Gene Ther Mol Biol Vol 4, 291-296. December 1999.

3-aminobenzamide: a novel drug to induce in vivo DNA hypermethylation

Review Article

Giuseppe Zardo[1], Anna Reale[2], Mariagrazia Perilli[1], Adriana de Capoa[3] and Paola Caiafa[1]

Department of Biomedical Sciences and Technologies[1], University of L'Aquila, Italy.
Department of Cellular Biotechnologies and Haematology[2] and of Genetics and Molecular Biology[3], University of Rome "La Sapienza", Italy.

Correspondence: Prof. Paola Caiafa, Dipartimento di Biotecnologie Cellulari ed Ematologia, Sezione di Biochimica Clinica, Facoltà di Medicina e Chirurgia, Università di Roma "La Sapienza", Viale Regina Elena, 324 (Policlinico), 00161, Roma, Italia. Tel: +39-06-49910900, Fax: +39-06 4440062, E-mail caiafa@bce.med.uniroma1.it

Key Words: histone hypoacetylation, gene silencing, histone deacetylation, 5-azacytidine, trichostatin A, chromatin

Received: 9 August 1999; accepted: 19 August 1999

Summary

Both DNA methylation and core histone hypoacetylation are associated with gene silencing but only recent experiments allowed the interlocking of these two processes. Through such experiments it was shown that the two processes are united in inducing gene silencing through a "shuttle-system" involving the methyl CpG binding protein (MeCP2). In this scenario, it is not clear whether DNA methylation or histone deacetylation is the leader in inducing down regulation of gene expression. Trichostatin A (TSA), a potent inhibitor of histone deacetylase, is usually used to clarify this point. As far as DNA methylation is concerned, only the 5-azacytidine (5-AzaC), able to induce hypomethylation, has been described until now. The aim of this paper is to suggest the use of 3-aminobenzamide (3-ABA) as a method capable of inducing in vivo DNA hypermethylation, so that new experiments could be performed in both directions to clarify the chronology by which the influence on gene expression takes place and to pinpoint the structure of methylated condensed chromatin.

I. Introduction

DNA methyltransferase (EC 2.1.1.37) is a nuclear enzyme that, by transferring methyl groups from S-adenosyl methionine (S-AdoMet) to cytosine (C) converts these residues into 5-methylcytosine (5mC) (Bestor and Ingram, 1983), the best substrate being the cytosine located in the CpG dinucleotide (Gruenbaum et al, 1981). This epigenetic modification is proposed to have an active role in the modulation of gene expression (Keshet et al, 1985; Boyes and Bird, 1992; Li et al, 1993; Hsiet, 1994). This role was confirmed by experiments in which an anomalous methylation, caused by targeted mutation of DNA methyltransferase gene in mice, results in embryonic lethality (Li et al, 1992). The DNA methylation pattern, which is defined during embryonic development (Brandeis et al, 1993), is very important since its characteristic is that some DNA regions, located in the 5' promoter region of housekeeping genes - termed CpG islands (Bird et al, 1985; Bird, 1986; Bird, 1987) - are present in their unmethylated state, this condition being essential for the expression of related genes (Keshet et al, 1985).

A second mechanism by which DNA methylation may be involved in down regulation of gene expression has recently been shown (Jones et al, 1998; Nan, et al, 1998) and debated (Bestor, 1998; Razin, 1998). This mechanism foresees that histone deacetylase, via its association with methyl-CpG binding protein (MeCP2) (Boyes and Bird, 1991; Meehan et al, 1992) reaches methylated DNA allowing the methylation-dependent chromatin condensation favoring gene silencing. Until now it is not clear how cytosine methylation might affect chromatin structure and much still has to be done to clarify the mechanism by which this influence takes place and to identify whether DNA methylation or histone deacetylation is the post-synthetic modification "leader" in inducing gene silencing (Selker,

1998; Eden et al, 1998; Cameron et al, 1999).

Trichostatin A (TSA), a potent inhibitor of histone deacetylase (Yoshida et al, 1995), is usually used to clarify this point. As far as DNA methylation is concerned, only the treatment of cells with 5-azacytidine (5-AzaC), able to induce hypomethylation, has been described until now (Adams and Burdon, 1985). The aim of this paper is to propose the treatment of cells with 3-aminobenzamide as a method to induce in vivo DNA hypermethylation so that new experiments can be performed in two directions in order to both clarify the order by which the influence on gene expression takes place and to pinpoint the structure of methylated condensed chromatin.

II. Treatment of cells with 3-aminobenzamide induces in vivo DNA hypermethylation

The 3-aminobenzamide is a specific inhibitor (Huletsky et al, 1989; Rankin et al, 1989) of the poly(ADP-ribose) polymerase (EC 2.4.2.30), an enzyme able to build and/or transfer ADP-ribose polymers onto chromatin proteins (Jacobson and Jacobson, 1989; de Murcia, et al, 1995).

The statement that following inhibition of poly(ADP-ribose) polymerase DNA methyltransferase becomes able to methylate the unmethylable cytosines on DNA is based on our experiments showing that a block of poly(ADP-ribosyl)ation introduces an anomalous hypermethylated pattern in genomic DNA (Zardo et al, 1997; de Capoa et al, 1999). Although our research was performed in order to demonstrate that poly(ADP-ribosyl)ation is an important process involved in controlling the expression of housekeeping genes (Zardo and Caiafa, 1998), the aim of this review is to point out that the 3-ABA induced block of this enzymatic process introduces an anomalous hypermethylated pattern on genomic DNA. All experiments were carried out on L929 and NIH\3T3 mouse fibroblast cells and poly(ADP-ribose) polymerase was inhibited by treatment of cells with 2 and/or 8 mM 3-aminobenzamide for 24 hours.

The first evidence came from experiments on endogenous DNA methyl-accepting ability (Zardo et al., 1997). In these experiments the methyl-accepting ability of isolated nuclei, obtained from 6.5 x 10^5 L929 mouse fibroblasts, previously preincubated with or without 3-ABA for 24 hours, was performed in the presence of [^{3}H]-S-AdoMet. After one hour of incubation at 37° C, we compared the ability to incorporate labeled methyl groups in their DNA in the absence of any exogenous DNA methyltransferase (i.e. by a process catalyzed by the endogenous enzyme). The level of incorporated methyl groups, evaluated on the total DNA purified from cells, was found to be 60% higher in the DNA from 3-ABA treated cells than in DNA from control cells whose methyl-acceptance value was taken as 100%, **Figure 1**.

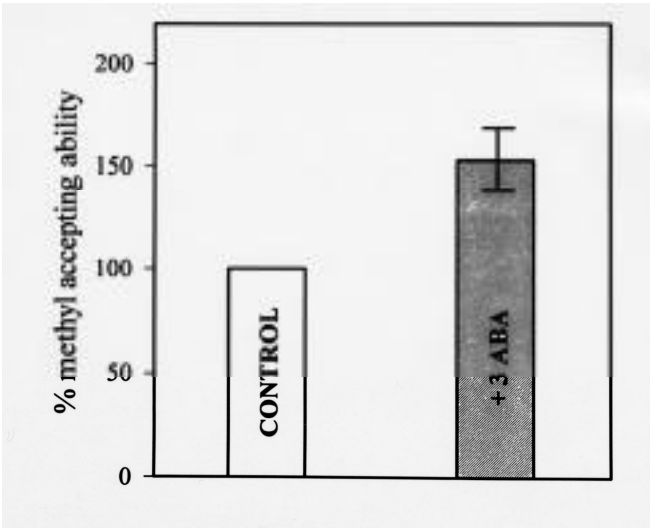

Figure 1. Methyl-accepting ability experiment. The endogenous methyl-accepting ability of native nuclei, obtained from 6.5 x 10^6 L929 fibroblasts preincubated for 24 h without (control) and with 8 mM 3-ABA, was performed in the presence of 16 μM [^{3}H]-S-AdoMet. The level of methyl groups has been evaluated on the total DNA purified from cells Control DNA, whose incorporation was 2.8±0.1 pmol of [^{3}H]-S-AdoMet, was considered as 100%. *Zardo et al. (1997) Biochemistry 36, 7937-7943.*

More recently (de Capoa et al, 1999) we have been able to show that during the 24 hours of 3-ABA treatment, interphase nuclei had already incorporated some methyl groups. The cells were indirectly immunolabeled with anti-5-methylcytosine (anti-5mC) monoclonal antibodies (de Capoa et al, 1996), microscope analysis was performed on a cell-by-cell basis and the images of the nuclei were recorded by a b/w CCD camera. A computer-assisted quantitative analysis of the methylation state of individual interphase nuclei was performed by dedicated software (de Capoa et al, 1998). Cells preincubated with 3-ABA consistently showed increased levels of anti-5mC antibody binding to heterochromatic regions, **Figures 2 and 3**.

Thus, both the DNA methyl-accepting assay and monoclonal anti 5-methylcytosine antibodies allowed us to show that reduced levels of poly(ADP-ribosyl)ation result in DNA hypermethylation.

III. Possible interpretation of the mechanism by which poly(ADP-ribosyl)ation controls DNA methylation

These results indicate that poly(ADP-ribosyl)ation protects in some way genomic DNA from full methylation although much still has to be done to explain the molecular mechanism(s). As for the CpG islands, our recent research (Zardo and Caiafa, 1998) has shown that, at least for the Htf9 promoter region, the inhibition of poly(ADP-ribosyl)ation allows the new methyl groups to position

themselves on DNA. Further experiments have shown that this inhibition also changes the methylation pattern of plasmid transfected in its unmethylated form (Zardo et al, 1999 in press).

Poly(ADP-ribose) polymerase that is dimeric in its catalytic form (Mendoza-Alvarez and Alvarez-Gonzales, 1993), has three domains which play specific roles in the poly(ADP-ribosyl)ation process. The N-terminal domain contains the zinc-finger motifs which are responsible for binding to DNA (Gradwohl et al, 1990), the C-terminal domain contains the catalytic site (de Murcia and Menissier de Murcia, 1994; Rolli et al, 1997) and the central domain is the domain that undergoes automodification (Desmarais et al, 1991). The enzyme starts its automodification following binding of the enzyme to DNA and needs breaks on DNA strands to be activated (de Murcia and Menissier de Murcia, 1994). During the automodification process the ADP-ribose polymers - up to 200 residues – are built in the 28 automodification sites (Kawaichi et al, 1981; Desmarais et al, 1991) located in this domain. Following automodification, the enzyme can start heteromodification reactions allowing interactions between ADP-ribose polymers and chromatin proteins (Boulikas, 1989; Scovassi et al, 1993). Several proteins (Wesierska-Gadek et al, 1996; Malanga et al, 1998) can be modified both in a covalent and non-covalent way, the best substrate being H1 histone (Poirier and Savard, 1980; D`Erme et al, 1996; Panzeter et al, 1992; Panzeter et al, 1993; Malanga et al, 1998).

Our in vitro findings show that the H1 histone, in its poly(ADP-ribosyl)ated isoform (Zardo et al, 1997) and through its genic variant H1e (Santoro et al, 1995; Zardo et al, 1996) could be a nuclear proteic trans-acting factor involved in maintaining the unmethylated state of CpG islands. We cannot exclude that other presently unknown proteic factor(s) could also play a regulatory role in the control of DNA methylation by means of this post-synthetic modification.

Acknowledgements

This work was supported by the Italian Ministry of University and Scientific and Technological Research (40% Progetti di Interesse Nazionale, 60% Ricerca Scientifica Università di L'Aquila e di Roma, "La Sapienza") and by the Consiglio Nazionale delle Ricerche (CNR). We thank Alessandra Spanò for technical assistance.

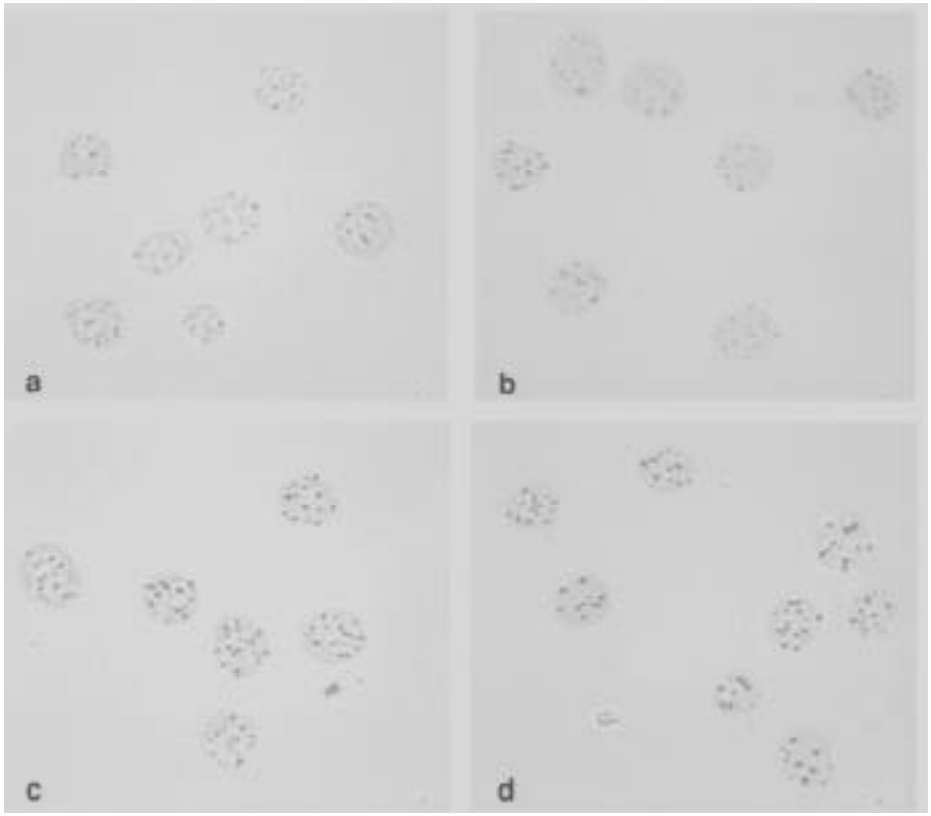

Figure 2. Increased levels of heterochromatin methylation in 3-ABA treated nuclei from mouse fibroblast cell lines as shown by indirect immunolabeling with anti-5MeC antibodies. de Capoa et al. (1999) The FASEB J. 13, 89-93; b/w CCD camera images of control (**a,b**) and treated (**c,d**) nuclei from L929 (left) and NIH/3T3 cell lines (right).

293

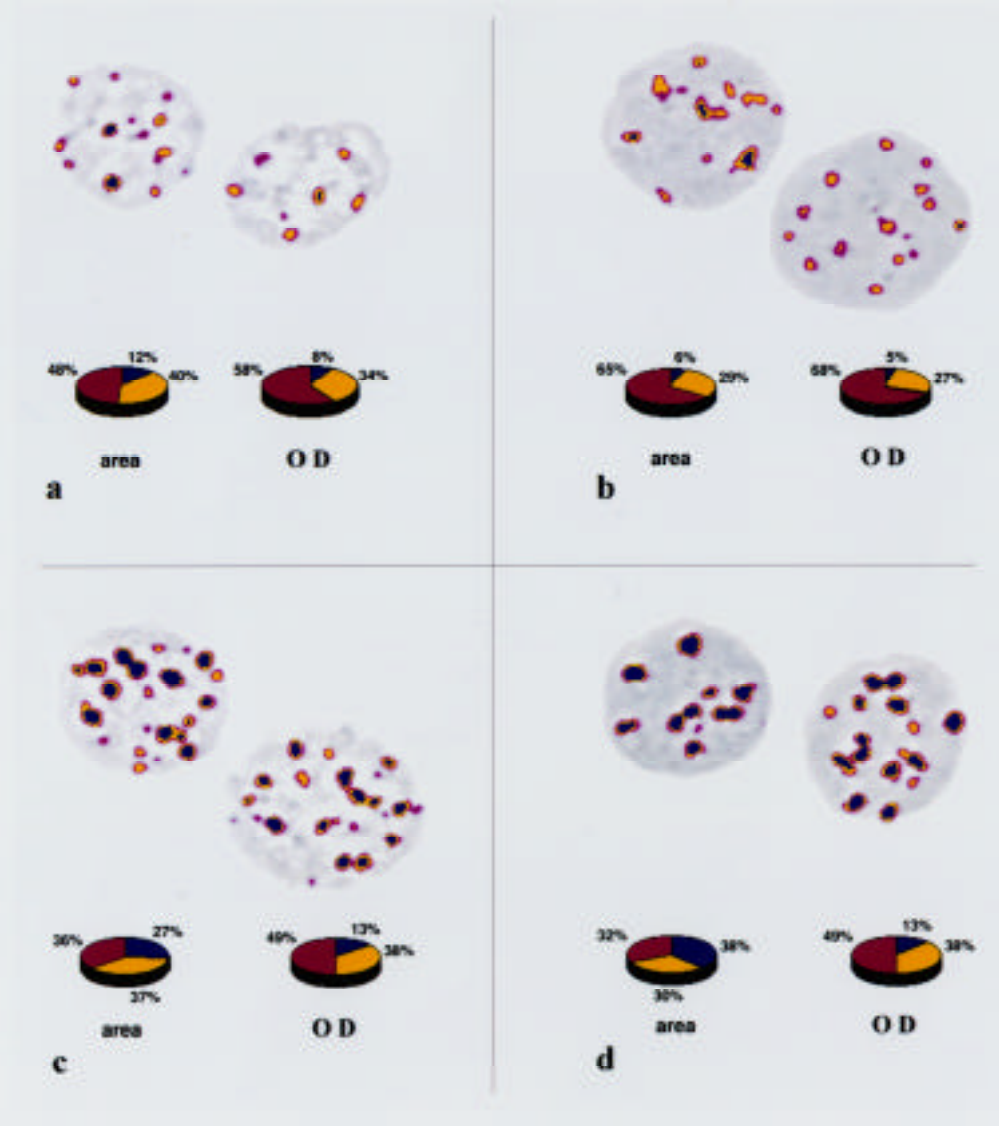

Figure 3. Methylation levels of control and 3-ABA treated mouse fibroblasts in samples of 20 nuclei each. Left: L929 and, right: NIH/3T3 cells. **Upper rows**: Examples of pseudocolored heterochromatic regions in some control (a, b) and treated (c, d) nuclei. A computer-assisted quantitative analysis of methylation levels in b/w CCD camera images of control and treated nuclei was performed. For each nucleus the methylation state was expressed as area of the methylated regions (μ^2) and different levels of optical densities (ODs, 0-185 in the gray scale range). Blue, yellow and red staining indicate, respectively, the heavily, medium and lightly methylated regions per nucleus. **Lower rows**: Percentages of differentially labeled areas and different optical densities in control and treated nuclei from each sample. Blue, yellow and red staining indicate, respectively, the heavily, medium and lightly methylated regions per sample.

References

Adams RLP and Burdon RH (**1985**). Molecular biology of DNA methylation. **Springer Series in Molecular Biology**, (Rich, A. ed), Springer-Verlag, New York.

Bestor TH (**1998**). Methylation meets acetylation. **Nature** *393*, 311-312.

Bestor TH and Ingram VM (**1983**). Two DNA methyltransferases from murine erythroleukemia cells: purification, sequence specificity and mode of interaction with DNA. **Proc Natl Acad Sci USA** *80*, 5559-5563.

Bird AP (**1986**). CpG islands and the function of DNA methylation. **Nature** *321*, 209-213.

Bird AP (**1987**). CpG islands as a gene markers in the vertebrate nucleus. **Trends Genet** *3*, 324-347.

Bird AP, Taggard M, Frommer M, Miller OJ and Macleod D (**1985**). A fraction of the mouse genome that is derived from islands of nonmethylated CpG-rich DNA. **Cell** *40*, 91-99.

Boyes J and Bird AP (**1991**). DNA methylation inhibits

transcription indirectly via a methyl-CpG binding protein. **Cell** *64*, 1123-1134.

Boyes J and Bird AP (**1992**). Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl CpG binding protein. **EMBO J** *11*, 327-333.

Brandeis M, Ariel M and Cedar H (**1993**). Dynamics of DNA methylation during development. **BioEssays** *15*, 709-713.

Cameron EC, Bachman KE, Myohanen S, Herman JG and Baylin SB (**1999**). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. **Nature Gen** *21*, 103-107.

D'Erme M, Zardo G, Reale, A and Caiafa P (**1996**). Co-operative interactions of oligonucleosomal DNA with the H1e histone variant and its poly(ADP-ribosyl)ated isoform. **Biochem J** *316*, 475-480.

de Capoa A, Febbo FR, Giovannelli F, Niveleau A, Zardo, G, Marenzi S and Caiafa P (**1999**). Reduced levels of poly(ADP-ribosyl)ation result in chromatin compaction and hypermethylation as shown by cell-by-cell computer-assisted quantitative analysis. **The FASEB J** *13*, 89-93.

de Capoa A, Menendez F, Poggesi I, Giancotti P, Grappelli C, Marotta MR, Di Leandro M, Reynaud C and Niveleau A (**1996**). Cytological evidence for 5-azacitidine-induced demethylation of the heterochromatic regions of human chromosomes. **Chromosome Res** *4*, 271-276

de Capoa A, Menendez F, Poggesi I, Giancotti P, Grappelli C, Marotta MR, Di Leandro MR, Spano A, Rocchi M, Archidiacono N and Niveleau A (**1998**). Computer-assisted analysis of methylation status of individual interphase nuclei in human cultured cells. **Cytometry** *31*, 85-92.

de Murcia G and Mènissier de Murcia J (**1994**). Poly(ADP-ribose) polymerase: a molecular nick sensor. **Trends Biochem Sci** *19*, 172-176.

Desmarais Y, Menard L, Lagneux J and Poirier GG (**1991**). Enzymological properties of poly(ADP-ribose) polymerase: characterization of automodification sites and NADase activity. **Biochim Biophys Acta** *1078*, 179-186.

Eden S, Hashimshony I, Keshet I and Cedar H (**1998**). **Nature** *394*, 842.

Gradwohl G, de Murcia JM, Molinete M, Simonis F, Koken M, Hoeijmakers JH and de Murcia G (**1990**). The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single stranded breaks in DNA. **Proc Natl Acad Sci** *87*, 2990-2994.

Gruenbaum Y, Stein R, Cedar H. and Razin A (**1981**). Methylation of CpG sequences in eukaryotic DNA. **FEBS Lett** *124*, 67-71.

Hsiet CL (**1994**). Dependance of transcriptional repression on CpG methylation density. **Mol Cell Biol** *14*, 5487-5494.

Huletsky A, de Murcia G, Muller S, Hengartner M, Menard L, Lamarre D and Poirier GG (**1989**). The effect of poly(ADP-ribosyl)ation on native and H1-depleted chromatin. **J Biol Chem** *15*, 8878-8886.

Jones PL, Veenstra GJC, Wade PA, Vermaak D, Kass SU, Landsberger N, Stouboulis J and Wolffe AP (**1998**). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. **Nature Gen** *19*, 187-191.

Kawaich, M, Ueda K and Hayaishi O (**1981**). Multiple auto-poly(ADP-ribosyl)ation of rat liver poly(ADP-ribose syntetase. **J Biol Chem** *256*, 9483-9489.

Keshet I, Yisraeli J and Cedar H (**1985**). Effect of regional DNA methylation on gene expression. **Proc Natl Acad Sci USA** *82*, 2560-2564.

Li E, Beard C and Jaenisch R (**1993**). Role of DNA methylation in genomic imprinting. **Nature** *366*, 362-365.

Li E, Bestor TH and Jaenisch R (**1992**). Targeted mutation of DNA methyltransferase gene results in embryonic lethality. **Cell** *69*, 915-926.

Malanga M, Pleschke JM, Kleczkowska HE and Althaus F (**1998**). Poly(ADP-ribose) binds to specific domains of p53 and alters its DNA binding functions. **J Biol Chem** *273*, 11839-11843.

Malanga M, Atorino L, Tramontano F, Farina B and Quesada P (**1998**). Poly(ADP-ribose) binding properties of histone H1 variants. **Biochim Biophys Acta** *1399*, 154-160

Meehan RR, Lewis JD and Bird AP (**1992**). Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. **Nucl Acids Res** *20*, 5085-5092.

Mendoza-Alvarez H and Alvarez-Gonzales R (**1993**). Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular. **J Biol Chem** *268*, 22575-22580.

Nan X, Ng H-H, Johnson CA, Laherty CD, Turner BM, Eisenman RN and Bird AP (**1998**) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. **Nature** *393*, 386-389.

Panzeter PL, Realini CA and Althaus FR (**1992**). Noncovalent interactions of poly(adenosine diphosphate ribose) with histones. **Biochemistry** *31*, 1379-1385.

Panzeter PL, Zweifel B, Malanga M, Waser SH, Richard MC and Althaus F (**1993**). Targeting of histone tails by poly(ADP-ribose)polymerase. **J Biol Chem** *268*, 17662-17664.

Poirier GG and Savard P (**1980**). ADP-ribosylation of pancreatic histone H1 and other histones. **Can J Biochem** *58*, 509-515.

Rankin PW, Jacobson EL, Benjamin RC, Moss J and Jacobson MK (**1989**). Quantitative studies of inhibitors of ADP-ribosylation in vitro and in vivo. **J Biol Chem** *264*, 4312-4317.

Razin A (**1998**). CpG methylation, chromatin structure and gene silencing a three-way connection. **The EMBO J** *17*, 4905-4908.

Rolli V, O'Farrel M, Ménissier-de Murcia J and de Murcia G (**1997**). Random mutagenesis of poly(ADP-ribose) polymerase catalytic domain reveals amino acids involved in polymer branching. **Biochemistry** *36*, 12147-12154.

Santoro R, D'Erme M, Mastrantonio S, Reale A, Marenzi S, Saluz HP, Strom R and Caiafa P (**1995**). Binding of histone variants H1e-c to CpG-rich DNA correlates with the inhibitory effect on enzymic DNA methylation. **Biochem J** *305*, 739-744.

Selker EU (**1998**). Trichostatin A causes selective loss of DNA methylation in Neurospora. **Proc Natl Acad Sci USA** *95*, 9430-9435.

Wesierska-Gadek J, Schmid G and Cerni C (1996) ADP-Ribosylation of wild-type p53 protein to specific p53 consensus sequence prevents its modification. **Biochem Biophys Res Commun** *224*, 96-102.

Yoshida M, Horinouchi S and Beppu T (**1995**). Tricostatin A and tropoxin: novel chemical probe for the role of histone acetylation in chromatin structure and function. **BioEssays** *17*, 423-430.

Zardo G, D'Erme M, Reale A, Strom R, Perilli M and Caiafa P (**1997**). Does poly(ADP-ribosyl)ation regulate the DNA methylation pattern? **Biochemistry** *36*, 7937-7943.

Zardo G and Caiafa P (**1998**). The unmethylated state of CpG islands in mouse fibroblasts depends on the poly(ADP-ribosyl)ation process. **J Biol Chem** *273*, 16517-16520.

Zardo G, Marenzi S and Caiafa P (**1998**). H1 histone as a trans-acting factor involved in protecting genomic DNA from full methylation. **Biol Chem** *379*, 647-654.

Zardo G, Marenzi S, Perilli S and Caiafa P (**1999**). Inhibition of poly(ADP-ribosyl)ation introduces an anomalous methylation pattern in transfected foreign DNA. **The FASEB J** 13, 1518-1522.

Zardo G, Santoro R, D`Erme M, Reale A, Guidobaldi L, Caiafa P and Strom R (**1996**) Specific inhibitory effect of H1e histone somatic variant on in vitro DNA methylation process. **Biochim Biophys Res Comm** *220*, 102-107.

(From left) Anna Reale, Paola Caiafa & Giuseppe Zardo