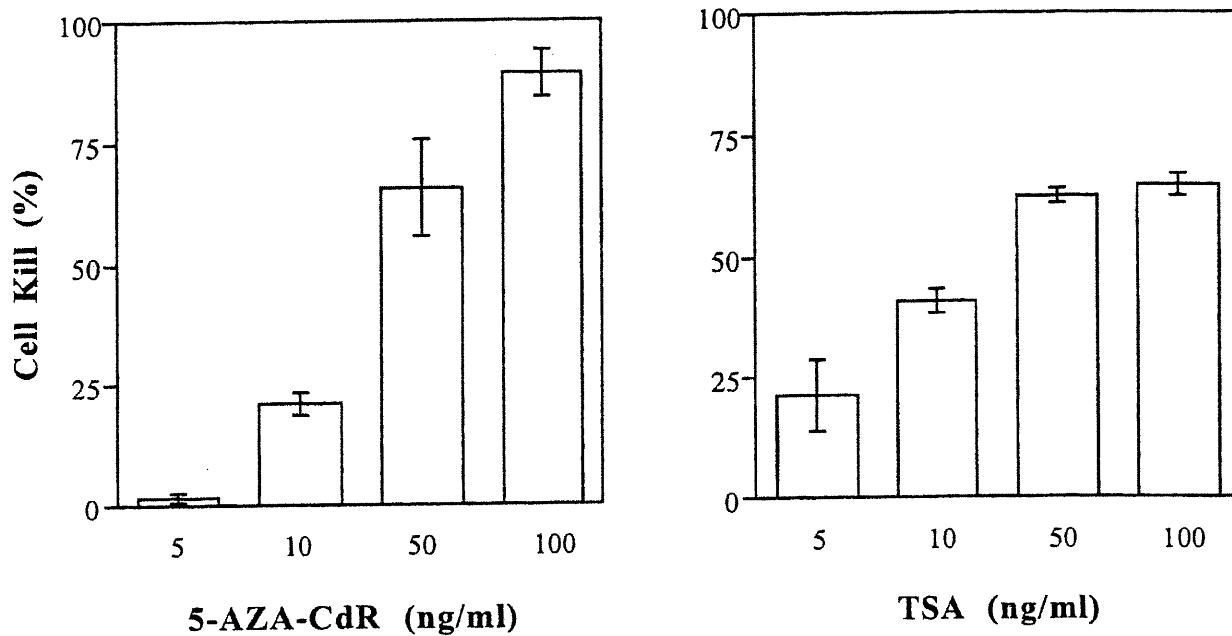

Fig. 8.1. Dose-response curve for the cytotoxic action of 5-AZA-CdR or TSA on MDA-MB-231 breast carcinoma cells. Drug exposure was 48 hr and cell survival determined by colony assay. Values represent the mean ± SE for 3 or more separate experiments.

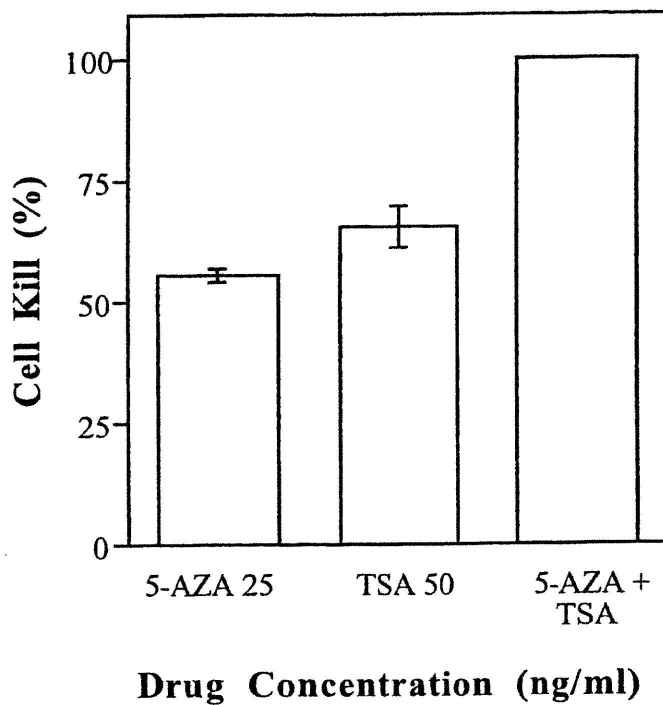

Fig. 8.2. Cytotoxic action of 5-AZA-CdR (25 ng/ml) and TSA (50 ng/ml) alone and in combination on MDA-MB-231 breast carcinoma cells. A sequential 48 hr drug exposure was used and cell survival determined by colony assay. Values represent the mean ± SE for 3 or more separate experiments.

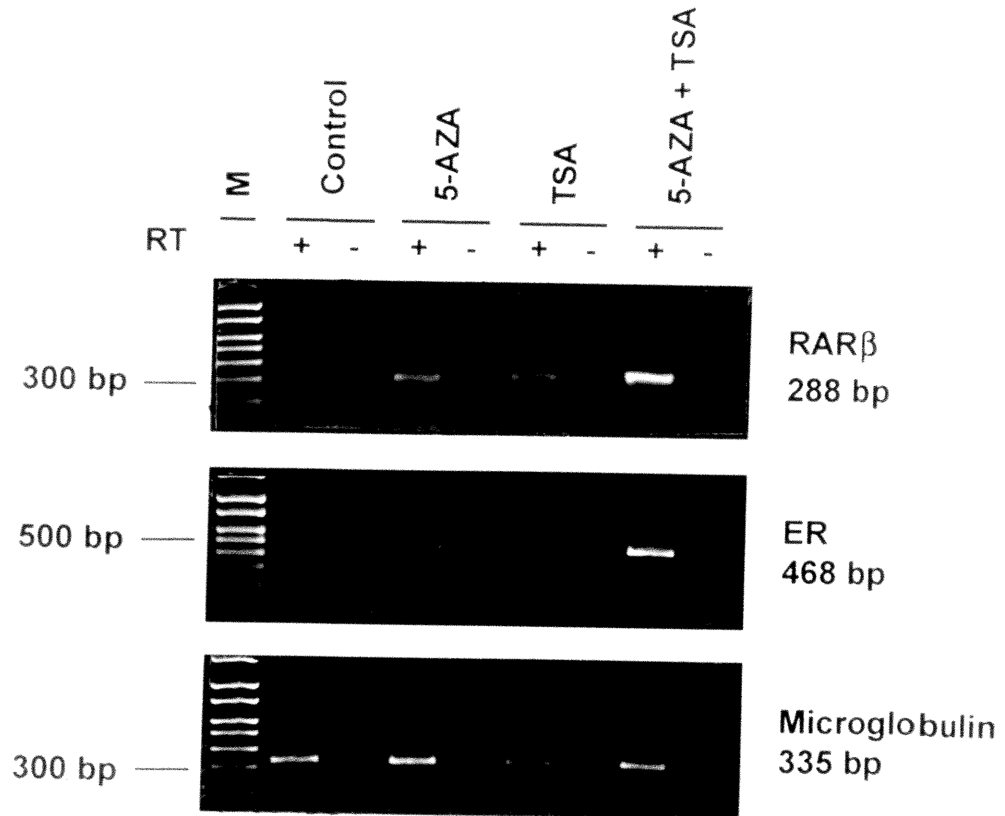

Fig. 8.3 RT-PCR analysis of activation of expression of RARβ and ER by 5-AZA-CdR and TSA. Total RNA was isolated 15- 20 hr after treatment with 5-AZA-CdR 50 ng/ml for 48 hr and/or TSA 181 ng/ml for 48 hr. PCR was performed using the cDNA with specific primers for RARβ, ER and microglobulin as described under "Methods". The amplified DNA was separated on agarose gel and stained with ethidium bromide. M, DNA size marker.

PART 3: GENERAL DISCUSSION

CHAPTER 9: DISCUSSION

9.1 Identification of an additional target for breast cancer therapy with 5-aza-2'-deoxycytidine: RARβ gene.

Silencing of tumor suppressor genes by aberrant methylation has been widely described for breast cancer (see **Table 1.1**) and other types of cancer (see **Table 2.1**). The products of tumor suppressor genes are involved in the control of cell proliferation and the monitoring the entry of normal cells into the cell cycle. When tumor suppressor activity is absent, cells lose the brakes that keep them from proliferating out of control and from progressing to cancer. Many cancer-related genes have also been found to be concomitantly silenced, in addition to tumor suppressor genes, in different types of tumors (Costello et al., 2000; Melki et al., 1999).

We detected methylation of RARβ gene in 30% of breast cancer biopsies analyzed by MSP (**Fig. 6.1**). Several tumor biopsies showed positive signals with primers specific for unmethylated DNA which may be have been due to the presence of traces of normal tissue in the biopsy such as blood vessels, white blood cells, fibrous tissue, etc. In addition, tumor cell populations may show heterogeneous methylation patterns for tumor suppressor genes which can result in the positive signal for unmethylated sequences (Gonzalgo et al., 1998). Another possibility to consider is the incomplete conversion of cytosine residues by sodium bisulfite. Since MSP is a PCR based technique, it is very sensitive and it may amplify those molecules that underwent incomplete conversion resulting in false positive bands (Harrison et al., 1998)

The detection of positive signals for methylation in normal tissue adjacent breast tumor may suggest that methylation takes place very early in tumorigenesis and be indicative of premalignant tissue surrounding the tumor (**Fig. 6.1**).

Bisulfite DNA sequencing revealed that the sites of methylation for the RARβ gene in breast tumors are almost identical to those found in DLD1 colon carcinoma cells (**Table 7.1**). The relevance of this finding is that methylated sites are conserved among different tumor types. This may be indicative of the importance of a specific

methylation pattern to silence the same tumor suppressor gene, such as RARβ, in different tumor types.

9.2 Activity of 5-AZA-CdR on MDA-MB-231 breast carcinoma cells.

The reports that treatment with 5-AZA-CdR can reactivate several tumor suppressor genes and cancer-related genes silenced by aberrant methylation in breast cancer (**Table 1.1**) suggest a possible potential of this analog as an antineoplastic agent for this type of tumor. Our *in vitro* studies reveal that 5-AZA-CdR is a potent inhibitor of growth and clonogenicity for the MDA-MB-231 breast carcinoma cells (**Figs. 6.1** and **6.2**). Both the growth inhibition and loss of clonogenicity increased with the exposure time and the concentration of the drug. It is interesting to note that the pharmacological concentrations of 5-AZA-CdR that showed significant *in vitro* antitumor activity (~100 ng/ml) are in the same range of the plasma levels of this analog used in pilot clinical studies with responses in patients with leukemia and lung cancer (Momparler et al., 1984; 1997). These observations provide the rationale for future clinical trials on 5-AZA-CdR in patients with breast cancer.

9.3 Modification of the SNuPE method for quantitation of DNA methylation.

The Single Nucleotide Primer Extension (SNuPE) assay was first described by Kuppuswamy et al (1991) to detect mutations in abnormal alleles. Gonzalgo and Jones (1997) modified this method to assess methylation differences at specific CpG sites of the p16 tumor suppressor gene and named it the methylation-sensitive SNuPE (Ms-SNuPE).

In this method, genomic DNA was treated with bisulfite to convert all the unmethylated cytosine residues to uracil and then the desired sequence was amplified by PCR using primers specific for the modified DNA. Since it is a PCR based method, only small amounts of bisulfite converted DNA are needed. The amplified DNA was then used as a template for single nucleotide extension, using a primer that allows the incorporation of just one molecule of [32P]dCTP if the target sequence is

methylated, or a molecule of [32P]dTTP if the target is unmethylated. The reaction products were loaded on a denaturing polyacrylamide gel and radioactivity quantified by phosphorimage analysis.

We modified the Gonzalgo and Jones method by omitting the polyacrylamide gel electrophoresis step and replaced it by counting radioactivity on paper discs. In addition, we used semi-nested PCR to amplify the bisulfite converted DNA prior to nucleotide extension reaction. These modifications made the method simpler to perform without losing its specificity and precision.

Bisulfite modification of DNA does not result in complete conversion of cytosine residues to uracil. It has been documented that some cytosines in DNA can be partially resistant to bisulfite conversion, leading to methylation artifacts (Harrison et al., 1998). This will leave us with a mixed population of DNA molecules containing some traces of unconverted cytosines. In order to overcome this problem, we designed primers that would selectively amplify converted DNA. In order to accomplish this we designed sense primers which contained 3 or more modified cytosine residues. In addition, we performed seminested PCR. The use of a second, semi-nested PCR was particularly important since it eliminated unspecific amplification of unmodified DNA.

The standard curve was established by using human WBC genomic DNA which has 0% methylated cytosine (0% 5MC) for RARβ and cancer-related genes. We methylated all the CpG dinucleotides in this genomic DNA by Sss1 methylase or used genomic DNA from DLD-1 colon cancer to obtain a 100% 5MC sample of RARβ. The DNA was treated with sodium bisulfite and amplified by the use of semi-nested PCR with primers specific for the converted target sequence. Different amounts of 100% and 0% 5MC were mixed to obtain 25, 50 and 75% 5MC samples. The results revealed very good precision and reproducibility of the modified method (**Figs. 7.1** and **Table 7.1**)

One of the major applications of the Ms-SNuPE method is that it permits the easy and quantitative detection of the methylation status of specific sites in a target sequence in tumors. This may be a valuable tool in selecting the patients that can be treated with demethylating agents like 5-AZA-CdR. Detection of methylation of key

tumor suppressor genes in a tumor biopsy can make a patient a candidate for therapy with 5-AZA-CdR. Using the SNuPE assay, it would also be possible to easily monitor the changes in the methylation status of target genes after 5-AZA-CdR therapy.

One can speculate that demethylation at only a few CpG sites is capable of restoring tumor suppressor gene functions (Gonzalgo et al., 1998). One should select carefully the specific CpG sites for SNuPE analysis. It was widely reported that hypermethylation of the promoter CpG island correlates with transcriptional silencing (Gonzalez-Zulueta et al., 1995). On other hand, aberrant methylation of CpG island in the exon regions of genes can be permissive for expression (Issa et al., 1996). Thus, it is important to select sites whose methylation correlates with gene silencing.

In conclusion, SNuPE analysis of tumor tissues may facilitate the development of more effective therapies for breast cancer.

9.4 Cytotoxicity of 5-Aza-CdR and TSA on MDA-MB-231 cells.

5-AZA-CdR is a potent demethylating agent. Hypomethylation of cancer-related genes silenced by aberrant methylation by this analog can lead to gene activation and terminal differentiation. The use of 5-AZA-CdR as an antineoplastic agent is limited by its severe hematopoietic toxicity. Combination of 5-AZA-CdR with other agents that also induce differentiation could lead to more effective therapy without increasing drug toxicity.

Histone deacetylase inhibitors (HDI) are a new class of antineoplastic agents which maintain histone acetylation in nucleosomes which can result in gene activation. The possible interaction between inhibitors of DNA methylation and HDI is currently under intensive investigation. It was reported by Cameron et al. (1999) that TSA, a specific histone deacetylase inhibitor, act synergistically with 5-AZA-CdR to reactivate tumor suppressor genes. These observations suggested to us a possible interaction between the two drugs, with respect to their antineoplastic activity.

We performed clonogenic assay on human MDA-MB-231 breast carcinoma cells with both 5-AZA-CdR and TSA, alone and in combination (**Fig. 8.1** and **8.2**). We found synergy between the two drugs when used in combination. These results provide the rationale for combination therapy of breast cancer with demethylating agents and HDIs. Other HDIs that can be used clinically should be investigated in combination with 5-AZA-CdR both *in vitro* and in animal models with breast cancer.

9.5 Reactivation of gene expression by 5-AZA-CdR and TSA.

In order to understand the mechanisms of interaction between 5-AZA-CdR and TSA, we studied the effect of these two agents on gene reactivation. We chose to study two genes that have been shown to be silenced by aberrant methylation in MDA-MB-231 breast carcinoma cells and that have been reported to be downregulated in primary breast cancer. The two genes are RARβ and ER.

For RARβ, gene reactivation was observed after treatment with 5-AZA-CdR (**Fig. 8.3**). SNuPE analysis confirms that reactivation involved demethylation of the two sites analyzed in the promoter region of RARβ. (**Table 8.1**). This observation is in accord with the report of Côté et al. (1997) on the activation of RARβ in DLD-1 colon carcinoma cells. The mechanism of silencing of expression of RARβ is probably due to the inhibition of the binding of transcription factors to its promoter by methylated CpGs in this region (Robertson and Jones, 2000). The demethylation of these CpGs by 5-AZA-CdR reversed this inhibition.

Reactivation of RARβ was observed also after treatment of MDA-MB-231 breast carcinoma cells with TSA alone **(Fig. 8.3).** This finding suggests that histone acetylation can also play an important role in the silencing of expression of RARβ. It is interesting to note that the combination of these two agents enhanced the expression of RARβ. Thus, it appears that the two processes, DNA methylation and histone deacetylation, are both involved in silencing RARβ expression. The mechanisms that lead to this interaction are not clear. If histone deacetylation be a non-specific process, some proteins (methylated DNA binding proteins) must be involved to direct its specificity. The changing of chromatin to a more open structure

due to histone deacetylation could permit more accessibility of transcription factors to gene promoters. More research is required to clarify this point.

The ER gene has been found silenced aberrant methylation in MDA-MB-231 breast carcinoma cells and primary tumors (Lapidus et al., 1998). Expression of the ER gene in breast tumor is a favorable prognostic factor for responsiveness to hormonal therapy with antiestrogens, such as tamoxifen. After treatment of MDA-MB-231 tumor cells with 5-AZA-CdR we observed reactivation of gene expression (**Fig. 8.3**). This event was probably due to demethylation of its promoter region by this analog. It is interesting to note that exposure to TSA alone did not show reactivation of ER. This finding indicates that different mechanisms are involved to silence ER expression as compared to RARβ expression. However, the combination of the two agents showed greater reactivation than 5-AZA-CdR alone. For the ER gene is appears that DNA methylation plays a more dominant role to control its expression than histone deacetylation.

9.6 Different epigenetic mechanisms of gene silencing.

The different effects of TSA on the genes studied, RARβ and ER, underlines the possible different mechanisms that contribute to gene silencing. As already mentioned above (see also Chapter 3) DNA methylation and histone deacetylation both cause silencing of gene expression. In a simplified view, the presence of methylated cytosines in the promoter region of genes could interfere with the binding of transcription factors to their response elements. Deacetylation of histones restores the positive charge on the lysine residues leading to a more compact chromatin structure which is transcriptionally inactive probably due to its inaccessibility to transcription factors.

Immediately after DNA replication, DMTase1 methylates the daughter strand, whereas deacetylation of the histones takes place later. It is possible that methylated DNA binding proteins (MDBP), such as MeCP2 (Jones et al., 1988; Nan et al., 1998), recognize and bind the methylated CpGs. This is followed by the formation of a complex with HDAC which will enhance the silencing of gene expression. The

relative importance of the two epigenetic changes in maintaining the silenced state of specific cancer-related genes remains to be clarified.

Our results shed some light on this question for the RARβ and ER genes. For RARβ we showed that either 5-AZA-CdR and TSA alone are able to reactivate its expression. These results seem to suggest that loss of expression of RARβ requires both DNA methylation and histone deacetylation. Neither change alone can lead to complete silencing of the gene. By inducing demethylation or histone acetylation, we reactivated the silent RARβ gene. Concomitant inhibition of DNA methylation and histone deacetylation appeared to synergistically reexpress this gene.

On the other hand, the ER gene was observed to be reactivated by 5-AZA-CdR alone, but not by TSA. The combination of these two agents lead to a higher degree of expression than produced by 5-AZA-CdR alone. In this scenario, we can hypothesize that methylation of the ER gene is the primary epigenetic change that leads to its silencing. Chromatin conformation changes alone are not able to silence this gene, even though they have some effect on the reactivation of gene expression after treatment with both 5-AZA-CdR and TSA.

9.7 General discussion.

The major objective of the experimental work presented in this thesis is to provide a rationale for the use of the hypomethylating agent, 5-AZA-CdR, as an antineoplastic agent in the therapy of breast cancer. We showed that this analog has a potent antineoplastic *in vitro* activity against a human breast cancer cell line, MDA-MB-231, with respect to their clonogenic potential and their growth.

In order to understand the molecular mechanisms of this potent antineoplastic activity, we studied the effects of 5-AZA-CdR on the RARβ gene which is known to be frequently silenced by aberrant methylation in breast cancer. We analyzed the methylation status of breast cancer biopsies and the normal adjacent tissue. We found methylation of RARβ in 30% of the tumor biopsies. Detection of positive signals in some of the adjacent tissue surrounding the tumor may be indicative that methylation of RARβ is an early event in tumorigenesis. Bisulfite DNA sequencing of two

biopsies revealed that the methylation sites were almost identical to those found in DLD1 colon carcinoma cells and MDA-MB-231 breast carcinoma cells. These findings suggest that for specific tumor suppressor genes a similar pattern of aberrant methylation is involved to silence its expression in different tumor types.

Since many cancer-related genes have been reported to be silenced by aberrant methylation in different tumors, inhibitors of DNA methylation may be good candidates for chemotherapy. The experimental methods to evaluate the methylation status of cancer-related genes can provide useful information for patient selection and to monitor therapy. Methods like MSP or SNuPE can be used for rapid screening. The advantage of the SNuPE assay is that being a quantitative method, it allows to monitor the effect of 5-AZA-CdR on demethylation of promoter regions of key tumor suppressor genes. This will provide important data for the analysis of the therapeutic efficacy of this analog.

One of the major limitations for the use of 5-AZA-CdR in cancer therapy is its hematopoietic toxicity. The interval between cycles of therapy with this agent can be 4 or more weeks to permit recovery from toxicity. It would be desirable to use 5-AZA-CdR in combination with other agents that do not produce leukopenia, but can enhance its therapeutic efficacy. One of the newer classes of antineoplastic agents that are of interest for this approach are the HDAC inhibitors. They induce differentiation in cancer cells by activating gene expression.

We tested the antineoplastic activity of TSA, a specific histone deacetylase inhibitor, on MDA-MB-231 breast carcinoma cells. It showed remarkable inhibition of clonogenicity and cell growth. Combination of 5-AZA-CdR and TSA were found to act synergistically on cell kill of this tumor cell line. It was reported by Cameron et al. (1999) that the two drugs act synergistically in reactivation of some tumor suppressor genes. Our objective was to investigate the effect of 5-AZA-CdR and TSA on reactivation of RARβ and ER genes, both of which are downregulated in the MDA-MB-231 breast carcinoma cell line by aberrant methylation.

Our results reveal a very interesting interaction between these two agents. The inhibition of DNA demethylation and histone acetylation produced different types of gene activation which depends on the gene analyzed. It appears that at least

two types of genes can be identified. For some genes silencing depends mostly on DNA methylation and for others histone deacetylation is also involved in this process.

The tumor suppressor gene, RARβ, was reactivated by either 5-AZA-CdR and TSA alone. This indicates that both DNA methylation and histone deacetylation are needed to silence this gene. On the other hand, the ER gene is not reexpressed after treatment with only TSA. That indicates that DNA methylation is essential for suppression of its activity. Histone acetylation may contribute to the silenced state, but not be able to maintain it in the absence of hypermethylation.

Future investigations on the use of inhibitors of DNA methylation and histone deacetylation on different cancer-related genes will help clarify the role that these two epigenetic mechanism play in tumorigenesis.

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APPENDIX I: Detailed protocol for Ms-SNuPE

Protocol Overview

1. Bisulfite conversion of genomic DNA over night (16-18 hours)
    - Solutions: -10 mM Hydroquinone
      - 3.65 M Sodium Bisulfite pH 5
      - 3M NaOH

2. Prep-A-Gene Protocol (clean DNA using the Prep-A-Gene DNA purification kit from Stratagene, 4-5-hours)

3. Precipitation of bisulfited DNA at -20°C over night

4. First PCR with primers specific for bisulfite-converted DNA (3-4 hrs)
    - Use Qiagen HotStar Taq kit (Taq-AntiTaq)

5. Semi-nested PCR with primers specific for bisulfite-converted DNA (3-4 hrs)

6. Agarose gel electrophoresis (1hr)
    - Extract DNA from gel: use QIAquick gel extraction kit (1hr)
    - Quantify DNA

7. Ms-SNuPE with dCTP[α^{32}P] and TTP[α^{32}P](1/2 hour)
    - Use Taq-Platinum (BRL) (Taq-AntiTaq)
    - SNuPE primers

8. Spot PCR products on DEAE discs and wash (1-2 hours)

9. Count radioactivity

I. Sodium bisulfite conversion of DNA

- Use 2μg of genomic DNA in 40μl of water
- Denaturate DNA at 95°C for 5 min
- Make fresh: 10 mM Hydroquinone
    - 3.65 M Sodium Bisulfite pH 5.0
    - 3.0 NaOH
- Add 2μl of 6N NaOH (final concentration 0.3N)
  total volume: 42μl
- Keep samples at 45°C for 20 min
- Add to the denatured solution:
    - 14μl of 10mM Hydroquinone (final concentration 2.5mM) total volume 56μl
    - 260μl of 3.65M Sodium Bisulfite pH5 (final conc. 3M)
      total volume 316μl
- Flush with Nitrogen gas and place in a thermal cycler:
    - Program:

Step				
	1	55°C	4h	
	2	90°C	2m	go to step 1 3 more times
	3	20°C	10m	
	4	4°C	hold	

II. Prep-A-Gene DNA purification kit (Stratagene).

- To the DNA solution add:
    - 3V of binding buffer (3x316μl)
    - 5μl of Prep-A-Gene DNA matrix (vortex well before using)
- Place at 20°C for 20 min, shake continuously to allow DNA to bind to matrix.
- Place on ice for 60 min, mix every 5 min; centrifuge at 5,000rpm for 30 sec; remove supernatant and discard
- Add 0.5ml of 75% EtOH to wash pellet (3 times)

- Remove all liquid to the last drop and let matrix dry (air-dry or vacuum)
- Elute DNA in 50µl TE pH 7.5 at 60°C for 30 min; centrifuge 30 sec
- Elute in 50µl of water at 60°C for 10min, combine eluates.

III. Disulphanation and Precipitation of DNA

- To each sample (vol. approx.90µl) add:
    - 4.75µl 6N NaOH to a final concentration 0.3M
      approx. vol.: 95µl
- Incubate 15 min at 40°C to desulphonate cytosine residues in DNA
- To each sample add:
    - 105µl 6M ammonium acetate pH7 to a final conc. of 3M
      (to neutralize NaOH)
    - 1µl of glycogen (20mg/ml)
    - 500µl of 100% EtOH
      (to precipitate DNA)
- Precipitate at -20°C overnight
- Centrifuge at max speed (14,000rpm) for 20min at 4°C, discard supernatant; repeat wash, centrifuge at 14,000rpm, 4°C for 10min, discard supernatant
- Let pellet dry (do NOT overdry)
- Dissolve DNA precipitate in 70µl of 10mM Tris-HCl pH8.5
- Quantify DNA.

•

IV. 1st PCR

- Choose primers with ≥2 cytosine residues not in CpG dinucleotides (they will become T after bisulfite treatment and PCR).

  - This will increase the odds of amplifying modified DNA over unmodified sequences. Performing semi-nested amplification will provide more specificity for modified DNA and pure sample for the SNuPE assay. Amplification of small sized fragment is usually easier. As an example the protocol for RARβ gene is given.

Reaction Mix (1st PCR)

H_2O	17.5 μl
10x PCR buffer (Qiagen)	2.5 μl
$MgCl_2$ 25 mM	2.0 μl
dNTPs (10mM)	1.0 μl
Upper primer 20 μM	0.5 μl
Lower primer 20 μM	0.5 μl
Bisulfite converted DNA	1.0 μl
HotStar Taq (Qiagen) 5U/μl	0.2 μl
Total Volume	25.0 μl

1st PCR program:

step:		
1	95C	15m
2	94C	1m
3	50C	1m
4	72C	3m go to 3, 4 times
5	94C	30sec
6	50C	30sec
7	72C	1m go to 5, 34 times
8	72C	10m
9	4C	hold

V. Semi-nested PCR

- Make 1000 fold dilution of 1st PCR mix, and use 1 or 2 μl for semi-nested PCR

Reaction Mix (semi-nested PCR)

H_2O	16.5 μl
10x PCR buffer (Qiagen)	2.5 μl
$MgCl_2$ 25 mM	2.0 μl
dNTPs (10mM)	1.0 μl
Upper primer 20 μM	0.5 μl
Lower primer 20 μM	0.5 μl
1000 fold dil. 1st PCR products	2.0 μl
HotStar Taq (Qiagen) 5U/μl	0.2 μl
Total Volume	25.0 μl

Semi-nested PCR program: step:

step	temp	time
1	95C	15m
2	94C	1m
3	50C	1m
4	72C	1m go to 3, 2 times
5	94C	1m
6	50C	30sec
7	72C	1m go to 5, 35 times
8	72C	10m
9	4C	hold

VI. Gel extraction of PCR products

- Run 6 PCR tubes for each sample and run the PCR products on a 2% agarose gel. Stain with ethidium bromide.

- Cut out the DNA fragment from the agarose gel with clean blade and place in 1.5 ml tube for DNA extraction.

- Use QIAquick Gel Extraction Kit:

•

VII. MS-SNuPE

- Use 10-20ng of amplified DNA sample per reaction
- Prepare reaction mixtures:

Rxn Mix C		**Rxn Mix T**
10x buffer (Gibco)	2.5 μl	10x buffer (Gibco)
$MgCl_2$ (25 mM)	1.0 μl	$MgCl_2$ (25 mM)
dCTP[α^{32}P] (25 μCi/μl)	0.1 μl	**dTTP**[α^{32}P] (25μCi/μl)
SNuPE primer 20 μM	1.25 μl	SNuPE primer 20 μM
Taq Platinum (Gibco)	0.2 μl	Taq Platinum (5U/μl)
DNA template (20 ng)	variable	DNA template (20 ng)
H_2O	variable	H_2O

Total volume 25 μl

SNuPE PCR program:

step:		
1	95C	5m
2	94C	1m
3	50C	2m
4	72C	1m
5	4C	hold

- Stop reaction by adding 1μl of EDTA 0.5M to each sample.

VIII. Cleaning steps of SNuPE PCR products.

- Prepare solution:

3X SSC:	NaCl	0.45 M	
	Sodium citrate	0.045 M	pH 7

- Apply SNuPE PCR products on DEAE circles (DE81), let stand for 10min

- 3X SSC washes:

1	10 min	shaking
2	10 min	shaking
3	10 min	shaking
4	10 min	shaking
5	10 min	shaking

- H_20 washes:

1	10 min	shaking
2	10 min	shaking
3	10 min	shaking

- Dry discs in oven at 80C
- Place discs in scintillation liquid and count radioactivity in counter.

APPENDIX II

TABLE Design of primers from the promoter region of the human RARß gene for PCR amplification of a bisulfite-modified DNA fragment for DNA sequencing to identify the positions of 5-methylcytosine.

	5′-END OF AMPLIFIED DNA (pos. 544-568)		3′-END OF AMPLIFIED DNA (pos. #1148-1172)
original	5′-GAG-AAG-TTG-GTG-CTC-AAC-GTG-AGC-C-3′	...	5′-GCT-GGC-TTG-TCT-GTC-ATA-ATT-CAT-G-3′
modified	5′-GAG-AAG-TTG-GTG-UTU-AAU-GTG-AGU-U-3′	...	5′-GUT-GGU-TTG-TUT-GTU-ATA-ATT-UAT-G-3′
primer	5′-GAG-AAG-TTG-GTG-TTT-AAT-GTG-AGT-T-3′ (5′-PRIMER)	...	5′-GTT-GGT-TTG-TTT-GTT-ATA-ATT-TAT-G-3′
			3′-CAA-CCA-AAC-AAA-CAA-TAT-TAA-ATA-C-5′ (3′-PRIMER)

DNA amplified 629 bp

The DNA sequence is from GenBank accession no. X56849. The bisulfite reaction of DNA converts C to U as shwon by the underlined base. The PCR reaction amplifies the DNA so the U is replaced by T. The primers must be designed from only one strand of DNA since bisulfite treatment modifies both strands of DNA so that they are no longer complementary. The example shown above the primers are designed to amplify the sense strand. A similar approach is used to design primers for methylation specific PCR (MSP). When 5-methylcytosine is present in the sequence the corresponding C is not converted to U. The MSP primers speccific for methylated DNA will contain C at the 5-methylcytosine sites whereas the primers specific for unmethylated DNA will contain T at these sites.

TABLE DNA sequence of human RARß promoter region and the the positions of 5-methylcyctosine for DLD-1 human colon carcinoma cells

501-	GTTTTCCAAG	CTAAGCCGCC	GCAAATAAAA	AGGCGTAAAG	GGAGAGAAGT
551-	TGGTGCTCAA	CGTGAGCCAG	GAGCAGCGTC	CCGGCTCCTC	CCCTGCTCAT
601-	TTTAAAAGCA	CTTCTTGTAT	TGTTTTTAAG	GAGAGAAATA	GGAAAGAAAA
651-	CGCCGGCTTG	TGCGCTCGCT	GCCTGCCTCT	CTGGCTGTCT	GCTTTTGCAG
701-	GGCTGCTGGG	AGTTTTTAAG	CTCTGTGAGA	ATCCTGGGAG	TTGGTGATGT
751-	CAGACTAGTT	GGGTCATTTG	AAGGTTAGCA	GCCCGGGTAG	GGTTCACCGA
801-	AAGTTCACTC	GCATATATTA	GGCAATTCAA	TCTTTCATTC	TGTGTGACAG
851-	AAGTAGTAGG	AAGTGAGCTG	TTCAGAGGCC	AGGAGGGTCT	ATTCTTTGCC
901-	AAAGGGGGGA	CCAGAATTCC	CCCATGCGAG	CTGTTTGAGG	ACTGGGATGC
951-	CGAGAACGCG	AGCGATCCGA	GCAGGGTTTG	TCTGGGCACC	GTCGGGGTAG
1001-	GATCCGGAAC	GCATTCGGAA	GGCTTTTTGC	AAGCATTTAC	TTGGAAGGAG
1051-	AACTTGGGAT	CTTTCTGGGA	ACCCCCCGCC	CCGGCTGGAT	TGGCCGAGCA

GenBank accession. no. X56849; transcription start site, 844; RARE, 792-97; 803-08; TATA box, 814-20; Sp1 element 1074-81; positions of 5-methylcytosine indicated by double underline.

APPENDIX III: Additional manuscript

JOURNAL OF CELLULAR PHYSIOLOGY 183:145–154 (2000)

REVIEW ARTICLE

DNA Methylation and Cancer

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The methylation of DNA is an epigenetic modification that can play an important role in the control of gene expression in mammalian cells. The enzyme involved in this process is DNA methyltransferase, which catalyzes the transfer of a methyl group from S-adenosyl-methionine to cytosine residues to form 5-methylcytosine, a modified base that is found mostly at CpG sites in the genome. The presence of methylated CpG islands in the promoter region of genes can suppress their expression. This process may be due to the presence of 5-methylcytosine that apparently interferes with the binding of transcription factors or other DNA-binding proteins to block transcription. In different types of tumors, aberrant or accidental methylation of CpG islands in the promoter region has been observed for many cancer-related genes resulting in the silencing of their expression. How this aberrant hypermethylation takes place is not known. The genes involved include tumor suppressor genes, genes that suppress metastasis and angiogenesis, and genes that repair DNA suggesting that epigenetics plays an important role in tumorigenesis. The potent and specific inhibitor of DNA methylation, 5-aza-2'-deoxycytidine (5-AZA-CdR) has been demonstrated to reactivate the expression most of these "malignancy" suppressor genes in human tumor cell lines. These genes may be interesting targets for chemotherapy with inhibitors of DNA methylation in patients with cancer and this may help clarify the importance of this epigenetic mechanism in tumorigenesis. J. Cell. Physiol. 183:145–154, 2000.
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GENERAL COMMENTS ON DNA METHYLATION

In mammalian cells, approximately 3% to 5% of the cytosine residues in genomic DNA are present as 5-methylcytosine (Ehrlich et al., 1982). This modification of cytosine takes place after DNA replication and is catalyzed by DNA methyltransferase using S-adenosyl-methionine as the methyl donor. The maintenance DNA methyltransferase (Dnmt 1) was cloned from mammalian cells (Bestor et al., 1988) and uses hemimethylated DNA as a preferential template (Bouchard and Momparler, 1983). The function of this methylase is to maintain the identical methylation pattern after DNA replication that is characteristic for each type of differentiated cell (Tucker and Bestor, 1997).

Three other DNA methyltransferases (Dnmt 2, Dnmt 3a, and Dnmt 3b) have been also cloned from mammalian cells. Dnmt 3a and Dnmt 3b appears to function as a de novo methylase since they can methylate hemimethylated and unmethylated DNA with equal efficiencies (Xie et al., 1999). The expression of Dnmt 3b was significantly increased in tumors suggesting that it may have a role in tumorigenesis (Robertson et al., 1999). Dnmt 2 was suggested to have the function of methylating integrated retroviral sequences (Yoder and Bestor, 1998). Another group reported that Dnmt 2 was not required for maintenance methylation of viral DNA and de novo methylation (Okano et al., 1998). The role of all these DNA methyltransferases in tumorigenesis still remains to be clarified. A specific mammalian demethylase that uses methylated CpG DNA as a substrate has been identified (Bhattacharya et al., 1999). The role of this interesting enzyme with respect to maintenance of DNA methylation patterns and gene expression remains to be elucidated.

Approximately 70% to 80% of 5-methylcytosine residues are found in the CpG sequence (Bird, 1986). This sequence, when found at a high frequency, in the genome, is referred to as CpG islands. Unmethylated CpG islands are associated with housekeeping genes, while the islands of many tissue-specific genes are methylated, except in the tissue where they are expressed (Yevin and Razin, 1993). This methylation of DNA was initially proposed to play an important role in the control of expression of different genes in eukaryotic cells during embryonic development (Scarano et al., 1967). The experimental findings that support this hypothesis were the observation that inhibitors of DNA methylation could induce differentiation in mammalian cells (Jones and Taylor, 1980).

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DNA methylation is involved in embryonic development (Kafri et al., 1992), a process that involves sequential activation and deactivation of different classes of genes. Genomic imprinting in which specific genes are only expressed by the paternal or maternal chromosome also involves DNA methylation (Falls et al., 1999). The methylation of cytosine is also responsible for the inactivation of specific genes on the X chromosome (Riggs and Pfeifer, 1992). DNA methylation has also been proposed to be involved in senescence and aging (Cooney, 1993). Inactivation of viral DNA that has integrated into the genome of the host mammalian cell by methylation illustrates the function of this process as a protective mechanism against infective agents (Schaefer et al., 1997). Although these aspects of DNA methylation are very interesting, they are not discussed here because the focus of this review is cancer.

DNA METHYLATION AND REGULATION OF GENE EXPRESSION

How does DNA methylation inhibit transcription? Because the 5-methylcytosine protrudes into the major groove of the DNA helix (Tate and Bird, 1993), it is possible that this modified cytosine interferes with the binding of transcription factors (Kass et al., 1997). It has been reported in an in vitro system that specific transcription factors bind with less affinity to methylated target sequences (Iguchi-Ariga and Schaffner, 1989; Mancini et al., 1998).

Another plausible mechanism through which DNA methylation can regulate gene expression is via proteins that bind preferentially to methylated promoters, thus preventing the binding of transcription factors to their target sequences. Two sequence-specific methylated DNA binding proteins have been identified, MDBP-1 and MDBP-2 (Huang et al., 1984; Jost and Hofsteenge, 1992).

Another class of methylated CpG-binding proteins (MeCPs) have been identified according to their ability to bind DNA containing highly methylated CpG dinucleotides. These proteins appear to act as transcription repressors. Two members of the MeCP family have been identified, MeCP1 (Meehan et al., 1989) and MeCP2 (Lewis et al., 1992). MeCP1 requires 12 methyl-CpGs for preferential binding to methylated DNA and it is able to repress transcription in vitro and in vivo (Boyes and Bird, 1991). MeCP2 is an abundant chromosomal protein that requires a single methylated CpG site for preferential binding to DNA. It can also repress transcription in vitro (Nan et al., 1997).

One interesting finding is that MeCP2 coprecipitates with histone deacetylase, suggesting a link between DNA methylation and chromatin structure (Jones et al., 1998; Nan et al., 1998). It is known that gene expression can be influenced by chemical modification of histones with subsequent changes in chromatin structure (Davie and Hendzel, 1994). The chromatin in mammalian cells consists of a series of nucleosomes arranged in a compact configuration. The nucleosome consists of 146-bp DNA wrapped around a protein octamer containing two molecules each of histone H2A, H2B, H3, and H4. At sites where transcription takes place, this chromatin structure becomes more "open" and accessible to transcription factors and the DNA sensitive to cleavage by nuclease digestion. Histone acetylation precedes transcription and results in decondensation of the chromatin to permit binding of transcription factors to DNA. Histone acetyltransferase and histone deacetylase play an important role in this process. Histone acetylation is associated with active transcription (Wolffe, 1996). Apparently, acetylation of the ε-amino group of the lysine residues reduces the positive charge of the histones and decreases their binding affinity for DNA that favors the "opening" of the chromatin. In support of this model is that the histone deacetylase inhibitor, trichostatin A (TSA), can activate the transcription of certain genes (Cameron et al., 1999).

How do histone acetylation and DNA methylation interact (Bestor, 1998; Eden et al., 1998)? It appears that methylated inactive genes contain underacetylated histones whereas unmethylated active genes preferentially associate with highly acetylated histones. The mechanism for this interaction apparently follows the path where the methylated gene binds MeCP2, which in turn recruits histone deacetylase resulting in a suppression of transcription (Jones et al., 1998; Nan et al., 1998). TSA can activate some genes that are methylated but not all methylated genes (Jones et al., 1998; Cameron et al., 1999), indicating that the interaction between the methylated DNA, MeCP2, and histone deacetylase is complex and probably involves other factors which are not fully understood at the present time. It is of interest to note that partial demethylation of some genes with the DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-AZA-CdR) interacts synergistically with TSA to activate methylated genes that were previously insensitive to this histone deacetylase inhibitor alone (Cameron et al., 1999).

ROLE OF DNA METHYLATION IN CANCER

General comments

The global level of 5-methylcytosine in tumor cells is often less that in normal cells (Gama-Sosa et al., 1983; Feinberg et al., 1998). This hypomethylation was proposed to be involved in tumor promotion (Counts and Goodman, 1994). However, it is not clear if the overall 5-methylcytosine content has any relationship to neoplasia because tumors can show higher levels of DNA methyltransferase than normal tissue (Issa et al., 1993; Ottaviano et al., 1994). In fact, specific methylation of the estrogen gene increases with age and during the development of neoplasia in human colon tissue (Issa et al., 1994). It should be noted that hypermethylation of CpG islands in colon tumors was reported not to be associated with the overexpression of DNA methyltransferase (Eads et al., 1999). These findings suggest that the decreased overall levels of DNA methylation in tumors is probably not related to neoplasia. In fact, during tumorigenesis there is an increase in the specific methylation of growth regulatory genes (see below). The reason for this paradox is not known.

5-Methylcytosine and mutations

5-Methylcytosine can undergo spontaneous deamination to form thymine at a rate much higher than the deamination of cytosine to uracil (Shen et al., 1994). If the deamination of 5-methylcytosine is unrepaired, it

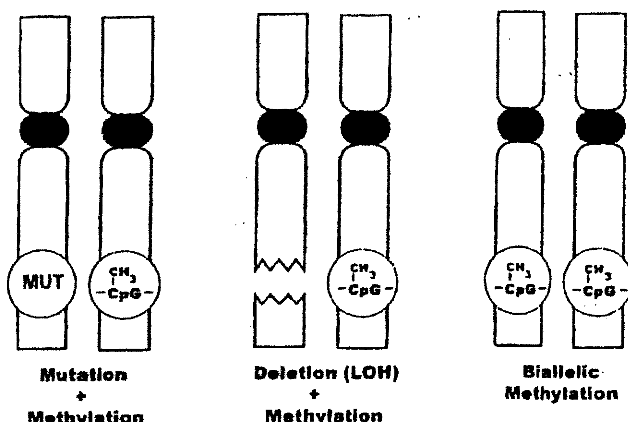

Fig. 1. Model for biallelic inactivation of tumor suppressor genes implicating aberrant methylation alone or with mutation or deletion. Biallelic gene inactivation by only mutation and/or deletion are not shown. Mut, mutation; horizontal broken line indicates deletion; CH_3-CpG, indicates methylation.

will result in a C to T transition mutation. This phenomenon was used to explain the high incidence of CpG to TpG transition mutations observed in the p53 tumor suppressor gene (Rideout et al., 1990). However, these phenomena may be more complex and may involve additional events (Schmutte and Jones, 1998). For example, the carcinogen, benzopyrene, was observed to preferentially form adducts at the methylated CpG sites of the p53 gene; these sites are hot spots for mutation (Denissenko et al., 1997). The relative importance of spontaneous deamination of 5-methylcytosine and carcinogen adduct formation at CpG sites in producing mutations remains to be clarified.

Aberrant DNA methylation of cancer-related genes

The mammalian cell, if unchecked, has enormous growth potential. One cell with a doubling time of 24 h can potentially form a mass of 1 kg (10^{12} cells) in only about 40 days. It is not surprising that cells contain many genes that can suppress this growth potential. The presence of two copies of these suppressor genes, one on the paternal and the other on the maternal chromosome, reduces the risk of unregulated growth due to the inactivation of a single gene copy. Knudsen (1971), in his analysis of the familial incidence of retinoblastoma (Rb), proposed that both alleles of the Rb tumor suppressor gene have to be inactivated to give rise to the malignant phenotype. Initially, point mutations and chromosomal deletions were considered to be the major events involved in the inactivation of tumor suppressor genes. The discovery that many tumor suppressor genes can also be inactivated by aberrant methylation of the CpG islands in their promoter region clearly indicates that epigenetic events also play a very important role in tumorigenesis (Jones and Laird, 1999). A model for the biallelic inactivation of tumor suppressor genes by aberrant DNA methylation alone or in combination with mutations or deletions is shown in Figure 1. This model is supported by the reports that some cancer-related genes were found to be inactivated by biallelic methylation of CpG sequences (Batova et al., 1997; Veigl et al., 1998).

The molecular mechanisms by which the aberrant methylation of DNA takes place during tumorigenesis are still not clear. It is possible that the DNA methyltransferase can make mistakes by methylating CpG islands in the nascent strand of DNA without a complementary methylated CpG in the parental strand. It is also possible that aberrant methylation may be due to the removal of CpG binding proteins that "protect" these sites from being methylated. Whatever the mechanism, the frequency of aberrant methylation is a rare event in mammalian cells.

Tumor-suppressor and other cancer-related genes that have been found to be hypermethylated in human cancer cells and primary tumors are summarized in Table 1. The different classes of genes that are silenced by DNA methylation include tumor suppressor genes, genes that suppress tumor invasion, and metastasis; DNA repair genes; genes for hormone receptors; and genes that inhibit angiogenesis. An example of each class of gene is summarized below. The genes that were activated in vitro by 5-AZA-CdR are also indicated.

p16 INK4A tumor suppressor gene

p16 codes for a constitutively expressed cyclin-dependent kinase inhibitor, which plays a vital role in the control of cell cycle by the cyclin D-Rb pathway (Hamel and Hanley-Hyde, 1997). p16 is located on chromosome 9p, a site that frequently undergoes loss of heterozygosity (LOH) in primary lung tumors (Merlo et al., 1994). In these cancers, it is plausible that the mechanism responsible for the inactivation of the nondeleted allele is aberrant methylation. For lung carcinoma cell lines that did not express p16, 48% showed signs of methylation of this gene (Otterson et al., 1995). Merlo et al. (1995) reported that 26% of primary nonsmall cell lung tumors showed methylation of p16. Primary tumors of the breast and colon displayed 31% and 40% methylation of p16, respectively (Herman et al., 1995). These results indicate that silencing of this gene is frequently associated with aberrant methylation in many different types of cancers.

E-cadherin invasion suppressor gene

E-cadherin is an adhesion molecule that suppresses tumor cell invasion and metastasis in experimental models. The CpG islands of E-cadherin are hypermethylated in breast and prostate carcinoma cell lines that do not express this gene (Graff et al., 1995). These latter investigators also reported that primary breast tumors showed greater than 45% methylation of E-cadherin. In a series of human breast carcinoma cell lines, Hiraguri et al. (1998) observed that both deletions and methylation were responsible for the loss of expression of E-cadherin. Methylation of E-cadherin was only observed in different tumor cell lines that did not express the mRNA for this gene (Yoshiura et al., 1995).

Estrogen receptor

Hypermethylation of CpG islands in the 5′-region of the estrogen receptor gene has been found in multiple tumor types (Issa et al., 1994). The lack of estrogen receptor expression is a common feature of hormone

TABLE 1. Genes silenced by aberrant DNA methylation and activated by 5-azadeoxycytidine (5-AZA-CdR) in human tumor cell lines

Gene	Activation 5-AZA-CdR	Reference
Tumor suppressor		
p15 INK4B (cyclin kinase inhibitor)	+	Herman et al., 1996b
p16 INK4A (cyclin kinase inhibitor)	+	Otterson et al., 1995; Merlo et al., 1995
p73 (p53 homology)	+	Corn et al., 1999
ARF/INK4A (regulate level p53)	+	Robertson and Jones, 1998
Wilms tumor	+	Laux et al., 1997
von Hippel Lindau (VHL)	+	Herman et al., 1994
Retinoic acid receptor-β (RARβ)	+	Côté and Momparler, 1997; Côté et al., 1998
Estrogen receptor	+	Ferguson et al., 1995
Androgen receptor	+	Jarrard et al., 1998
Mammary-derived growth inhibitor	+	Huynh et al., 1996
Hypermethylated in cancer (HIC1)	nd	Ahuja et al., 1997
Retinoblastoma (Rb)	nd	Ohtani-Fujita et al., 1993
Invasion/metastasis suppressor		
E-cadherin	+	Graff et al., 1995
Tissue inhibitor metalloproteinase-3 (TIMP-3)	+	Bachman et al., 1999
mts-1	+	Tulchinsky et al., 1995
CD-44	+	Verkaik et al., 1999
DNA repair/detoxify carcinogens		
Methylguanine methyltransferase	+	Qian and Brent, 1997
hMLH1 (mismatch DNA repair)	+	Herman et al., 1998; Deng et al., 1999
Glutathione S-transferase	nd	Esteller et al., 1998; Millar et al., 1999
BRCA-1	nd	Dobrovic and Simpfendorfer, 1997; Mancini et al., 1998
Angiogenesis inhibitor		
Thrombospondin-1 (TSP-1)	+	Li et al., 1999
TIMP-3	+	Bachman et al., 1999
Tumor antigen		
MAGE-1	+	Weber et al., 1994; Coral et al., 1999

nd, not done.

unresponsive breast cancers, even in the absence of gene mutation (Roodi et al., 1995). Using Southern blot analysis, Lapidus et al. (1998) reported that 25% of primary breast tumors that were estrogen receptor-negative displayed aberrant methylation at one site within this gene. Estrogen receptor expression was demonstrated to be reactivated after treatment of breast carcinoma cells with demethylating agents (Ferguson et al., 1995). Breast carcinoma cell lines that do not express the mRNA for the estrogen receptor displayed increased levels of DNA methyltransferase and extensive methylation of the promoter region for this gene (Ottaviano et al., 1994). Age-related hypermethylation of estrogen receptor gene is a common event in colon cells, but how relevant this hypermethylation is to the increased possibility of developing cancer is not yet fully understood (Issa et al., 1994).

Retinoic acid receptor

Retinoic acid receptors are nuclear transcription factors that bind to retinoic acid responsive elements (RAREs) in DNA to activate gene expression. Lack of expression of the retinoic acid receptor-β (RARβ), a putative tumor suppressor gene, has been reported for breast cancers (Swisshelm et al., 1994; Widschwendter et al., 1997) and other types of cancers (Caliaro et al., 1994; Crowe, 1998). The RARβ gene is located at chromosome 3p24, a site that shows frequent loss of heterozygosity in breast cancer (Deng et al., 1996). Additional evidence that RARβ is a tumor suppressor gene includes the reports that transfection of RARβ cDNA into some tumor cells induced terminal differentiation (Caliaro et al., 1994) and reduced their tumorigenicity in nude mice (Houle et al., 1993). In the DLD-1 human colon carcinoma cells, RARβ was shown to be inactivated by methylation of its promoter and reactivated by treatment with 5-AZA-CdR (Côté and Momparler, 1997; Côté et al., 1998). The activation of RARβ in these tumor cells resulted in a synergistic antineoplastic effect by the combination of 5-AZA-CdR with retinoic acid (Côté and Momparler, 1997). Using the methylation-sensitive PCR (MSP) assay, methylation of RARβ was detected in 43% of primary colon carcinomas (Côté et al., 1998) and in 30% of primary breast carcinomas (Bovenzi et al., 1999).

Mismatch repair gene (hMLH-1)

Mismatch repair is used by the cell to increase the fidelity of DNA replication during cellular proliferation. Lack of this activity can result in mutation rates that are much higher than that observed in normal cells (Modrich and Lahue, 1996). Methylation of the promoter region of the mismatch repair gene (hMLH-1) was shown to correlate with its lack of expression in primary colon tumors, whereas normal adjacent tissue and colon tumors that expressed this gene did not show signs of its methylation (Kane et al., 1997). Expression on hMLH1 in colorectal cancer cells was restored after treatment with 5-AZA-CdR (Herman et al., 1998; Deng et al., 1999).

Thrombospondin-1

Hypermethylation of the thrombospondin-1 gene, an angiogenesis inhibitor, was observed in 33% of primary brain tumors (Li et al., 1999).

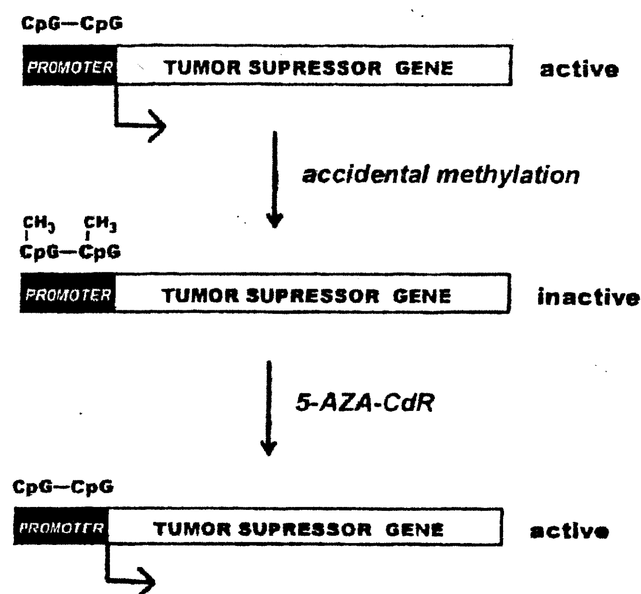

Fig. 2. Model for inactivation of the expression of tumor suppressor gene by accidental methylation of its promoter region. Reactivation of gene expression by treatment with 5-aza-2′-deoxycytidine (5-AZA-CdR) is shown.

CANCER THERAPY WITH INHIBITORS OF DNA METHYLATION

The large number of target genes related to cancer development that are silenced by aberrant DNA methylation (Table 1) suggests that inhibitors of this process may have interesting potential in cancer therapy. 5-AZA-CdR shows promising activity as an anticancer agent. 5-AZA-CdR is a potent and specific inhibitor of DNA methyltransferase. A model for the activation by 5-AZA-CdR of tumor suppressor genes that have been silenced by aberrant methylation is shown in Figure 2. The presence of 5-AZA-CdR in DNA has been proposed to produce an inactivation of DNA methyltransferase by the irreversible formation of a covalent bond between this enzyme and the analog (Santi et al., 1984; Jüttermann et al., 1994). The inhibition of DNA methylation as a target for cancer therapy is supported by the interesting observation that antisense to DNA methyltransferase shows in vitro antitumor activity and some potential to reverse the malignant phenotype (MacLeod and Szyf, 1995). This antisense oligonucleotide also inhibits tumor growth in an animal model (Ramchandani et al., 1997).

Interest in the chemotherapeutic potential of inhibitors of DNA methylation has been retarded by the reports that analogs of 5-azacytosine are carcinogenic (Carr et al., 1984) and can activate oncogenes by hypomethylation (Counts and Goodman, 1994). There has been some confusion in this field since results obtained with the riboside analogue, 5-azacytidine, have also been assumed to apply to 5-AZA-CdR. There are large differences in the pharmacology of these two analogues. Most of 5-azacytidine is incorporated into RNA, interfering with its function (Momparler et al., 1976) and only a small fraction of this analogue is incorporated into DNA, producing hypomethylation. Due to this dual action of 5-azacytidine, it cannot be assumed that all its pharmacologic action is due to its effects on DNA methylation, making data obtained with this analogue difficult to analyze. An example of this is the report that 5-azacytidine was carcinogenic in rats, whereas 5-AZA-CdR was not carcinogenic (Carr et al., 1988). In fact, in the mouse model 5-AZA-CdR was demonstrated to suppress intestinal neoplasia (Laird et al., 1995). It has been reported that 5-AZA-CdR can produce mutations in a bacterial gene in a mouse model (Jackson-Grusby et al., 1997). However, in vitro mutagenesis assays on 5-AZA-CdR in mammalian cells indicate that 5-AZA-CdR is negligibly or not mutagenic (Landolph and Jones, 1982; Momparler et al., 1984c).

5-AZA-CdR is a S-phase specific agent with a short in vivo half-life and pharmacologic activity that is very dose-schedule–dependent (Momparler, 1986). In clinical therapy with 5-AZA-CdR an intensive dose with short interval of treatment appears to the optimal schedule for this agent. Clinical investigations on 5-AZA-CdR were stimulated by the observations that this analogue was a much more potent antileukemic agent in the mouse model that cytosine arabinoside (Momparler et al., 1984b). Also, in support of clinical trials on leukemia with this analogue were the observations that it could induce in vitro differentiation of human leukemic cells (Pinto et al., 1984; Momparler et al., 1985). In the mouse model, the antileukemic activity of 5-AZA-CdR correlates with its inhibition of DNA methylation (Wilson et al., 1983). The first clinical trials on 5-AZA-CdR showed that this analogue could induce complete remissions in patients with acute leukemia in relapse (Momparler et al., 1986). The dose schedule of 5-AZA-CdR used in this trial produced a significant inhibition of DNA methylation in the leukemic blasts (Momparler et al., 1984a). Currently, 5-AZA-CdR shows interesting clinical activity in patients with chronic myeloid leukemia in blast crisis (Kantarjian et al., 1997) and in patients with myelodysplastic syndrome, a preleukemic disease (Zagonel et al., 1993).

Several pilot clinical trials on the antitumor activity of 5-AZA-CdR have been performed. Preclinical studies on human tumor xenografts in the mouse model showed that this analogue is a potent antitumor agent (Braakhuis et al., 1988). 5-AZA-CdR also shows interesting in vitro antitumor activity against breast cancer (Bovenzi et al., 1999). In preliminary studies on patients with head and neck cancer and patients with prostate cancer, 5-AZA-CdR showed minimal to moderate antitumor activity (van Groeningen et al., 1986; Thibault et al., 1998) due to the suboptimal dose schedule used. Interesting antitumor activity was observed with 5-AZA-CdR using a more intense dose schedule in patients with stage IV nonsmall cell lung cancer, including one patient who survived more than 6 years; this response was remarkable due to the short life expectancy of patients with this disease (Momparler et al., 1997). The clinical antitumor activity of 5-AZA-CdR was complex to evaluate because of its delayed antineoplastic action, a characteristic of an agent that induces terminal differentiation. In fact, after treatment some tumor progression was observed for some patients followed by growth arrest. It is interesting to note that a similar phenomenon occurred in some leukemic pa-

tients after treatment with 5-AZA-CdR (Momparler et al., 1986).

More clinical studies have to be performed to evaluate the antitumor activity of 5-AZA-CdR fully. The development of tests that can detect the methylation of tumor suppressor genes in DNA present in the serum (Esteller et al., 1999) and the tests to quantitate the extent of inhibition of DNA methylation (Gonzalgo and Jones, 1997; Lo et al., 1999; Bovenzi and Momparler, in press) will permit the monitoring of these events during therapy with 5-AZA-CdR and help clarify its antineoplastic activity. Hematopoietic toxicity is the major side effect produced by 5-AZA-CdR and limits the dose intensity (Momparler et al., 1986, 1997). It may be possible to overcome this problem by using gene therapy to insert the drug resistance gene, cytidine deaminase, into hematopoietic cells to protect them from the toxicity produced by 5-AZA-CdR (Momparler et al., 1996). Elevated expression of cytidine deaminase in cells confers drug resistance to 5-AZA-CdR (Eliopoulos et al., 1998).

CONCLUSION

The silencing of many genes involved in tumorigenesis by DNA methylation suggests that this event plays an important role in cancer development (Baylin et al., 1998; Jones and Laird, 1999). More experimentation is needed to comprehend the relationship between this epigenetic mechanism and cancer fully. The clinical potential of inhibitors of DNA methylation in cancer therapy still remains to be evaluated (Szyf, 1996). There are preliminary data that suggest that these inhibitors have the potential to be potent antitumor agents (Momparler et al., 1997; Ramchandani et al., 1997). It is possible that investigations on inhibitors of DNA methylation in cancer therapy may help us to further understand the role of this epigenetic process in tumorigenesis.

METHODS FOR THE EVALUATION OF DNA METHYLATION

A variety of methods are used to evaluate the methylation status of genes: Southern blot analysis, bisulfite genomic DNA sequencing, restriction enzyme-PCR, MSP, and the methylation-sensitive single nucleotide primer extension (MS-SNuPE). A report on the comparison of some of these methods has been published (Gonzalgo et al., 1997). A brief description of each of these methods is given below.

Southern blot

The Southern blot is the most frequently used method for DNA methylation analysis. In this method, the genomic DNA is cleaved with methylation-sensitive and insensitive endonucleases specific for the same sequence, such as *Hpa*II and *Msp*I. An analysis of the target sequence for potential methylated CpG sites should be performed to allow proper selection of the suitable restriction enzymes. The restriction fragments are then separated on an agarose gel, transferred to a membrane, and hybridized with a probe specific for the target sequence. Autoradiography will reveal the presence of bands of the predicted size that will allow to assess the methylation status of the target sequence. The limitations of this technique are: (1) the amount of DNA required for the Southern blot analysis (5–10 μg per sample) that becomes prohibitive if the tumor sample is small and (2) the possibility of incomplete enzymatic digestion can lead to results that are difficult to interpret.

Restriction enzyme PCR

Genomic DNA is cleaved with methylation-sensitive and -insensitive restriction enzymes, and the digested DNA is then amplified using primers flanking the target region. Using a methylation-sensitive endonuclease, it should be possible to amplify DNA of a predicted size if the target sequence contains methylated CpG sites. With the use of a methylation-insensitive endonuclease prior to PCR, no amplification will be possible regardless of the methylation status of the target DNA sequence. The major limitation of this method is that the enzyme digestion must be complete. Another limitation is that if several CpG sites are present in the target region, some methylated and others not methylated, the results obtained will be inconclusive. This technique is nevertheless very helpful as a primary screening of DNA methylation in a target sequence (Kane et al., 1997).

Bisulfite DNA sequencing

The bisulfite method for sequencing DNA containing 5-methylcytosine has led to the development of new techniques to analyze the methylation status of genes (Grigg and Clark, 1994). Bisulfite genomic sequencing was first described by Frommer et al. (1992). This technique provides an excellent tool for the detection of single 5-methylcytosine (5-MC) residues in genomic DNA. The method is based on the ability of sodium bisulfite to deaminate all the cytosines to uracil in single-stranded DNA, while leaving the 5-MC unchanged (Hayatsu et al., 1970). The modified DNA is then amplified by PCR, using a set of primers specific for the converted sequence; these primers will amplify a fragment in which all the uracils (converted cytosines) are amplified as thymine (T) and only the 5-MC remain as C. The amplified fragments can then be sequenced directly or after cloning of individual molecules. The cloning strategy is useful for the analysis of the methylation differences between alleles (Clark et al., 1994, 1995). It is very important to verify if the bisulfite conversion is complete, because it has been reported that C adjacent to methylated CpG sites can be resistant to bisulfite treatment (Harrison et al., 1998).

MSP

MSP is a technique that was first described by Herman et al. (1996a). It takes advantage of the sequence difference existing between methylated and unmethylated DNA after bisulfite treatment. Cytosines are deaminated to uracil, which replicates as thymine (T) during PCR, allowing the design of primers that will specifically anneal to sequences that contain either methylated (C) or modified (T) sites. After PCR, amplified DNA will be obtained with the pair of primers specific for unmethylated or methylated sequences (Herman et al., 1996a). MSP is a rapid and qualitative method for the analysis of presence of methylation in a given region of DNA. Careful selection of primers is

very important because it is possible to obtain false-positives with both methylated and unmethylated primer pairs, making it difficult to interpret the results. Incomplete bisulfite modification of genomic DNA will also give false-positives for methylated C. Another approach that can be used is to determine if the PCR product is sensitive to digestion by restriction enzymes that will only digest the DNA if their recognition sequence was methylated prior to treatment with bisulfite (Li et al., 1999).

Methylation-sensitive single nucleotide primer extension

The single nucleotide primer extension assay was first described by Kuppuswamy et al. (1991) for the detection of mutations in abnormal alleles. Gonzalgo and Jones (1997) modified this method for the quantitation of methylation differences at specific CpG sites. After bisulfite treatment of DNA and amplification of the target sequence with primers specific for the converted DNA, the resulting amplified DNA can be used as a template for the methylation-sensitive single nucleotide primer extension (MS-SNuPE) reaction. The primers used for this single nucleotide extension reaction are designed so that the primer ends just one nucleotide before the incorporation site designated for methylation analysis. The purified amplified DNA is incubated with either radioactive dCTP or dTTP and DNA polymerase. If the target site is methylated, a C will be incorporated during nucleotide extension, if the site is unmethylated, a T will instead be incorporated. Quantitation of the relative C and T incorporated will allow the determination of the methylation status of the target site. As discussed above, primer design and complete modification of DNA are important in order to obtain good results with this assay.

Other assays for evaluation of DNA methylation

The above assays are the most widely used for DNA methylation analysis. Other interesting assays have been developed including the COBRA assay (Xiong and Laird, 1997) and the creation of new specific sites for restriction enzymes by bisulfite modification of genomic DNA (Sadri and Hornsby, 1996). Several novel methods have been developed for the detection of methylated sequences and for the cloning of differentially methylated genes (Gonzalgo et al., 1997; Huang et al., 1997; Akama et al., 1998; Ushijima et al., 1997; Toyota et al., 1999). We apologize for the omission in this review of other interesting assays that can be useful for methylation analysis.

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APPENDIX IV: Additional results and discussion

Objectives:

1. Determine if different concentrations of 5-AZA-CdR and TSA produce an additive antineoplastic action as shown by clonogenic assay against MDA-MB-231;
2. Determine if similar antineoplastic interaction also occurs in a second human breast cancer carcinoma cell line (MCF-7);
3. Confirm, using a different assay (inhibition of DNA synthesis), similar additive interactions between 5-AZA-CdR and TSA in MDA-MB-231 cells;
4. Perform a time course on the inhibition of DNA synthesis by 5-AZA-CdR and TSA in this cell line;
5. Perform a time course on the inhibition of DNA methylation of RARβ promoter by 5-AZA-CdR in MDA-231 cells.

Experimental methods:

Clonogenic assay: Clonogenic assay for MDA-MB-231 and MCF-7 cells was performed as described at page 124 (Bovenzi and Momparler, submitted manuscript). Cells were treated with 5-AZA-CdR at concentrations ranging from 10 to 50 ng/ml for 48 hours, and/or with TSA at concentrations ranging between 2.5 and 100 ng/ml for 48 hours.

DNA synthesis inhibition: MDA-MB-231 cells were plated at 5,000 cells/well on a 6-well dish containing 2ml of media and treated with 5-AZA-CdR at concentrations of 1, 5, 10 and 25 ng/ml or TSA at concentrations of 6, 11, 23, 45 and 91 ng/ml, for 48 hours. For drug combinations cells were treated sequentially with 5-AZA-CdR 48 h and TSA 48 h at different concentrations. At 24h from beginning of treatment, ^{3}H-thymidine (0.6μCi/well) was added to the media for 24 hours. Cells were harvested at 48 h from the beginning of treatment and collected on A/C glass fibre filters (25mm), washed with 3ml of 0.9% NaCl, then with 2 x 3ml of cold 5% TCA (trichloroacetic acid), and finally with 3ml of 100% ethanol. Discs were dried at 80°C and the radioactivity was measured in a Beckman LS 6000IC scintillation counter. For the time course experiment,

MDA-MB-231 cells were plated at 75,000 cells/well and treated with 5-AZA-CdR 50 ng/ml for 12, 24, 30, 36 and 48 hours.

DNA methylation analysis: For the DNA methylation analysis, MDA-MB-231 cells were treated with 5-AZA-CdR 50 ng/ml at different time points: 0, 12, 24, 30, 36, and 48 hr. DNA methylation analysis was performed by SNuPE as published (Bovenzi and Momparler, 2000).

Results

Our results show that combination of 5-AZA-CdR and TSA at different concentrations results in additive inhibition of clonogenicity on both MDA-MB-231 and MCF-7 breast cancer cell lines (**Fig. A1 and A2**).

Inhibition of DNA synthesis by either 5-AZA-CdR and TSA in MDA-MB-231 cells increases at increasing concentrations of the drugs with an IC_{50} of about 2.5 ng/ml for 5-AZA-CdR and 9 ng/ml for TSA (**Fig. A3**). Combination of these two drugs leads to additive inhibition of DNA synthesis as shown in **Fig. A4**. Inhibition of DNA synthesis increases linearly with time starting at 12 hours of drug exposure and reaching IC_{50} at 33h for 5-AZA-CdR and at 26h for TSA as shown in **Fig. A5**.

Analysis of DNA methylation of the promoter region of RARβ by MSP showed that significant demethylation takes place in this region already at 12h of 5-AZA-CdR treatment (data not shown). In order to quantify the observed decrease in DNA methylation, we performed a SNuPE assay on the bisulfite-converted DNA from MDA cells treated as described in *methods*. **Fig. A6** shows that at 12 h significative decrease in methylation takes place. From time 12 to 48h the extent of inhibition of DNA methylation reaches a plateau for both primers. At 48 hours with primer A, a significant decrease in inhibition seems to take place.

Discussion

Using a clonogenic assay and different drug concentrations, we observed an additive antineoplastic action of 5-AZA-CdR in combination with TSA against MDA-MB-231 (**Fig. A1**). In order to determine if this interaction also occurs in other breast tumor cell lines, we performed a similar experiment of MCF-7 (ER+) breast carcinoma cells. With this latter cell line we also observed and additive antitumor effect between 5-AZA-CdR and TSA (**Fig. A2**).

Our next objective was to determine if the additive interaction could also be demonstrated by a different assay. We investigated the effect of 5-AZA-CdR and TSA on DNA synthesis: after 48 hours of exposure, each drug was able to inhibit DNA synthesis in MDA-231 cells at concentrations ranging from 1 to 90 ng/ml (**Fig. A3**). Combination of both agents produced a synergistic inhibition of DNA synthesis (**Fig. A4**). This interaction may be due to the differentiating effects of both drugs. Both 5-AZA-CdR (Pinto et al., 1984; Momparler et al., 1985) and histone deacetylase inhibitors (Yoshida et al. 1990) have been shown to lead to *in vitro* terminal differentiation of leukemic cells.

An alternative explanation may be provided by the new model for DNA methyltransferase (MTase) protein-protein mechanism of action (Szyf, 2000). According to this model, MTase is present at the replication fork in order to ascertain that the methylation pattern of the parental strand is maintained in the nascent strand. MTase protein has a domain that targets the replication fork, and it binds the PCNA (proliferating cell nuclear antigen) protein (Leonhardt et al., 1992). The interaction of MTase and PCNA occurs at the same site of interaction of PCNA with the cyclin dependent kinase inhibitor, p21 (Leonhardt et al., 1992). The competitive binding of either MTase or p21 to PCNA may be a determinant for the initiation or arrest of the replication machinery. Interestingly, cancer cell lines and primary tumors have been shown to overexpress MTase (Kautiainen and Jones, 1986; El-Deiry et al., 1991). This favours binding of MTase to PCNA instead of p21, then allowing DNA synthesis and uncontrolled cell proliferation, characteristic of transformed cells.

Our data suggest a synergistic interaction between 5-AZA-CdR and TSA on inhibition of DNA synthesis in MDA-231 cells. One possible explanation to this observation may be that 5-AZA-CdR, by covalently binding and sequestering MTase favours the binding of p21 to PCNA, thus stopping the replication machinery. On the other hand, it has been widely reported that TSA, and other HDAC inhibitors, strongly up-regulate the p21 gene (Kim et al., 1999, DiGiuseppe et al., 1999; Richon et al., 2000). Increased amounts of p21 protein lead to a block in DNA synthesis. Both drugs cause the same final effect (favour binding of p21 to PCNA), through different mechanisms.

We next performed a time-course experiment for DNA demethylation and inhibition of DNA synthesis after exposure to 5-AZA-CdR. The results, shown in **Fig. A5**, clearly indicate that DNA synthesis inhibition increases linearly within the time lag considered in this experiment and reaches ≥90% of inhibition within 48 h for 5-AZA-CdR and TSA at the concentrations used. It seems quite improbable that the effects of 5-AZA-CdR on DNA synthesis at 12-24 hours of exposure are mediated through induction of genes involved in differentiation.

5-AZA-CdR needs to be incorporated into the DNA in order to induce inhibition of DNA methylation by covalently binding MTase. We tested the inhibition of DNA methylation of RARβ promoter at the same time points used for inhibition of DNA synthesis (0, 12, 24, 30, 36, and 48 hours) after treatment with 5-AZA-CdR. The doubling time of MDA-231 cells is 27 hours. We observed a significative decrease in DNA methylation at 12 hr following the beginning of 5-AZA-CdR treatment, equal to 22 and 24% of the control for primer A and B respectively (**Fig. A6**). This observation was quite interesting because it suggests that demethylation of RARβ is an early event following treatment of MDA-MB-231 cells with 5-AZA-CdR. The question still remains whether the observed demethylation is an effect of the incorporation of 5-AZA-CdR into the DNA, or if an active process of demethylation by a *bona fide* demethylase takes place.

Another interesting observation was that after the first significative decrease at 12 hours, DNA methylation seems to remains stable in the following

36 hours and did not decrease, as we would have expected (**Fig. A6**). These results are in agreement with the finding that DNA synthesis reaches 90% of inhibition within 48 hours of 5-AZA-CdR treatment. This inhibition in cell cycle may be due to different mechanisms: first, induction of genes involved in cell differentiation may be induced by the demethylation caused by 5-AZA-CdR; second, the recognition of the MTase-5-AZA-CdR complex by the DNA repair machine, may lead to cell cycle arrest in order to allow DNA repair. Third, inhibition of the replication machinery may be due to the altered balance between p21 and MTase binding to PCNA (Szyf, 2000). An interesting experiment would be treatment of cells with antisense oligonucleotides or oligonucleotide based DNMT agonists, in order to rule out the mechanism(s) involved in cell cycle arrest by direct inhibitors of DNA methylase, without the additional effects possibly caused by the incorporation of 5-AZA-CdR into the DNA.

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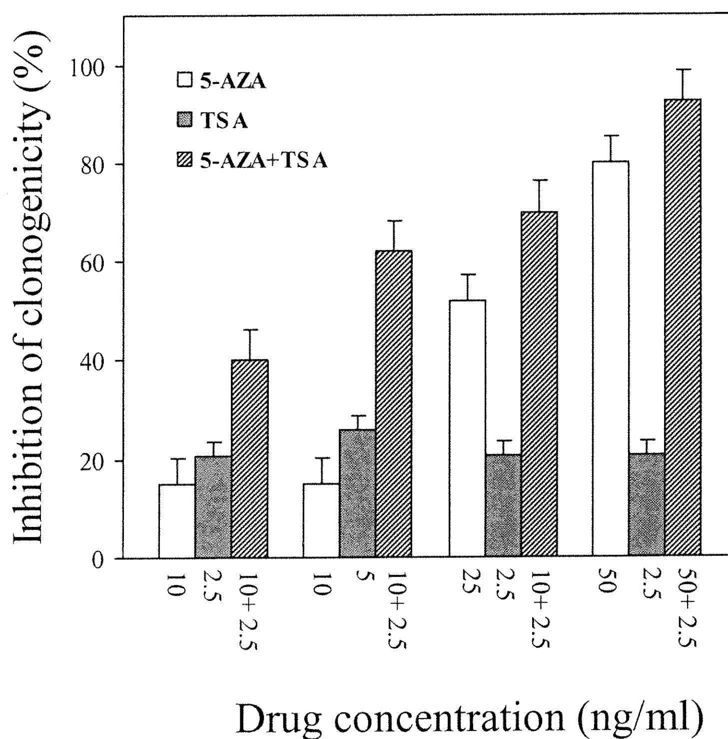

Fig. A1: Clonogenic assay on MDA-MB-231. Cells were treated with 5-AZA or TSA at different concentrations for 48 h. For drug combination, cells were treated sequentially for 48h with each drug. After drug removal, cells were left in culture and colonies counted at day 11-14.

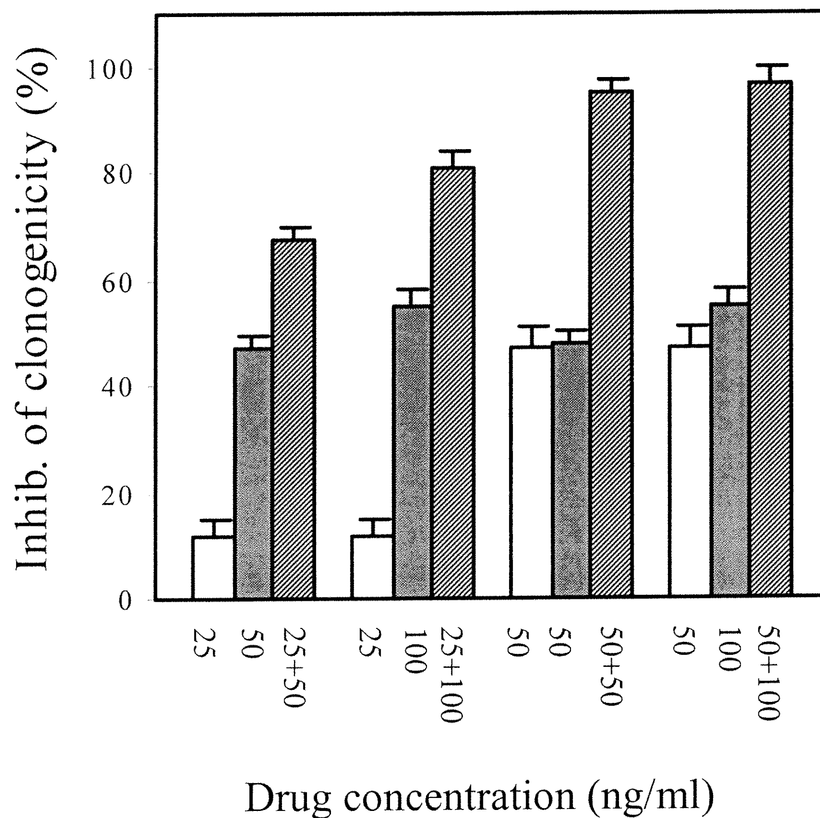

Fig. A2: Clonogenic assay on MCF-7. Cells were treated with 5-AZA or TSA at different concentrations for 48 h. For drug combination, cells were treated sequentially for 48h with each drug. After drug removal, cells were left in culture and colonies counted at day 11-14.

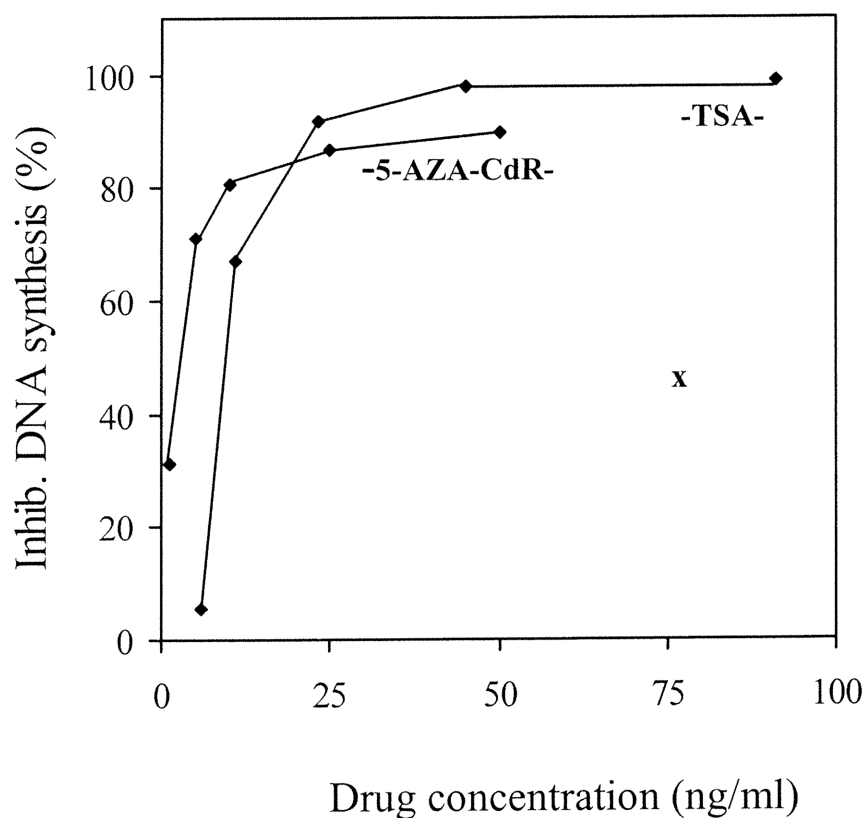

Fig. A3: Inhibition of DNA synthesis in MDA-MB-231 cells. Cells were treated with 5-AZA-CdR (1, 5, 10, and 25 ng/ml) or TSA (6, 11, 23, 45, and 91 ng/ml) for 48 h. Cells were pulsed with ^{3}H-thymidine for the last 24 hours of drug treatment. Inhibition of DNA synthesis was calculated as the decrease in ^{3}H-thymidine incorporation of treated cells compared to untreated cells.

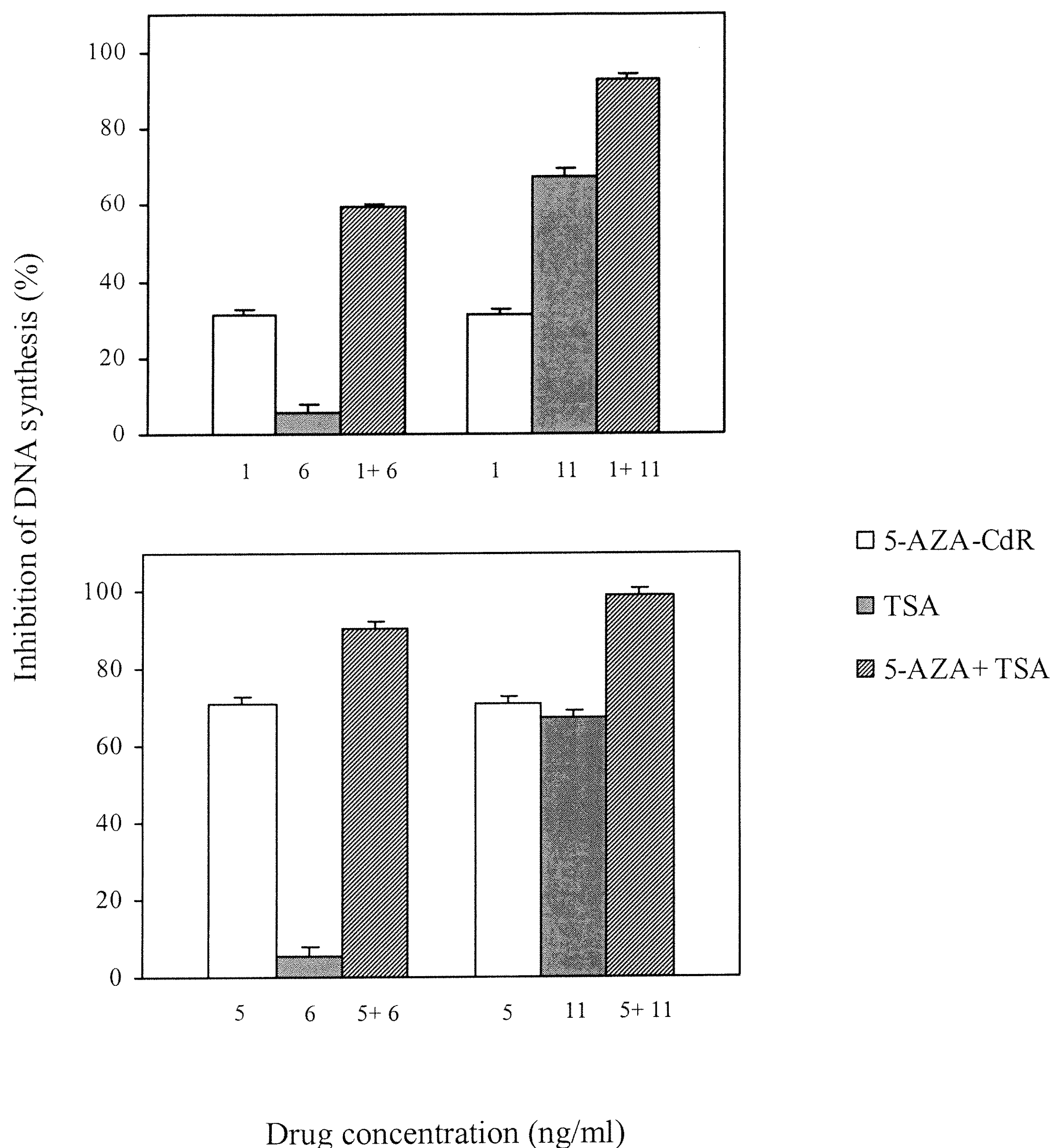

Fig. A4: Inhibition of DNA synthesis by 5-AZA-CdR and TSA. MDA-MB-231 cells were treated with different concentrations of 5-AZA-CdR or TSA for 48h. For drug combination, sequential exposure to both drugs was performed for a total of 96h. Cells were pulsed with ^{3}H-thymidine for the last 24 hours of drug treatment. Inhibition of DNA synthesis was calculated as the decrease in ^{3}H-tymidine incorporation of treated cells compared to untreated cells.

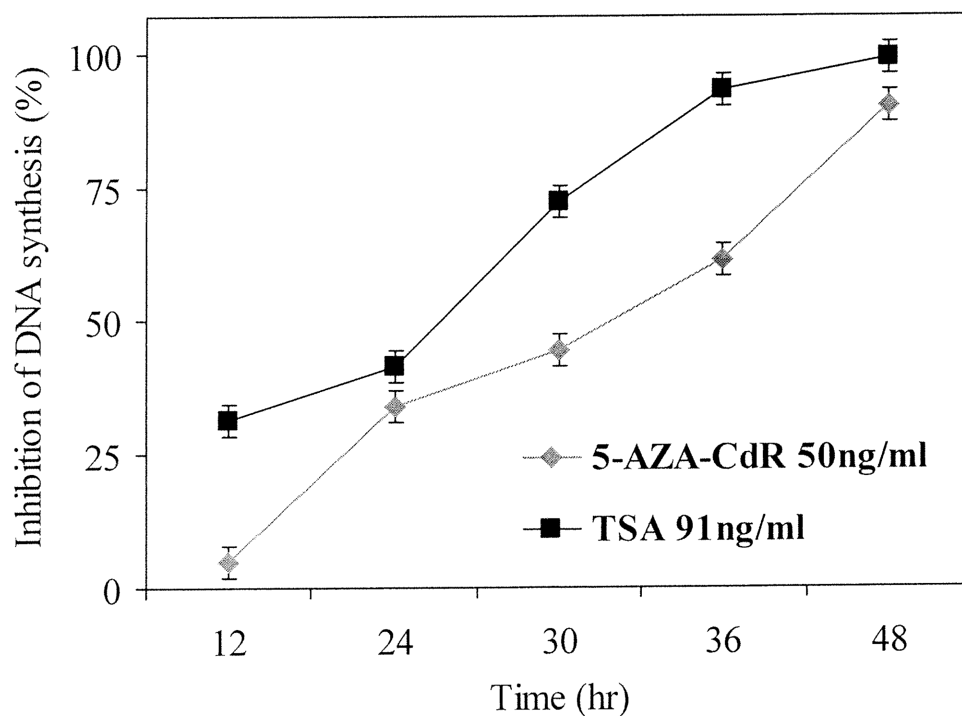

Fig. A5: Time-dependent inhibition of DNA synthesis. MDA-MB-231 cells were treated with 5-AZA-CdR 50 ng/ml or TSA 91 ng/ml at different times of exposure: 12, 24, 30, 36, and 48 h. Cells were pulsed with ^{3}H-thymidine for 12-24 hours. Incorporation of ^{3}H-thymidine was counted and compared to untreated cells to mesure the inhibition of DNA synthesis.

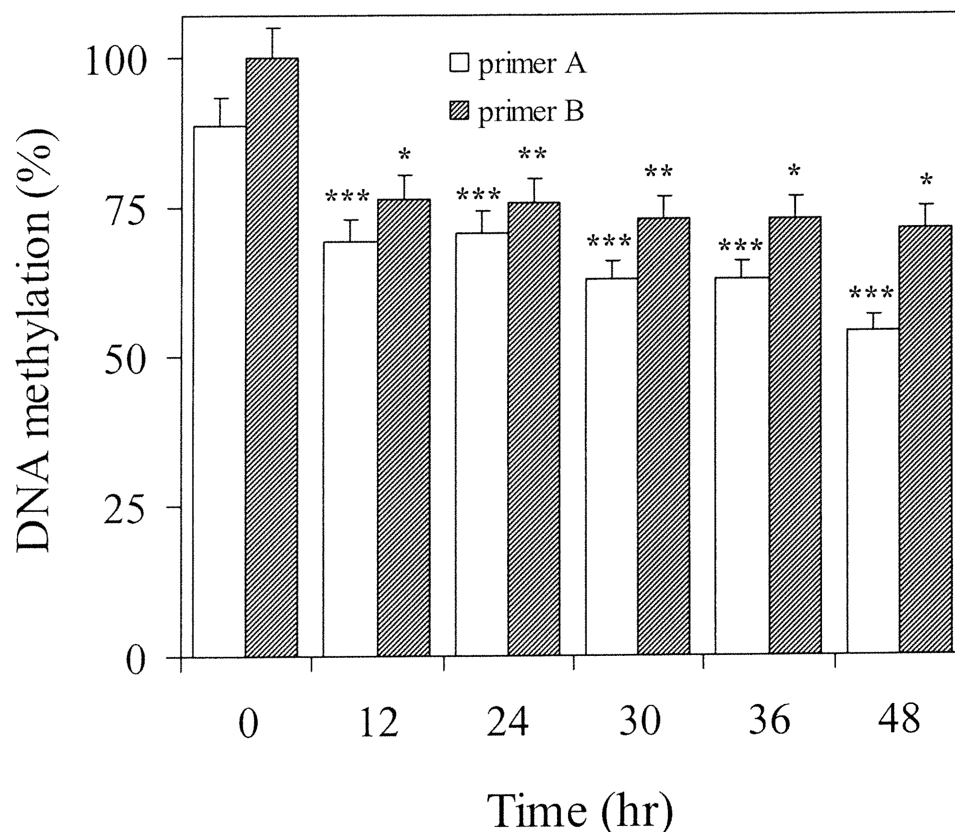

Fig. A6: Time dependent decrease in DNA methylation of RARβ by 5-AZA-CdR. MDA-MB-231 cells were treated with 5-AZA-CdR 50 ng/ml for 0, 12, 24, 30, 36, and 48 h. Genomic DNA from these cells was treated with sodium bisulfite as described previously (Bovenzi and Momparler, 2000). Bisulfite converted DNA was used to amplify a region of the RARβ promoter. Quantification of DNA methylation at specific sites was performed using the SNuPE assay as described in *methods*. Student t test for independent samples was performed for each time point versus control (t=0). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.