

DNA methyltransferase: a downstream effector of oncogenic programs; implications for therapy.

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Summary

DNA MeTase is an attractive anticancer target. A molecular analysis of its regulation suggests that it is a downstream effector of many oncogenic pathways and that its down modulation can inhibit tumor growth. It is possible that DNA MeTase inhibitor will be effective in a broad spectrum of cancers because it lies downstream to nodal cellular checkpoints that could be activated by multiple ways. An important challenge is to design novel inhibitors of DNA MeTase that are highly specific. Such inhibitors are potentially important pharmacological agents with wide therapeutic applications.

I. Introduction

A. Working hypothesis: DNA MeTase is an anticancer target

This review summarizes recent findings demonstrating that the cytosine DNA Methyltransferase (DNA MeTase) is a downstream effector of cellular pathways leading either to oncogenesis or a change in the state of differentiation of vertebrate cells (Rouleau et al., 1992; Rouleau et al., 1995; MacLeod et al., 1995; MacLeod and Szyf, 1995; Szyf et al., 1992). It is becoming clear that control of gene expression by pharmacological means is one of the great challenges and hopes of current therapeutics. DNA MeTase is a master regulator of gene expression programs. This review suggests that genomic programs could be specifically modulated by pharmacological inhibition of DNA MeTase. Since DNA MeTase is believed to be involved in similar processes in a broad group of animals and plants, my hypothesis is that agents that inhibit DNA MeTase specifically will have broad pharmacological applications (Szyf 1994; Szyf 1996). Recent data from our laboratory using different classes of DNA MeTase inhibitors supports this hypothesis.

II. Background

The goal of this section is to illustrate the logical

progression of concepts and data leading to our working hypothesis and to the proposal that DNA MeTase inhibitors could serve as important pharmacological agents.

A. What is DNA methylation?

DNA methylation is a postreplicative covalent modification of DNA that is catalyzed by the DNA methyltransferase enzyme (DNA MeTase) (Razin and Szyf, 1984; Bestor et al., 1988). The main concept in DNA methylation is the idea of a pattern of DNA methylation and its correlation with the state of activity of genes (Yisraeli and Szyf, 1984). In vertebrates, the cytosine moiety at a fraction of the CpG sequences is methylated (60-80%); the non methylated CpGs are distributed in a nonrandom manner generating a pattern of methylation that is gene and tissue specific (Yisraeli and Szyf, 1984). Plant DNA is also methylated at CG as well as CXG sequences (Gruenbaum et al., 1984). DNA methylation plays an important role in development of plant cells and might be a critical element involved in silencing transgene expression in plants (Meyer, 1995).

B. DNA Methylation patterns encode epigenetic information

1. Correlation of gene expression and DNA

methylation patterns

Does the pattern encode epigenetic information? A large number of papers published in the last two decades

have shown a correlation between the pattern of methylation and the state of activity of genes (Yisraeli and Szyf, 1984). That is, some or all of the CpG sites in

Figure 1. DNA methylation patterns fix gene expression programs, DNA MeTase inhibitors alter gene expression programs .

The genome of a vertebrate (first line) bears many potential sites for modification by methylation (open circles). However, a subset of these sites is methylated (indicated by an M in the circle) in different tissues. When one looks at the methylation pattern of different genes (a to e) in different tissues (for example tissues A and B), one observes that they bear a different pattern of methylation. One also observes that in general inactive genes are modified whereas active genes bear sites of methylation that are not modified. It is proposed that both binding of transcription factors to regulatory sites of the genes (indicated by the triangles and ovals-activators in green and methylated-DNA binding factors-repressors) as well as the pattern of methylation (methylated sites attract methylated-DNA dependent repressors) define the state of activity of vertebrate genes. The pattern of methylation is maintained because of limiting level of DNA MeTase, thus the level of DNA MeTase locks the gene expression program of a tissue. Inhibition of DNA MeTase by DNA MeTase inhibitors results in transient demethylation and unlocking of the gene expression program. Demethylation enables reorganization of the interactions of transcription factors with DNA and resetting of a new program of gene expression. The direction that this reorganization will take is limited by the repertoire of transcription factors in the cell.

regulatory regions of a specific gene will be methylated in all tissues where the gene is silenced but the same sites will be nonmethylated in tissues that express the gene (Yisraeli and Szyf, 1984) (**Fig. 1**).

2. DNA methylation and gene expression: cause or effect?

There is a longstanding and unresolved discussion whether the state of methylation of genes is a cause or

effect of their state of expression. Whereas a series of experiments demonstrated that inactivation of a gene precedes its repression (Lock et al., 1987), other studies have shown that methylation of genes before they are introduced into cells can suppress their activity (Stein et al., 1982, for a review of this question see Szyf, 1996). One possible solution to this dilemma is the suggestion that there is a dynamic interrelationship between DNA methylation and gene expression (Szyf, 1996). DNA methylation can play both a primary and secondary role in gene expression. That is, methylation of certain sites

precipitates gene repression whereas in other instances the chromatin structure of a repressed gene can trigger DNA methylation. The combination of these processes should result in formation of a stable state of gene repression by a

covalent modification of the DNA structure itself. Thus, the pattern of methylation in a cell will stabilize a gene expression program for this cell (**Fig. 1**).

Figure 2. Two mechanisms for gene repression by methylation.

DNA methylation can inhibit gene expression by two different mechanisms. The model gene described in this figure is activated (horizontal arrow indicates mRNA transcript) by interaction of transcription factors (triangle) with a cis acting sequence (shaded box) located in front of the transcription initiation site. The gene has a number of methylatable sites (open circles), one of which is located at the transcription factor recognition sequence. Mechanism A describes a case where the methylatable site located at the transcription recognition site is methylated (M). This methylation inhibits the recognition of the cis acting sequence by the transcription factor. Mechanism B describes a case where a regional methylation occurred in the body of the gene. This methylation results in binding of methylated-DNA binding protein(s) to the methylated region (open oval). The binding of this protein precipitates the spreading of an inactive chromatin structure, the gene becomes inaccessible to transcription factors and is not transcribed.

3. What is the mechanism of gene repression by methylation?

A series of publications suggest that DNA methylation can repress gene expression directly, by inhibiting binding of transcription factors to regulatory sequences (Becker et al., 1987), or indirectly, by signaling the binding of methylated-DNA binding factors that repress gene activity or by precipitating an inactive chromatin structure (Razin and Cedar, 1977; Keshet et al., 1986). Two methylated DNA binding proteins that can repress transcription in a methylation dependent manner have been recently characterized MeCP2 and MeCP1 (Cross, et al., 1997; Nan et al., 1997). The carboxy terminal half of MeCP2 contains a repressor domain which can interact with the transcriptional machinery. MeCP2 is also suggested to

precipitate or stabilize an inactive chromatin structure. Kass et al., have shown that methylated DNA is assembled into an inactive chromatin structure (Kass et al., 1997). It is not clear yet whether MeCP2 is generally involved in the precipitation of an inactive chromatin structure on methylated DNA or whether other mechanisms are involved in building of inactive chromatin around methylated DNA (**Fig. 2**).

4. Summary: methylation plays an important role in control of genomic functions.

A long list of data supports the hypothesis that DNA methylation plays an important role in the control of genomic functions. It is well established that regulated

changes in the pattern of DNA methylation occur during development (Brandeis et al., 1993), parental imprinting (Peterson and Sapienza, 1993) and cellular differentiation (Razin et al., 1985) and that aberrant changes in the pattern of methylation occur in cellular transformation (Feinberg et al., 1983; de Bustros et al., 1988; Baylin et al., 1991). A targeted mutation, by homologous recombination in ES cells, of the DNA MeTase gene results in embryonic lethality (Li et al., 1992) and inhibition of DNA MeTase by expression of an antisense to the DNA MeTase results in a change in the identity of a cell from a fibroblast to a cell of myogenic lineage (Szyf et al., 1992).

Whereas the most studied biological process regulated by DNA methylation is gene expression, it is possible that DNA methylation directly regulates other genome functions such as replication and recombination. These processes might be as important as gene expression in the events leading to oncogenesis (Szyf, 1996). If a DNA methylation pattern locks certain gene expression programs, then modulating the pattern should unlock these programs and play important therapeutic roles (Szyf, 1994; Szyf, 1996) (Fig. 1). To be able to design therapeutic strategies to unlock a pattern of methylation and gene expression, one has to understand the mechanisms that control DNA methylation patterns.

C. The level of DNA MeTase is an important determinant of DNA methylation patterns.

1. The semiconservative model of methylation inheritance

The accepted model in the field has been that patterns of methylation are maintained because the DNA MeTase is very efficient in methylating hemimethylated DNA generated in the process of replication (maintenance methylation) but very inefficient in methylating nonmethylated DNA (de novo methylation) (Razin and Riggs, 1980). However in spite of the simplicity of this model, both the cloned DNA MeTase (Tollefsbol and Hutchinson, 1995) and a putative new enzyme (Lei et al., 1996) have been shown to bear de novo methylation activity.

2. The role of cis acting signals

If de novo methylation is possible, what determines the specificity of DNA methylation? One important factor is cis signals contained in the sequence and putative cellular factors recognizing these signals (Szyf et al., 1989; Szyf, 1991) (Fig. 3). These signals possibly direct the general DNA methylation machinery to specific regions. Several experiments have shown that the presence of certain cis-acting sequences protect adjacent sequences from methylation (Szyf et al., 1990) while other sequences target adjacent sequences to become methylated (Szyf et al., 1989).

3. DNA methylation patterns are regulated by an interplay between local signals and the level of DNA MeTase activity. The role of cell legacy

Could the pattern of DNA methylation be determined also by central cellular signals? Very limited attention has been given to the role that the cellular level of the enzyme catalyzing DNA methylation might play in determining and controlling DNA methylation patterns. One obvious reason why this level of regulation was not considered is because it had been difficult to explain how a general change in the level of the enzyme could lead to discrete changes in DNA methylation. To address that question I have previously suggested that the pattern of methylation is determined by an interplay between local signals, as suggested for example by the de novo methylation of the C21 gene in Y1 cells (Szyf et al., 1989), and the level of DNA MeTase (Szyf et al., 1984) and demethylase activities (Szyf 1994; Szyf et al., 1995) in the cell (Fig. 3). The affinity of each CpG site to DNA MeTase is determined by either the properties of the sequence or the DNA binding proteins interacting with it in specific cell types. Thus, the final pattern of methylation will reflect the legacy of the cell, its specific repertoire of DNA binding factors and resulting chromatin structure.

According to this hypothesis we predict that a general change in DNA MeTase will result in a predictable change in DNA methylation pattern that is specific per cell type. In accordance with this hypothesis it has been shown that a limited inhibition of DNA MeTase results in specific

Figure 3. What determines DNA methylation patterns? A model.

DNA methylation patterns are determined by an interplay between: Transacting factors (triangle-factors

enhancing methylation, oval-factors enhancing demethylation), Signals in DNA (red-enhancing methylation, green-enhancing demethylation), Levels of DNA MeTase and DNA demethylase activities. The pattern of methylation could be altered by modulation of any of these factors. The ideal targets for pharmacological intervention are the enzymatic activities.

Figure 4: Regulation of the DNA MeTase, a model.

The DNA MeTase promoter and 5' upstream region: the filled boxes indicate the first exons. Two translation initiation sites were identified, potentially resulting in a long and a short form of DNA MeTase (indicated by ATG) which might exhibit different transforming capabilities.

Each of these initiation sites is regulated by a different promoter. The lower translation initiation site is a product of a transcript (transcription initiation sites indicated by horizontal arrows) initiating downstream of a promoter that is regulated by the Ras signaling pathway. The AP-1 recognition sequences located at (~-1.7) upstream of the lower transcription initiation sites are indicated as ovals. The expression of DNA MeTase is regulated at multiple levels: First, by choice of promoter resulting in two different DNA MeTase forms. Second, the basal promoter which maintains limiting levels of DNA MeTase expression and is possibly regulated by tumor suppressors. Third, the mRNA is destabilized at G0 phase of the cell cycle.

This is regulated by the retinoblastoma protein. T antigen can remove this regulation and stabilize the DNA MeTase mRNA. Fourth, a cluster of AP-1 sites can mediate transactivation of DNA MeTase by signal transduction pathways and by oncogenic signals. The activation by AP-1 could be repressed by glucocorticoid receptor.

alterations in DNA methylation patterns and differentiation of the cell to the next stage in differentiation rather than a chaotic loss of identity (Szyf et al., 1992). Animal experiments and clinical trials

performed two decades ago have shown that general inhibition of DNA MeTase by the DNA MeTase inhibitor 5-Aza-CdR resulted in specific activation and demethylation of γ globin gene in animals and patients

(Ley et al., 1982; DeSimone et al., 1982).

4. DNA MeTase is regulated by central cellular signals at the transcriptional and posttranscriptional level

a. Transcriptional regulation:

During the last five years, we have shown that the DNA MeTase is regulated at both the transcriptional and posttranscriptional level by nodal cellular signaling pathways (Szyf, 1994; Szyf, 1996). Cloning and characterizing the promoter of the DNA MeTase enabled us to determine that it bears AP-1 sites which are transactivated by Jun, a downstream effector of the nodal cellular and oncogenic Ras signaling pathway (Rouleau et al., 1992; Rouleau et al., 1995) (**Fig. 4**). Down regulation of the Ras-Jun pathway in the mouse adrenocarcinoma cell line Y1 leads to inhibition of DNA MeTase expression, inhibition of DNA methylation, alteration of DNA methylation patterns and reversal of oncogenesis (MacLeod and Szyf, 1995; MacLeod et al., 1995). Recently, an additional start site upstream to the one identified by us which encodes a new translation initiation site resulting in a larger protein has been reported (Tucker et al., 1996). This start site is located in a CG island which is characteristic of housekeeping genes. My hypothesis is that differential utilization of these two promoters, an AP-1-regulated activity versus a housekeeping basal regulation plays an important role in determining the cellular level of DNA MeTase and its substrate specificity. Our recent data demonstrates that the human DNA MeTase promoter region bears as well a large number of AP-1 sites and is also upregulated by the Ras signaling pathway (Ramchandani et al., unpublished data).

b. Posttranscriptional regulation:

The DNA MeTase is also regulated with the phase of the cell cycle (Szyf et al., 1991). Whereas transcription of the message continues throughout the cell cycle, DNA MeTase mRNA is absent in Go cells which is consistent with posttranscriptional control of DNA MeTase mRNA. It has been recently shown that posttranscriptional regulation of DNA MeTase is also associated with muscle differentiation (Liu et al., 1996). Our recent data has linked the posttranscriptional regulation of DNA MeTase to basic cellular pathways that are known to play a critical role in cellular transformation (**Fig. 4**). We have recently shown that ectopic expression of SV40 T antigen in nontransformed 3T3 cells results in induction of DNA MeTase mRNA, DNA MeTase protein levels and genomic DNA methylation. A T antigen mutant which has lost the ability to bind pRb does not induce DNA MeTase. Surprisingly, this upregulation of DNA MeTase by T antigen occurs mainly at the posttranscriptional level by

altering mRNA stability. Inhibition of DNA MeTase by 5-Aza-CdR reverses T antigen induced transformation suggesting a causal role for increased DNA MeTase activity in T antigen triggered transformation (Pinard et al., unpublished observations). This data links the Rb tumor suppressor pathway to the posttranscriptional regulation of DNA MeTase. It is interesting to note that Ras and T antigen can only transform primary cells when they are jointly expressed. It is tempting to speculate that their cooperative role in cellular transformation reflects the fact that they regulate DNA MeTase at two different levels.

An alternative mechanism that might be responsible for the specificity of DNA MeTase is regulated alternative splicing of exons encoded by the DNA MeTase gene. Recent data from my laboratory suggests that the human DNA MeTase is encoded by 40 exons and that there is a potential for in-frame alternative splicing that will result in different forms of DNA MeTase (Deng et al., unpublished results). Regulation of this process with different developmental stages might play an important role in specifying specific classes of sites for methylation.

In summary, our observations do not only establish the regulation of DNA MeTase by central cellular signaling pathways, but also suggest a potential molecular link between DNA methylation and oncogenic pathways (Szyf, 1994) (**Figures 4, 5**). The concept that DNA methylation patterns could be controlled by altering the level of the DNA MeTase leads to the idea that partial inhibition of DNA MeTase by pharmacological agents could result in altering gene expression programs (**Fig. 4**). One example of many possible applications of my hypothesis is the use of inhibition of DNA methylation to inhibit cellular transformation (**Fig. 5**). Inhibitors of DNA methylation could possibly be used to alter and control genetic programs in humans, plants and animals and might have broad application in clinical medicine, veterinary medicine and agriculture (Meyer, 1995).

III. DNA MeTase is an important therapeutic target.

A. DNA methylation and cellular transformation

1. Is methylation involved in oncogenesis?

The findings that the level of DNA MeTase as well as the pattern of DNA methylation might be controlled by oncogenic pathways leads to the question of whether DNA methylation plays an important role in cellular transformation? An activity that has a widespread impact on the genome such as DNA MeTase is a good candidate to play a critical role in cellular transformation. This hypothesis is supported by many lines of evidence that have demonstrated aberrations in the pattern of

methylation in transformed cells.

2. Induction of dMTase activity in cancer cells explains the hypomethylation observed in these cells.

Although it is clear that methylation patterns are altered in cancer cells, the direction that these changes take is perplexing. While many reports show hypomethylation of both total genomic DNA (Feinberg et al., 1988) and individual genes in cancer cells (Feinberg et al., 1983), other reports have indicated that hypermethylation of specific loci such as "tumor suppressor" genes is an important characteristic of cancer

cells (Makos et al., 1992; Baylin et al., 1988; Baylin et al., 1991). How can one resolve this contradiction? We have recently suggested (Szyf, 1994) that the hypomethylation observed in cancer cells is a consequence of increased DNA demethylation activity induced by oncogenic pathways such as Ras7 which acts on a different subset of sites than those that are hypermethylated (Szyf et al., 1995). We have recently characterized a bona fide demethylase activity that is especially abundant in all cancer cells (Bhattacharya and Szyf unpublished results). We suggest that the dMTase recognizes CpG sites with different specificity than the DNA MeTase resulting in concomitant hypomethylation of some sites and hypermethylation of other sites (Szyf, 1994).

Figure 5: DNA MeTase hyperactivation and tumorigenesis; reversal by DNA MeTase antagonists, a model.

Regulated expression of DNA MeTase (standing rectangle indicating MeTase level is partly filled) is critical for maintaining the pattern of methylation (open circles indicate methylatable sites and M indicates methylation) and locking a somatic cell in its program. Two facets of genome functions are regulated by DNA methylation. First, the profile of gene expression (the maintenance of the cognate program is indicated by a lock, expressed genes are indicated by horizontal arrows, transcription factors are indicated by ovals and triangles, methylated-DNA binding repressors are indicated by red ovals). Second, control of DNA replication is regulated by methylation (indicated by a stop sign). The replication control sequences (indicated by the open square) are not methylated in a resting somatic cell, signaling arrest of DNA replication. Oncogenic signaling pathways can induce the DNA MeTase resulting in hypermethylation of certain sequences, both genes and replication control regions. This results in loss of the original gene expression profile of the cell (open lock) and loss of the control over replication. Inhibitors of DNA MeTase can reduce the level of DNA MeTase, resulting in hypomethylation and activation of the replication control regions as well as restoration of some of the original gene expression program.

3. Induction of DNA MeTase by oncogenic pathways is a critical component of oncogenic programs

The critical remaining question is whether the "hypermethylation" observed in cancer cells is a programmed or random event? Random events are more difficult to control pharmacologically. However, if hypermethylation is a consequence of a programmed increase in DNA MeTase activity, the probability of reversing this state by inhibitors of DNA MeTase is high.

A possible explanation for this observed hypermethylation is that it is a consequence of the limited increase in DNA MeTase activity observed in many tumor cells (Kautiainen and Jones, 1986; el-Deiry et al., 1991). Recently Belinsky et al., have shown that increased DNA MeTase activity is an early event in carcinogen induced lung cancer in mice (Belinsky et al., 1996). Forced expression of exogenous DNA MeTase cDNA causes transformation of NIH 3T3 cells supporting the hypothesis that overexpression of DNA MeTase can cause cellular transformation (Wu et al., 1993). Our data demonstrating that the increase in DNA methylation activity in cancer cells is an effect of activation of either the oncogenic Ras-Jun signaling pathway (Szyf, 1994; MacLeod et al., 1995) or the oncogenic pathway induced by T antigen (Pinard and Szyf, unpublished data) supports the hypothesis that increased DNA MeTase is a critical component of diverse oncogenic programs. Several lines of evidence obtained by us support a causal role for increased DNA MeTase in oncogenesis. First, treatment of Y1 adrenocortical carcinoma cells with the DNA MeTase inhibitor 5-aza-CdR or stably expressing an antisense to DNA MeTase in these cells results in inhibition of tumorigenesis *in vitro* (MacLeod and Szyf, 1995). Second, when the DNA MeTase antisense transfected Y1 cells are injected into a syngeneic mouse, tumor formation *in vivo* is significantly inhibited (MacLeod and Szyf, 1995). Third, 5-Aza-CdR treatment of T antigen transformed 3T3 cells results in inhibition of cellular transformation *in vitro*. Fourth, intra peritoneal administration of phosphorothioate modified DNA MeTase antisense oligonucleotides inhibits tumorigenesis *in vivo* in LAF/1 mice (syngeneic strain) bearing Y1 tumors (Ramchandani et al., 1997). Similarly, Laird et al., (1995) have shown that treating mice bearing the Min mutation with 5-Aza-CdR significantly reduces the appearance of intestinal polyps.

Based on these data, our working hypothesis is that induction of the enzymatic machinery controlling DNA methylation is a critical component of oncogenic programs and that oncogenesis could be reversed by inhibiting this induction.

B. What is the mechanism by which overexpression of the DNA MeTase induces

tumorigenesis?

If induction of DNA MeTase is an important component of an oncogenic program, what is the mechanism? Based on what is known about the functions of DNA methylation in diverse biological systems, three alternative possible modes of actions emerge. Hypermethylation can result in stable mutations, can repress tumor suppressor genes or possibly directly control DNA replication.

1. DNA MeTase induces C to T transitions

The first mechanism proposed by Peter Jones is that hypermethylation can increase the probability of reversion of 5mC to T by deamination, resulting in mutagenesis (Jones et al., 1992). However, this is an irreversible mechanism which is inconsistent with recent data. Laird et al., have previously shown that treatment of mice bearing the Min allele of APC with the DNA MeTase inhibitor 5-Aza-CdR reduces the frequency of polyp formations in these mice suggesting that DNA MeTase is critical for tumor formation in Min mice. If the mechanism by which DNA MeTase induces tumorigenesis is an increase in mutation rate, then treatment with 5-azaCdR should have resulted in reduced mutagenesis. However, recent data by Jackson-Grusby et al., (1997) suggests that incorporation of 5-Aza-CdR into DNA increases the rate of mutagenesis when DNA MeTase is present in the cell. This data strongly suggests that the mechanism by which the DNA MeTase inhibitor inhibits polyp formation in mice does not involve inhibition of mutagenesis.

2. Inactivation of tumor suppressors

The second proposed mechanism discussed above is that hypermethylation results in silencing of "tumor suppressor" genes (Pokora and Schneider, 1992; Ohtani-Fujita et al., 1993; Merlo et al., 1995; Royer-Merlo et al., 1995; Herman et al., 1995). Although there is solid evidence that DNA methylation is an important mechanism involved in silencing "tumor suppressors", it is not clear whether methylation of tumor suppressor genes is a consequence of a programmed change in the level of DNA MeTase, such as that occurring in the Y1 system. An inherent problem in the "tumor suppressor" model is how can a general increase in DNA methylation result in site-specific methylation of specific genes. One possible model is that additional factors such as the "imprinters", proposed to function in parental imprinting of genes, might be involved in translating the increase in DNA methylation into site specific methylation events (Szyf, 1991). Alternatively, induction of DNA MeTase might directly activate cellular regulatory pathways that result in inactivation of tumor suppressor genes. Following inactivation, the tumor suppressors undergo methylation.

3. Direct control of cell growth

A third hypothesis is that DNA methylation directly controls the progression of the cell cycle (Szyf, 1996). Recent evidence suggests that hypermethylated CG clusters is a marker of active origins (Rein et al., 1997), and that methylation of origins of replication occurs concurrently with replication (Araujo et al. unpublished). I have therefore proposed (Szyf, 1996) that hypermethylation causes firing of normally silent origins, explaining the chromosomal abnormalities observed in cancer cells. One interesting question is what is the kinetics of transformation induced by methylation. One hypothesis is that the increased MeTase is required to maintain random events of de novo methylation of tumor suppressor genes. Cells that have acquired these methylations are selected. If this model is true, transformation induced by methylation should be slow and the number of transformed cells should increase with time. On the other hand if increased MeTase levels target central controls of cell growth, then transformation by methylation should be rapid. Future experiments will most probably resolve this question.

In summary, I will like to suggest this unifying hypothesis explaining the involvement of DNA methylation in cancer. The basic oncogenic programs in the cell trigger an induction of both DNA MeTase and dMTase activities resulting in hypermethylation of certain sites and hypomethylation of others. The specificity of this process is determined by the different affinities of the different sites to these respective enzymes. I suggest that the milieu of DNA binding factors in the cell directs the DNA MeTase to growth suppressor sites and the dMTase to growth stimulating sites. Thus, the coordinate induction of DNA MeTase and dMTase results in a simultaneous repression of all growth suppression functions and induction of growth activation functions (Fig. 5). If a general induction in DNA MeTase activity is indeed responsible for launching this program, then inhibition of the enzyme by pharmacological means should direct the cell towards the original program of a nontransformed cell.

IV. Therapeutic applications of DNA MeTase inhibitors

A. DNA MeTase inhibitors

An essential step in the developing of new pharmacological concepts is identifying novel targets. DNA MeTase was not considered a pharmacological target of importance because of the prevalent conception that inhibitors of DNA methylation might be carcinogenic (Platt, 1995). Because of this prevalent conception, no inhibitors to the cytosine DNA MeTase were developed

since the introduction of 5-azacytidine. 5-azacytidine was originally synthesized as a nucleoside analog and was later found to inhibit DNA methylation after its incorporation into cellular DNA by covalently trapping the DNA MeTase (Wu and Santi, 1985; Jones, 1985). Our basic research of the mechanisms involved in regulating DNA MeTase activity and DNA methylation reviewed in the previous sections introduces the DNA MeTase as an important and very broad pharmacological target.

1. 5-Aza-CdR a DNA MeTase inhibitor with serious side effects

The only specific inhibitor of DNA MeTase that is currently available is 5-Aza-CdR which is phosphorylated by cellular kinases, incorporated into DNA and traps DNA MeTase molecules by forming a covalent bond with the catalytic site of the protein (Wu and Santi, 1985). This mechanism of action results in potential toxicities and side effects that limit the utility of 5-azadC as a therapeutic agent as well as a research tool (see review in Szyf, 1996). Recent data suggests that the mutagenicity induced by 5-azaCdR is a consequence of the interaction of DNA MeTase with 5-azaCdR incorporated into the DNA (Juttermann et al., 1994; Jackson-Grusby et al., 1997). It is clear that new DNA MeTase inhibitors that are not incorporated into DNA should be developed (Fig. 6).

2. SAM analogs

S-adenosyl- homocysteine, an analogue of SAM and one of the products of the methylation reaction is an inhibitor of DNA methylation (Mixon and Dev, 1983). Inhibitors of SAH hydrolysis such as periodate-oxidized adenosine or 3-deazaadenosine analogs (Chiang et al., 1992) were used before as inhibitors of DNA methylation. However, SAH, its analogues and inducers will inhibit a large number of different methylation reactions in the cell and must have nonspecific side effects (Papadopoulos et al., 1987) (Fig. 6).

3. Antisense oligonucleotides

We have recently shown that a DNA MeTase antisense mRNA that is expressed in Y1 tumor cells inhibits tumorigenesis *ex vivo* and *in vitro* (MacLeod and Szyf, 1995). The advent of antisense oligodeoxynucleotides as specific inhibitors of protein expression *in vivo* offers new opportunities to test the therapeutic value of inhibition of DNA MeTase as well as to use these as novel therapeutic agents. We have recently shown that a phosphorothioate-modified antisense oligodeoxynucleotide directed against the DNA MeTase inhibits DNA MeTase as well as inhibits the growth of tumors in syngeneic mice *in vivo* (Ramchandani et al., 1997). These results have now been extended to xenografts of human cancer lines in nude mice. Active DNA MeTase antisense compounds that can inhibit

human DNA MeTase mRNA as well as growth of human tumor cell lines *in vivo* have been identified (MacLeod et al., unpublished). DNA MeTase antisense oligonucleotides are potential candidates for anticancer agents in humans (**Fig. 6**).

4. Direct inhibitors of DNA MeTase

Antisense oligonucleotides only inhibit *de novo* synthesis but not the existing DNA MeTase protein. There might be certain situations where the turn-over rate of the enzyme will be too slow. In addition, antisense compounds are species specific and can not be used in animal models as well as nonanimal models such as plants which will most probably be of significant commercial potential. It is clear that new inhibitors are required to fully realize the research and applied potential of DNA methylation.

Other approaches that are now tested in my laboratory is to use analogs of the CG substrate as direct inhibitors of DNA MeTase. DNA MeTase is an attractive candidate for DNA based antagonists since in distinction from other DNA binding protein it forms a covalent transition state intermediate with the DNA substrate (Wu and Santi, 1985). An ideal DNA based antagonists would therefore bind the MeTase but would not be an acceptor for methyl transfer. Thus, a stable complex would be formed between the enzyme and the substrate (**Fig. 6**). Recent unpublished data from our laboratory suggests that some analogs inhibit DNA MeTase activity at the nanomolar range and inhibit DNA MeTase and tumor growth in living cells (Bigey et al., unpublished).

B. Potential side effects of DNA MeTase inhibitors

One important issue that might challenge the utility of DNA MeTase inhibitors as therapeutics is potential side effects resulting from activation of unwanted genes. It is obviously impossible to assess the full systemic effect of DNA MeTase inhibitors at this stage. However, previous data as well as our understanding of the mechanisms of action of DNA methylation suggest that these effects will not be a major issue. First, demethylation is insufficient *per se* to activate genes, the presence of the proper transcription machinery is required. Demethylation will only activate those genes in the cell that have the appropriate transcription factors available. Second, the new pattern of methylation generated after demethylation will be dictated by the legacy of the cell. This is probably why extensive demethylation of cell lines results in activation of the next stage in differentiation rather than chaotic activation of many possible programs (Szyf et al., 1992). Third, DNA MeTase inhibitors will only have an effect on dividing cells since they passively inhibit methylation during replication but do not remove methyl groups from DNA. Fourth, chronic treatment of mice with 5-Aza-CdR (Laird et al., 1995) or DNA MeTase antisense (Ramchandani et al., 1997) does not result in apparent systemic toxicity.

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Figure 6: Inhibitors of DNA MeTase

DNA MeTase could be inhibited at different levels. The first line illustrates a scheme (not to scale) of the first exons and introns of the DNA MeTase gene. The second and third lines are schemes of mRNAs encoded by the DNA MeTase.

Antisense oligonucleotides: Antisense oligonucleotides (line under the mRNA) directed against some of the splice junctions, reduce DNA MeTase mRNA level (MacLeod et al., unpublished). Two different messages are transcribed from the DNA MeTase gene. Antisense oligonucleotides could be directed against the sequence encoding the ATG translation initiation site of each protein specifically. Once the protein is synthesized, it could be inhibited by either of three different ways. **Hairpin inhibitors:** First, a modified hairpin oligonucleotide substrate (left) bearing a hemimethylated CG sequence will bind the DNA MeTase and form a stable complex with the substrate. As the modification of the hairpin inhibits the transfer of a methyl group from SAM, the enzyme remains bound to the substrate and is unavailable for methylating genomic DNA.

SAM analogs: SAH and its analogs bind the SAM binding pocket of the DNA MeTase and inhibit methylation.

The first line describes the DNA methylation reaction. A double stranded DNA bearing a methylated C in a CG dinucleotide on the parental strand and a nonmethylated C in the CG dinucleotide on the nascent strand is reacted with S-adenosyl-methionine (SAM) in a reaction catalyzed by the DNA methyltransferase (DNA MeTase). The resulting products of the reactions are a double stranded methylated DNA and S-adenosyl homocysteine (SAH).

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