Cancer Chemother Pharmacol (2001) 48: 71–76
DOI 10.1007/s002800100294

ORIGINAL ARTICLE

Veronica Bovenzi · Richard L. Momparler

Antineoplastic action of 5-aza-2′-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor β and estrogen receptor α genes in breast carcinoma cells

Received: 3 October 2000 / Accepted: 28 February 2001 / Published online: 15 May 2001

Abstract *Purpose*: During tumorigenesis 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. The aim of this study was to determine the antineoplastic activities of 5-AZA-CdR and trichostatin A (TSA), either administered alone or in combination, in MDA-MB-231 breast carcinoma cells. The effects of these drugs (alone and in combination) on the expression of the tumor suppressor gene, retinoic acid receptor (RARβ) and of the estrogen receptor α gene (ERα), whose expression is lost in the cell line used in the study, were also investigated. *Methods*: MDA-MB-231 cells were treated with 5-AZA-CdR and TSA and the antitumor activity of these drugs was determined by clonogenic assay. Total RNA was extracted from the treated cells and RT-PCR was used to determine the effect of the treatment on the expression of RARβ and ERα. Methylation-sensitive PCR analysis was used to confirm that lack of expression of both genes was due to hypermethylation of their promoter regions. A single nucleotide primer extension assay was also used to quantify the reduction in DNA methylation following drug treatment. *Results*: Both 5-AZA-CdR and TSA alone showed significant antineoplastic activity. The combination of the two drugs was synergistic with respect to MDA-MB-231 cell kill. 5-AZA-CdR alone weakly activated the expression of both RARβ and ERα. TSA alone only activated RARβ, but not ERα. The combination of these agents appeared to produce a greater activation of both genes. *Conclusions:* The interesting interaction between 5-AZA-CdR and TSA in both cell kill and cancer-related gene reactivation provides a rationale for the use of inhibitors of DNA methylation and histone deacetylation in combination for the chemotherapy of breast cancer.

Keywords Breast cancer · 5-Aza-2′-deoxycytidine · Trichostatin A · Retinoic acid receptor β · Estrogen receptor α · DNA methylation

Abbreviations *5-AZA-CdR:* 5-aza-2′-deoxycytidine · *ERα:* estrogen receptor α · *HDAC:* histone deacetylase · *MSP:* methylation-sensitive PCR · *MS-SNuPE:* methylation-sensitive-single nucleotide primer extension · *RARβ:* retinoic acid receptor β · *TSA:* trichostatin A

Introduction

It has been widely shown that an epigenetic mechanism that involves the methylation of CpG islands in their promoter region may be responsible for the silencing of many cancer-related genes [2, 17, 25]. Another epigenetic mechanism that can regulate gene expression is the acetylation of chromosomal histones [15]. Highly acetylated regions of chromatin contain transcriptionally active regions while hypoacetylated chromatin is transcriptionally silent [19]. Methylated DNA is often found associated with deacetylated histones [1, 12]. These observations led to the hypothesis that these two phenomena could be linked and act in concert in the regulation of gene expression. Jones et al. [19] and Nan et al. [29] have found that a methyl-CpG binding protein, MeCP2, coprecipitates with HDAC activity. MeCP2 is found in multiprotein complexes that can include HDACs and transcriptional co-repressors [3, 21].

Supported by grant 9231 from the Canadian Breast Cancer Research Initiative

V. Bovenzi
Département de pharmacologie, Université de Montréal, Québec, Canada

R.L. Momparler (✉)
Centre de recherche pédiatrique, Hôpital Ste-Justine, 3175 Côte Ste-Catherine, Montréal, Québec H3T 1C5, Canada
Tel.: +1-514-3454691 ext. 6140
Fax: +1-514-3454801

The MeCP2 complex recognizes and binds the methylated CpG dinucleotides leading to gene silencing. Their interaction has been clarified by Cameron et al. [7] who observed a synergy between the DNA methylation inhibitor, 5-AZA-CdR, and the HDAC inhibitor, TSA, with respect to the reactivation of some 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 in tumor cells. 5-AZA-CdR is an experimental anticancer drug that has shown promising preclinical [4, 38] and clinical activity [27, 30]. It is a potent and specific inhibitor of the DNA methylase enzyme (DNMT), which catalyses the methylation of cytosine residues on DNA. Once incorporated in the DNA, 5-AZA-CdR forms covalent adducts with DNMT [20]. This results in depletion of DNMT from the cells resulting in demethylation of genomic DNA following treatment with this cytosine analog. HDAC inhibitors have also shown promising preclinical antineoplastic activity [22, 32, 36]. HDAC inhibitors induce G_1/S cell cycle arrest and morphological changes in various cell lines [13, 28]. Both classes of inhibitors can induce differentiation in neoplastic cells [18, 26, 36], suggesting that their combined administration may show enhanced antitumor activity.

In this study, we investigated the in vitro antineoplastic activity of 5-AZA-CdR and TSA in 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 α (ERα), two genes that play important roles in breast cancer progression. The MDA-MB-231 cells lack expression of the RARβ gene [34] and the ERα gene [11]. Lack of expression of RARβ has been reported for several primary tumors, including breast cancers [34, 37]. We have 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 [9, 10]. Loss of ERα expression occurs frequently in advanced breast cancer that becomes unresponsive to hormonal therapy [31]. Hypermethylation of CpG islands in the 5′-region of the ERα gene has been found in different tumor types, including breast cancer and its expression has been demonstrated to be reactivated after treatment with 5-AZA-CdR [14].

In the study reported here, using a clonogenic assay, we demonstrated that the combination of the two drugs was synergistic with respect to the antineoplastic activity in the MDA-MB-231 breast carcinoma cells. Our results also showed that both RARβ and ERα genes were weakly reactivated by 5-AZA-CdR whereas only RARβ was significantly reactivated by TSA. In addition, the combination of these agents produced greater gene activation than their individual effects. Furthermore, we showed that TSA did not produce an additional decrease in promoter demethylation produced by 5-AZA-CdR. A preliminary report of this work has been presented [5].

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 an incubator at 37°C in an atmosphere containing 5% CO_2.

Clonogenic assay

MDA-MB-231 cells were plated at 100 cells/well in a six-well dish in 2 ml medium. 5-AZA-CdR (Pharmachemie, Haarlem, The Netherlands) was dissolved in 0.45% NaCl containing 10 m*M* sodium phosphate (pH 6.8), and stored at –70°C. TSA (Wako Bio-Products, Richmond, Va.) was dissolved in 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 h. Due to its chemical instability, 5-AZA-CdR was added to the medium every 24 h. To assess the effect of a 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 h. Colonies were counted on day 10.

Reverse transcriptase PCR (RT-PCR) analysis

In order to study the reactivation of RARβ and ERα genes in MDA-MB-231 cells, we treated the cells with 5-AZA-CdR 50 ng/ml (250 n*M*) and TSA 181 ng/ml (600 n*M*), alone or in combination as a sequential exposure for 48 h. Cells were harvested 15–20 h after removal of the drugs. Total RNA was extracted from 1×10^6 cells using the RNeasy Mini Kit (Qiagen) and the remaining cells were pelleted and frozen for subsequent DNA extraction. For cDNA synthesis, 500 ng total RNA was used in 20 µl reaction mixture using the RT OmniScript (Qiagen). The reaction mixture also contained 1×RT buffer (Qiagen), dNTPs (0.5 m*M* each), random hexamers (1 µ*M*), and RNase inhibitor (0.5 µ*M*) with or without RT (4 U). The reaction was performed at 37°C for 1 h, 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 ERα. The primers of RARβ (accession no. NM_000965) were: sense 5′-AGA GTT TGA TGG AGT TGG GTG GAC-3′ (position 229–252) and antisense 5′-GAC GAG TTC CTC AGA GCT GGT G-3′ (position 495–516). For the ERα gene (accession no. NM_000125) the primers were: sense 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). The 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 1 min for five cycles, and then the annealing temperatures were lowered to 57–59°C for 30 more cycles for a total of 35 cycles. Fewer cycles were used for the internal control gene, microglobulin. The reactions were performed in a programmable minicycler. For each gene we ensured that the DNA amplification did not reach a plateau. The PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining.

Densitometric analysis

Agarose gel photographs were scanned at high resolution (600 d.p.i.) and densitometric analysis was carried out with the Adobe Photoshop 5.0 program. Band intensity of the RARβ and ERα transcripts was normalized to the intensity of the microglobulin signal in order to account for differences in input RNA or RT-PCR efficiency. Ratios of the RARβ and ERα signals in treated and

control cells were used to calculate the level or reactivation of both genes after drug treatment compared to the mock-treated cells.

Methylation-specific PCR (MSP) assay

MSP takes advantage of DNA sequence differences between methylated and unmethylated sequences after bisulfite modification [16]. A first round of PCR was performed using primers specific for bisulfite-converted DNA. For the RARβ gene (accession no. X56849) the primers were: sense 5′-TTA AG(C/T) TTT GTG AGA ATT TTG-3′ (position 717–736) and antisense 5′-CCT ATA ATT AAT CCA AAT AAT-3′ (position 1120–1141) amplifying a 426 bp fragment. PCR was performed under the following conditions: 95°C for 15 min to allow the antibody to detach from the Taq polymerase and activate it; 94°C for 1 min, 50°C for 1 min, 72°C for 3 min for five cycles, then 94°C for 30 s, 50°C for 30 s and 72°C for 1 min for 34 additional cycles for a total of 39 cycles. For the ERα gene (accession no. X62462) primers amplified a 632 bp region and the sequences were: sense 5′-GGG GTA TAT AAG GTA GTA TAT TAG-3′ (position 2442–2465) and antisense 5′- CCA ACT CAT TCC CTT AAA TC-3′ (position 3054–3072). Touchdown PCR was performed under the following conditions: 95°C for 15 min; 94°C for 1 min, 61°C down to 55°C annealing temperature with a decrement of 2°C per cycle, 72°C for 1 min, and then 34 more cycles at the lower annealing temperature (55°C) for a total of 38 cycles. All reactions contained 5–10 ng of bisulfite-converted DNA, 1×PCR buffer, 10 μM dNTP, 2.5 μM primers, 2.5 mM $MgCl_2$, and 1.2 U Qiagen HotStar Taq polymerase in a total volume of 25 µl. The DNA fragments resulting from the first PCR were used for MSP. Primer pairs for the RARβ promoter region (RARβ-M and RARβ-U) have been described by Côté et al. [10]. These primers were used to amplify a 146 bp fragment of the RARβ promoter region. MSP primer pairs for the ERα gene were ER1u and ER1m as described by Lapidus et al. [23], which amplified fragments of 199 and 198 bp, respectively. PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide and photographed.

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 [4]. Semi-nested PCR was performed to amplify a 404 bp fragment of the promoter region of the RARβ gene. This amplified DNA was used to perform the MS-SNuPE assay as previously described [6]. Briefly, 20 ng of the 404 bp amplified DNA was used in 25 µl reaction containing 1×buffer (Gibco/BRL), 1 mM $MgCl_2$, 1 μM primers A or B, 0.1–1 µCi of either [α^{32}P]dCTP or [α^{32}P]dTTP, and 1 U platinum Taq polymerase (Gibco/BRL). The conditions for primer extension were as described previously [6]. The reaction mixtures were applied to DEAE circles and the circles were washed five times with 3×SSC and then rinsed with water. The radioactivity was measured in a Beckman LS 6000IC scintillation counter.

Statistical analysis

Data from the clonogenic assay are presented as means ± SEM. Differences between groups were analyzed using Student's t-test for independent samples. The critical level of significance was set at $P < 0.05$.

Results

The dose-response curves of 5-AZA-CdR and TSA showed that 5-AZA-CdR at higher concentrations was a more potent antitumor agent than TSA against MDA-MB-231 breast carcinoma cells (Fig. 1A, B). At a concentration of 100 ng/ml, 5-AZA-CdR produced 89% cell kill, while TSA at the same concentration produced a cell kill of only 65%. We were interested in any 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 h to 5-AZA-CdR (25 ng/ml)

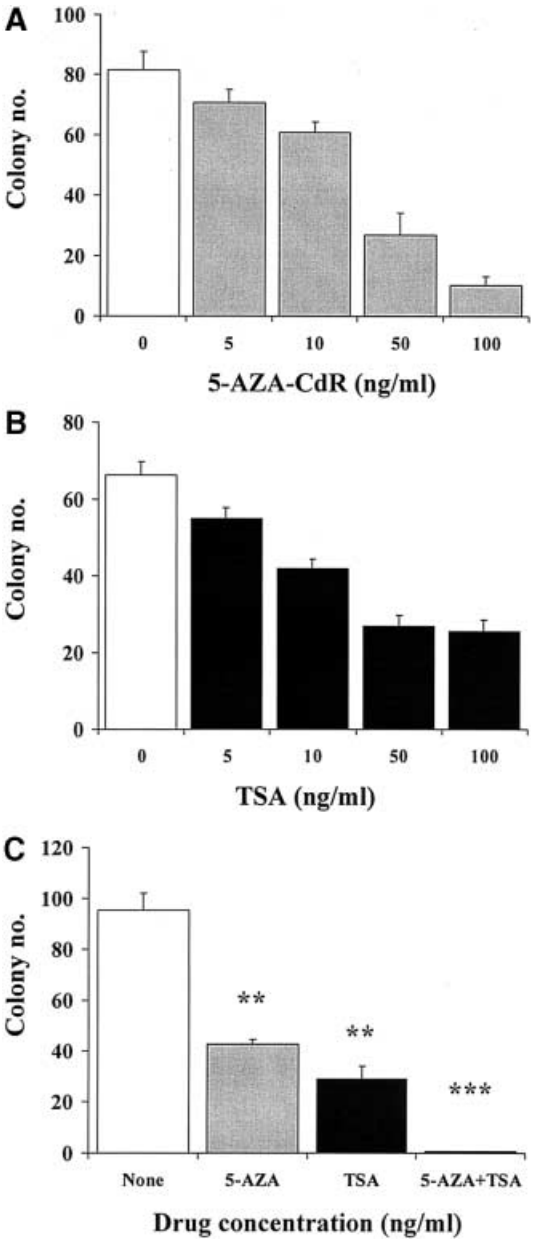

Fig. 1A–C Dose-response curves for the cytotoxic action of 5-AZA-CdR (**A**) and TSA (**B**) on MDA-MB-231 breast carcinoma cells. Drug exposure was 48 h and cell survival determined by colony assay. **C** Cytotoxic action of 5-AZA-CdR (25 ng/ml) and TSA (50 ng/ml) administered alone and in combination on MDA-MB-231 breast carcinoma cells. A sequential 48-h drug exposure was used and cell survival determined by colony assay. Values are the means ± SEM from three to five experiments (**$P < 0.01$ vs control, ***$P < 0.001$ 5-AZA-CdR + TSA vs 5-AZA-CdR)

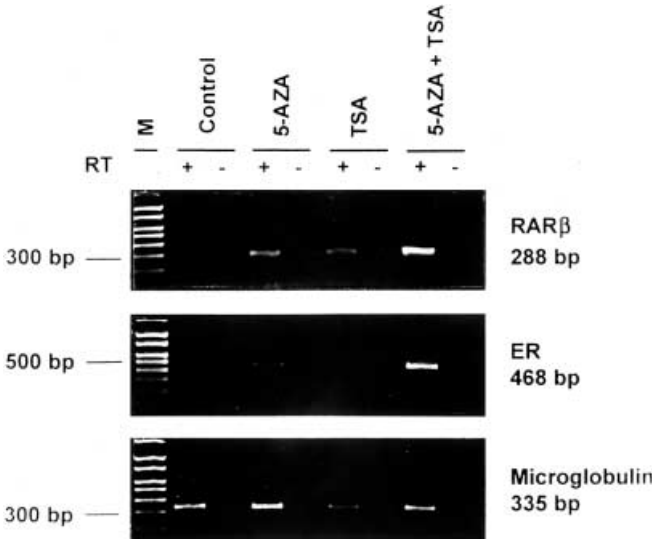

Fig. 2 RT-PCR analysis of activation of expression of RARβ and ERα by 5-AZA-CdR and TSA. Total RNA was isolated 15–20 h after treatment with 5-AZA-CdR 50 ng/ml for 48 h and/or TSA 181 ng/ml for 48 h. PCR was performed using the cDNA with specific primers for RARβ, ERα and microglobulin as described in Methods. The amplified DNA was separated on agarose gel and stained with ethidium bromide. The relative intensity of the bands, as determined by Adobe Photoshop analysis for RARβ are 5-AZA 7, TSA 23, 5-AZA + TSA 25, and for ERα are 5-AZA <1, TSA <1, 5-AZA + TSA 11 (*M* DNA size marker)

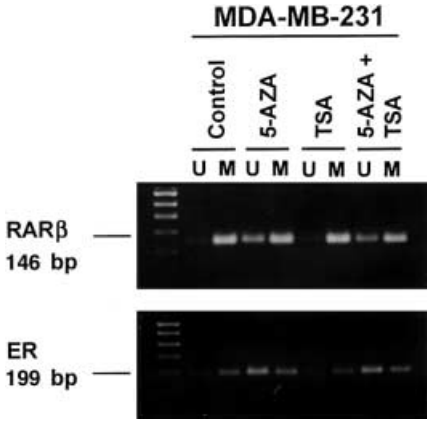

Fig. 3 MSP analysis of RARβ and ERα in human MDA-MB-231 breast cancer cells. MSP was used to assess the methylation status of promoter in both genes. The results show clear signs of promoter methylation in MDA-MB-231 cells for RARβ and ERα, and partial demethylation following treatment with 5-AZA-CdR alone or in combination with TSA. Treatment with TSA did not change the methylation status of DNA

and then to TSA (50 ng/ml) for an additional 48 h (Fig. 1C). 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 [35] analysis.

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 RT-PCR for two tumor suppressor genes, RARβ and ERα, reported to be silenced in MDA-MB-231 cells [11, 34]. The sensitivity of RT-PCR allowed the detection of bands for RARβ, a gene which is difficult to detect by Northern blot analysis due to the low copy number of its mRNA. After densitometric analysis, the results were normalized to the intensity of the microglobulin bands and ratios were calculated in order to express RARβ and ERα reactivation after drug treatment (see Methods). Treatment of the cells with 5-AZA-CdR (50 ng/ml) weakly reactivated both genes by demethylation of DNA (Fig. 2). Our results also indicated that RARβ, but not ERα, was reactivated by TSA. Treatment of MDA-MB-231 cells with both 5-AZA-CdR and TSA resulted in an increased reactivation of RARβ expression, and a robust reactivation of ERα. The MSP assay confirmed that lack of expression of RARβ and ERα was due to promoter methylation and that 5-AZA-CdR treatment led to partial demethylation (Fig. 3). In order to determine if the effect of increased reactivation of RARβ by TSA, in combination with 5-AZA-CdR, was not due to an enhancement of promoter hypomethylation produced by this nucleoside analog, we performed the MS-SNuPE assay to quantitate the methylation status of two specific CpG sites in the promoter region of RARβ after drug treatment (Table 1). Exposure of cells to TSA after treatment with 5-AZA-CdR did not further decrease DNA methylation.

Table 1 Effect of treatment of MDA-MB-231 cells with 5-AZA-CdR alone or with TSA on the methylation status of RARβ using the SNuPE assay. The cells were exposed sequentially for 48 h to the drugs. DNA was isolated at 15–20 h after treatment and treated with bisulfite. Semi-nested 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 in Methods. The values are the means ± SEM from three different experiments

Primer	Additions	Concentration (ng/ml)	Relative inhibition, DNA methylation (%)
A	5-AZA	50	36.0 ± 3.0
A	TSA	181	< 1
A	5-AZA + TSA	50181	30.8 ± 2.2
B	5-AZA	50	28.3 ± 2.0
B	TSA	181	< 1
B	5-AZA + TSA	50 + 181	33.2 ± 2.2

Discussion

5-AZA-CdR has been shown to be an interesting antineoplastic agent that shows clinical activity in both leukemia and lung cancer [27, 30]. 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 [14]. This mechanism of action of 5-AZA-CdR makes it a very promising agent for 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 agent which increase histone acetylation leading to a more "open" chromatin structure that is accessible to transcription factors [24]. 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 [28].

Interaction between DNA methylation and histone acetylation in the control of gene expression is currently under intensive investigation. Cameron et al. [7] recently reported that 5-AZA-CdR and TSA in combination produce a synergistic reactivation of tumor suppressor genes suggesting 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. 1A, B). Interestingly, sequential treatment of these cells led to a synergistic interaction between the two agents in which the combination produced 100% cell kill (Fig. 1C). This finding may have relevance in the clinical treatment of patients with breast cancer. The rationale for the use of 5-AZA-CdR first is that this drug is S-phase specific, and TSA can cause arrest in the G_0/G_1 phase of the cell cycle, thus decreasing the percentage of cells in the S phase [13]. We have performed clonogenic assays on MDA-MB-231 cells using both drugs simultaneously, but the results obtained were inconsistent (data not shown). For these reasons, treatment of cells with TSA prior to 5-AZA-CdR was not considered.

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 [34, 37]. We have shown previously that the RARβ gene is silenced by DNA methylation and reactivated after treatment with 5-AZA-CdR in human DLD1 colon carcinoma cells [9]. In this study, we demonstrated that in MDA-MB-231 breast cancer cells RARβ can be reactivated by treatment with either 5-AZA-CdR or TSA (Fig. 2). This finding confirms the report by Sirchia et al. [33]. The reactivation of RARβ by the combination of these two drugs was weakly greater than with either agent alone. One possible explanation for 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. Our results also seem to suggest that the two epigenetic modifications act independently in silencing RARβ.

The second gene investigated was the ERα gene which has been reported to be silenced by hypermethylation in the MDA-MB-231 breast cancer cell line [14]. In contrast to the RARβ gene, expression of the ERα gene could not be induced by TSA (Fig. 2). 5-AZA-CdR only produced a weak activation of ERα. However, after exposure of MDA-MB-231 cells to both 5-AZA-CdR and TSA, a synergistic gene activation was observed, which is in agreement with the reports of Cameron et al. [7] for several cancer-related genes and Chiurazzi et al. [8] for the FMR1 gene. These results suggest that methylation plays the dominant role in the silencing of ERα 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 partial demethylation occurs, histone deacetylation partly maintains the gene in the silenced state. Silencing of both RARβ and ERα genes by aberrant promoter methylation was confirmed by MSP (Fig. 3), and partial demethylation was evident after treatment of MDA-MB-231 cells with 5-AZA-CdR alone and in combination with TSA.

In order to determine whether TSA could indirectly affect the extent of demethylation produced by 5-AZA-CdR, we performed quantitative methylation analysis of two specific sites in the RARβ gene by the MS-SNuPE assay which showed that the increased re-expression of this gene after treatment of cells with 5-AZA-CdR and TSA was 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 in 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 show 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 that 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.

Acknowledgements We thank Mrs Louise F. Momparler for skilful technical support.

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