Interaction of 5-aza-2′-deoxycytidine and depsipeptide on antineoplastic activity and activation of 14-3-3σ, E-cadherin and tissue inhibitor of metalloproteinase 3 expression in human breast carcinoma cells

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Genes that suppress tumorigenesis can be silenced by epigenetic events, such as aberrant DNA methylation and modification of chromatin structure. Inhibitors of DNA methylase and histone deacetylase (HDAC) can potentially reverse these events. The aim of this study was to determine the *in vitro* antineoplastic activity of 5-aza-2′-deoxycytidine (5-AZA-CdR), a potent inhibitor of DNA methylase, in combination with depsipeptide (depsi), an inhibitor of HDAC, on human breast carcinoma cells. We observed a synergistic antineoplastic interaction between 5-AZA-CdR and depsi in their capacity to inhibit colony formation of Hs578T and MCF-7 breast carcinoma cells. In order to understand the molecular mechanism of this interaction, we investigated the effect of these drugs on the activation of the 14-3-3σ, E-cadherin and tissue inhibitor of metalloproteinase 3 (TIMP3) cancer-related genes, which were reported to be silenced by aberrant methylation in many breast tumor cell lines. 14-3-3σ was reported to produce G_2 cell cycle arrest following DNA damage. E-cadherin and TIMP3 function as suppressors of tumor metastasis. Semi-quantitative RT-PCR was used to determine the effect of the co-administration of 5-AZA-CdR and depsi on four breast carcinoma cell lines for the reactivation of these genes. We observed a synergistic activation of E-cadherin by the combination in Hs578T, MDA-MB-231 and MDA-MB-435 tumor cells. For 14-3-3σ, we demonstrated an additive to synergistic activation by the combination for Hs578T and MDA-MB-435 tumor cells, respectively. In the MCF-7 tumor cells, the drug combination produced a synergistic activation of TIMP3. The association between the synergistic antineoplastic activity and the synergistic activation of the target genes in this study suggests that the mechanism of anticancer activity of 5-AZA-CdR, in combination with depsi, is probably related to their enhanced activation of different types of tumor suppressor genes that have been silenced by epigenetic events. *Anti-Cancer Drugs* 14:193–202 © 2003 Lippincott Williams & Wilkins.

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Introduction

Breast cancer is one of the major causes of death in women. There is an urgent need to develop new approaches to increase the effectiveness of the treatments for this malignancy. Aberrant methylation of cancer-related gene promoters is an important epigenetic event that occurs during the development of breast tumors [1–3]. *In vitro* studies on human carcinoma cell lines have shown that most hypermethylated genes related to cancer can be reactivated by 5-aza-2′-deoxycytidine (5-AZA-CdR), a potent inhibitor of DNA methylation [2–4]. The demethylation produced by this agent can induce terminal differentiation and senescence of neoplastic cells [5]. In clinical trials, this agent has shown interesting antineoplastic activity in patients with leukemia [6–8] and lung cancer [9].

Another epigenetic event implicated in repression of transcription is the deacetylation of histones in chromatin. Highly acetylated histones facilitate the opening of chromatin structure, which allows the transcription factors to have readily access to the promoter region of the target genes [10,11]. Deacetylation of the histones by histone deacetylase (HDAC) leads to a compact structure of the chromatin that is unfavorable for gene transcription. Depsipeptide FR901228 (depsi) is a potent inhibitor of HDAC that can reactivate some cancer-related genes [12]. This agent showed antitumor activity

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in animal models [13] and is currently in phase I clinical trials in patients with cancer. Up to now, it has produced some positive responses [14].

Recent studies indicate that there is a link between methylation and histone acetylation in which a family of methyl-CpG-binding proteins is involved [15]. When these proteins bind to a methylated promoter, they recruit HDAC, and the interaction of these two epigenetic events inhibits gene expression by interfering with the function of transcription factors and the compaction of the chromatin structure [16–18]. Inhibitors of these epigenetic changes can lead to the reactivation of genes that suppress tumorigenesis. In accord with this hypothesis is the report on the synergistic interaction of 5-AZA-CdR and the HDAC inhibitor, trichostatin A (TSA), in the reactivation of tumor suppressor genes [19]. This same drug combination was also reported to induce a synergistic reactivation of the estrogen receptor-α in breast carcinoma cells [20].

Several cancer-related genes have been reported to be silenced by aberrant methylation in breast cancer. These include 14-3-3σ, E-cadherin and tissue inhibitor of metalloproteinase 3 (TIMP3) genes. The 14-3-3σ gene plays a role in the cell cycle as a G_2 checkpoint regulator [21] and its hypermethylation was detected frequently in breast tumors [22]. The E-cadherin gene is involved in cell–cell adhesion and functions as an invasion suppressor [23]. Inactivation of this gene by hypermethylation has been observed in breast carcinoma cells [24] and in primary breast tumors [25]. The TIMP3 gene shows some activity to inhibit tumor growth, angiogenesis and metastasis [26]. It has been recently suggested that the TIMP3 gene could have a tumor suppressor role [27]. This gene was found to be hypermethylated in some breast cancer cell lines and in breast primary tumors [28]. Treatment with 5-AZA-CdR activated the expression of 14-3-3σ [22] and E-cadherin genes [24] in breast carcinoma cells, and of TIMP3 in different tumor cell lines [19,28].

In this study, our first objective was to evaluate the antineoplastic activity of the combination of 5-AZA-CdR and depsi on MCF-7 and Hs578T human breast carcinoma cell lines. Previously, we have observed a synergistic antitumor interaction between 5-AZA-CdR and TSA [29] or depsi [30] on other breast carcinoma cell lines. Our second objective was to evaluate the effect of this combination on the activation of 14-3-3σ, E-cadherin and TIMP3 genes in four different breast carcinoma cell lines.

Our results showed that drug combinations produce a greater antineoplastic effect and a greater activation of some target genes in human breast carcinoma cells than either drug alone. These data suggest that 5-AZA-CdR plus depsi may be an interesting drug combination to investigate for the treatment of advanced breast cancer.

Materials and methods

Materials

5-AZA-CdR (Decitabine) was obtained from Pharmachemie (Haarlem, The Netherlands). Stock concentrations were made in phosphate-buffered saline and stored at -70°C. Depsi (FR901228) was obtained from Fujisawa Pharmaceutical (Osaka, Japan). Stock concentrations were made in absolute ethanol and stored at -20°C.

Cell culture

The Hs578T, MDA-MB-231 and MDA-MB-435 human breast carcinoma cell lines were obtained from ATCC (Manassas, VA), and the MCF-7 human breast carcinoma cell line was provided by Dr Wilson Miller (Lady Davis Institute, Montreal, Canada). The MDA-MB-231, MDA-MB-435 and MCF-7 cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent, St Bruno, Quebec, Canada) and, for MDA-MB-435 cells, containing 4 μl/ml of insulin/transferrin/selenium A supplement (ITS; Invitrogen, Burlington, Ontario, Canada). The Hs578T cell line was cultured in DMEM medium containing 10% FBS and ITS. The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Inhibition of DNA synthesis

The inhibition of DNA synthesis by the drugs was determined by measurement of the incorporation of radioactive thymidine into DNA after 48 h drug exposure as described previously [30].

Clonogenic assay

Clonogenic assay was performed by comparing the ability of 5-AZA-CdR and/or depsi to inhibit colony formation. The Hs578T and MCF-7 cells were plated in six-well culture dishes at a concentration of 150 and 125 cells/ml, respectively, in 2 ml of medium. The next day, 5-AZA-CdR and/or depsi were added to the medium at the indicated concentrations for 48 h exposure. The cells were then placed in drug-free medium for 14–17 days. The colonies (>50 cells) were then stained with 0.5% methylene blue in 50% methanol and counted.

RNA isolation and RT-PCR analysis

In order to study the reactivation of 14-3-3σ, E-cadherin and TIMP3 genes in Hs578T, MCF-7, MDA-MB-231 and MDA-MB-435 breast carcinoma cell lines, the cells were treated with 5-AZA-CdR (20 ng/ml) or/and depsi (1 ng/ml) as a simultaneous exposure for 48 h. Cells were harvested 24 h after the removal of the drugs. Total RNA was isolated using the Absolutely RNA RT-PCR miniprep kit (Stratagene, San Diego, CA). For cDNA synthesis,

1000 ng of total RNA was reverse transcribed using the OmniScript RT kit (Qiagen, Mississaugua, Ontario, Canada). The reaction was performed at 37°C for 1 h followed by 5 min at 93°C to inactivate the enzyme.

PCR amplifications were performed using HotStar Taq polymerase (Qiagen) and specific primers designed for a portion of the mRNA region of each gene: E-cadherin (GenBank accession no. NM004360), 14-3-3σ (accession no. AF029082), TIMP3 (accession no. XM009943) and β_2-microglobulin (accession no. XM_007650). The human β_2-microglobulin housekeeping gene was used as an internal control. The specific primers used were as follow: E-cadherin (length: 410 bp), sense 5′-CAA TCC CAC CAC GTA CAA G-3′ and antisense 5′-CTG GGC AGT GTA GGA TGT GA-3′; 14-3-3σ (length: 219 bp), sense 5′-TGG ACA TCA GCA AGA AGG AGA-3′ and antisense 5′-TCA GGT TGT CTC GCA GCA G-3′; TIMP3 (length: 252 bp), sense 5′-AGG ACG CCT TCT GCA ACT C-3′ and antisense 5′-CCG TGT ACA TCT TGC CAT CA-3′; β_2-microglobulin (length: 355 bp), sense 5′-CTC GCG CTA CTC TCT CTT TCT GG-3′ and antisense 5′-GCT TAC ATG TCT CGA TCC CAC TTA A-3′. The PCR amplifications were performed using the following conditions: 95°C for 15 min (activation of the Taq polymerase); 94°C for 40 s as denaturation step, 55°C (β_2-microglobulin), 58°C (TIMP3 and E-cadherin) and 60°C (14-3-3σ) for 30 s as annealing step, and 72°C for 40 s as elongation step. After 5 cycles, the annealing temperature was then lowered by 2°C for 14-3-3σ, TIMP3 and E-cadherin for an additional 30–35 cycles. For β_2-microglobulin, 28–32 cycles were used without a reduction in the annealing temperature. For each gene, the number of cycles was limited to the exponential phase of DNA amplification. The PCR products were resolved by electrophoresis in a 2% agarose gel and detected by ethidium bromide staining. The amount of DNA amplified in each sample was also quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) for quantitative detection of gene expression. The Bioanalyzer made very precise measurements of both, the quantity and size of DNA, in a small sample volume of 1 μl. For the PCR reactions, the amount of cDNA used for each sample was normalized to the DNA concentrations obtained with the β_2-microglobulin gene.

Standard curves were performed for each gene. The cDNA for E-cadherin, 14-3-3σ, TIMP3 and β_2-microglobulin genes were amplified from normal human breast cDNA (Clontech, Palo Alto, CA) using the PCR conditions described above. The amplified DNA was resolved by electrophoresis in a 2% agarose gel and the specific band of each gene was cut out from the gel. The gel fragments were then placed in Amicon Ultrafree-DNA columns (Amicon, Bedford, MA) and centrifuged at 5000 *g* for 10 min. The eluates were diluted 100-fold and 1 μl of each diluted DNA was used to perform a second PCR. The amplified DNA was purified using a QIAquick PCR purification kit (Qiagen). The concentration of each sample was then quantified using an Agilent 2100 Bioanalyzer, the number of molecules of each sample determined and dilutions were made in the range of 10^4–10^7 molecules. PCR reactions were performed using serial dilutions for the E-cadherin, 14-3-3σ, TIMP3 and β_2-microglobulin genes for 23, 26, 26 and 25 cycles, respectively. The size and concentration of each PCR product were then quantified using the Bioanalyzer. The standard curves showed an excellent correlation ($r > 0.9$) between concentration of DNA and the number of molecules of the target gene (Fig. 2).

Data analysis

Statistical comparisons were determined using the one-way ANOVA method, which compared the results obtained for each drug alone with those obtained for the combination of both agents. To find out whether the interaction of the drugs was additive or synergistic in the clonogenic assays, the survival fraction of the combination of 5-AZA-CdR and depsi was compared to the product of the survival fraction of the two drugs administrated alone as described by Valeriote and Lin [31,32]. In their analysis, if the product of the survival fraction of each drug alone is superior or equal to the survival fraction of the combination, the drug interaction is synergistic or additive, respectively.

Results

Clonogenic assays

The capacity of 5-AZA-CdR or depsi alone to inhibit colony formation by Hs578 T and MCF-7 tumor cells was determined after 48 h drug exposure (Table 1). Both drugs were potent agents to inhibit colony formation. From the 5-AZA-CdR dose–response data, we estimated that its IC_{50} was 50 ng/ml for the Hs578T cells and 30 ng/ml for the MCF-7 cells (Table 2). Depsi inhibited the colony formation in a dose-dependant manner and its IC_{50} was estimated to be about 0.80 ng/ml for the MCF-7 cells and 0.45 ng/ml for the Hs578T cells (Table 2). From previous work on the MDA-MB-231 and MDA-MB-435 cells [30], we determined an IC_{50} of about 1.5 and 30 ng/ml, respectively, for 5-AZA-CdR, and 0.35–0.45 ng/ml for depsi [30]. These data indicate that depsi is a much more potent cytotoxic agent than 5-AZA-CdR. The IC_{50} values for 5-AZA-CdR showed a greater range than the IC_{50} values for depsi for the breast carcinoma cell lines.

DNA synthesis inhibition

The IC_{50} to inhibit DNA synthesis was determined for the Hs578T cell line and is summarized in Table 2. We reported previously that the MDA-MB-231 tumor cells were more sensitive to the inhibition produced by 5-AZA-CdR than MDA-MB-435 cells [30]. The IC_{50} for

Table 1 Effect of different concentrations of 5-AZA-CdR or depsi on loss of clonogenicity by Hs578T and MCF-7 human breast carcinoma cells

Hs578T		MCF-7	
5-AZA-CdR (ng/ml)	Loss of clonogenicity (%)	5-AZA-CdR (ng/ml)	Loss of clonogenicity (%)
2.5	7.3 ± 6.8[a]	1	20.8 ± 8.5
25	24.7 ± 13	10	30.5 ± 8.4
50	49.6 ± 5.0	50	63.7 ± 6.4
125	69.1 ± 2.3	100	84.2 ± 11
250	84.4 ± 2.3	1000	97.3 ± 2.3
Depsi (ng/ml)	Loss of clonogenicity (%)	Depsi (ng/ml)	Loss of clonogenicity (%)
0.01	16.5 ± 10	0.1	3.2 ± 2.8
0.1	36.0 ± 8.7	0.2	15.9 ± 4.0
1	74.9 ± 6.9	0.6	23.2 ± 6.9
10	96.3 ± 3.1	0.8	46.3 ± 2.9
		1.0	59.1 ± 11

[a]The cells were exposed to the indicated concentrations of drug for 48 h. Loss of clonogenicity was determined by colony assay. Data are mean values ± SD, $n = 4$ relative to control cells without drug treatment.

Table 2 The 50% inhibitory concentrations (IC_{50}; ng/ml) of 5-AZA-CdR and depsi on human breast carcinoma cell lines for DNA synthesis and for loss of clonogenicity

Cell line	Drug	Inhibition of DNA synthesis (IC_{50})	Loss of clonogenicity (IC_{50})
Hs578T	5-AZA-CdR	100[a]	50[a]
MCF-7	5-AZA-CdR	ND	40
MDA-MB-435	5-AZA-CdR	50	30
MDA-MB-231	5-AZA-CdR	2.5	1.5
Hs578T	depsi	0.30	0.45
MCF-7	depsi	ND	0.80
MDA-MB-231	depsi	0.20	0.40
MDA-MB-435	depsi	0.25	0.40

Drug exposure was 48 h. The data for the MDA-MB-231 and MDA-MB-435 cell lines were reported previously by our laboratory [30].
[a]Estimated values ± 15%.
ND, not done.

5-AZA-CdR ranged from 2.5 ng/ml for the MDA-MB-231 cells up to 50 and 100 ng/ml for the MDA-MB-435 and Hs578T, respectively. For depsi, the IC_{50} was in the range between 0.2 and 0.3 ng/ml for all the three cell lines, indicating that this agent is a much more potent inhibitor of DNA synthesis than 5-AZA-CdR.

Combination chemotherapy

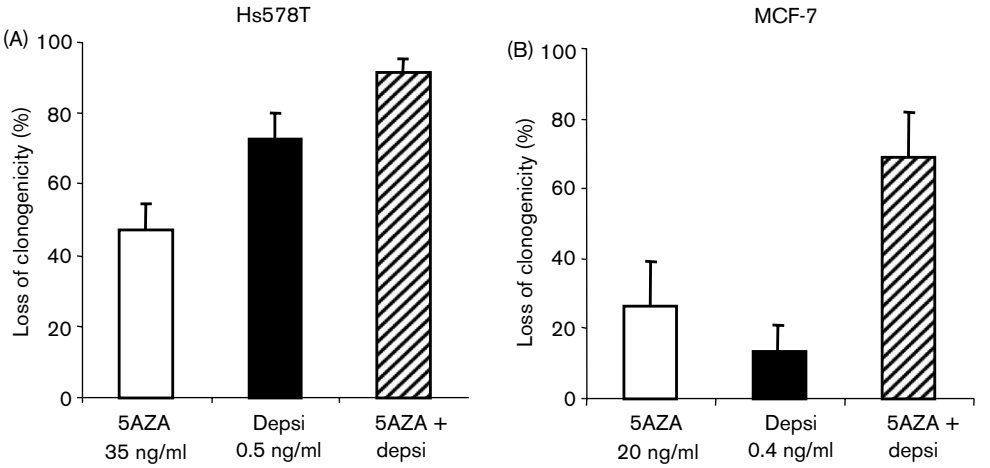
The effect of the combination of 5-AZA-CdR and depsi on the loss of clonogenicity in the MCF-7 and Hs578T breast carcinoma cells was evaluated (Fig. 1). For each cell line, we selected concentrations of 5-AZA-CdR and depsi that would produce less than 75% inhibition of colony formation to facilitate the analysis of drug interaction in terms of enhancement of antineoplastic activity. The co-administration of 5-AZA-CdR and depsi produced a significantly greater loss of clonogenicity than either drug alone for the two breast carcinoma cells (Fig. 1). In the Hs578T cells, 5-AZA-CdR (35 ng/ml) or depsi (0.5 ng/ml) alone produced 47 and 72% inhibition of colony formation, whereas the combination of both agents showed a 92% inhibition (Fig. 1A). This interaction between the two inhibitors was synergistic as defined by Valeriote and Lin [31,32]. For the MCF-7 cells, we also observed a synergistic interaction for the combination of both agents that produced 66% loss of clonogenicity, whereas 5-AZA-CdR (20 ng/ml) or depsi (0.4 ng/ml) alone showed 32 and 21% loss of clonogenicity (Fig. 1B). These results are in accord with data obtained previously for the MDA-MB-231 and MDA-MB-435 tumor cells [30].

Gene expression

In order to evaluate the capacity of 5-AZA-CdR and depsi, alone and in combination, to reactivate genes that have been silenced by epigenetic mechanisms, we investigated the effect of these methylation and HDAC inhibitors on the expression of E-cadherin, 14-3-3σ and TIMP3 cancer-related genes. These latter genes were reported to be frequently silenced in breast cancer [24,25,28]. Specific sets of primers were designed for each gene and their mRNA expression was determined by RT-PCR using two different end points: the ethidium bromide staining of the PCR products and the quantification of the amount of amplified DNA using an Agilent 2100 Bioanalyzer (Figs 3 and 4). Ratios of the amount of amplified DNA for the drug-treated samples as compared to the control were calculated in order to express the relative level of gene expression after drug treatment (Figs 3 and 4).

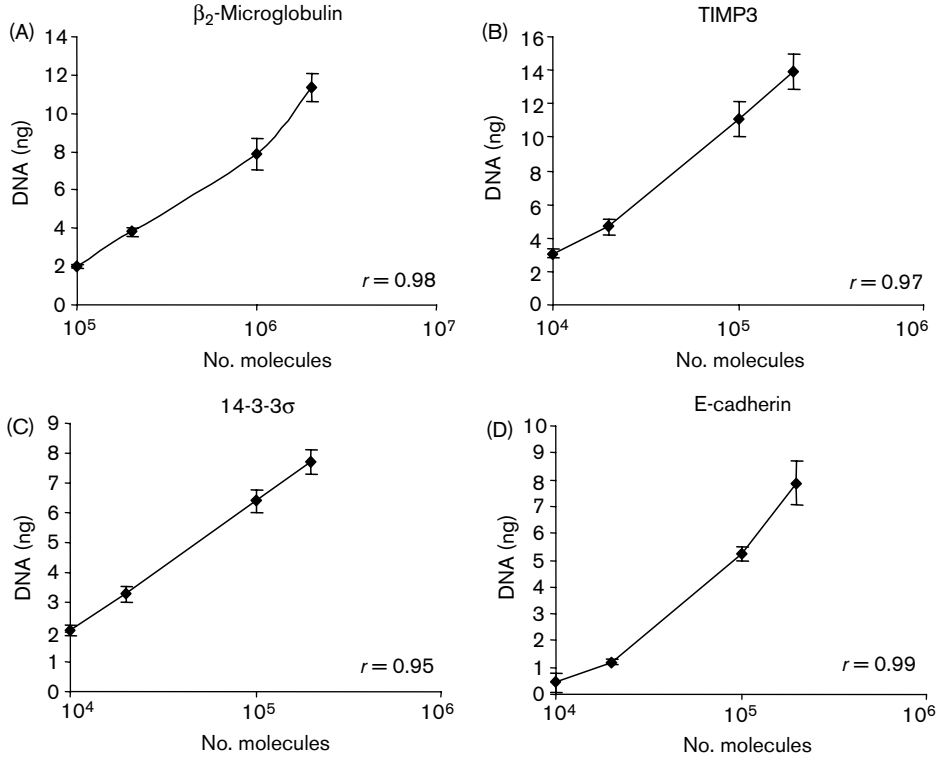
In our study on 14-3-3σ, we observed a synergistic reactivation of its expression by 5-AZA-CdR plus depsi in Hs578T and MDA-MB-435 tumor cells (Figs 3A and 4B). We detected a low level of expression of 14-3-3σ in the

Fig. 1



Effect of 5-AZA-CdR (5AZA) or depsi alone and in combination on loss of clonogenicity by Hs578T (A) and MCF-7 (B) human breast carcinoma cells. The cells were exposed to the indicated concentrations of drug for 48 h and cell survival determined by colony assay. Data shown are mean values ± SD, $n=4$. Statistical analysis (for both cell lines): 5-AZA-CdR or depsi alone versus combination: $p<0.001$.

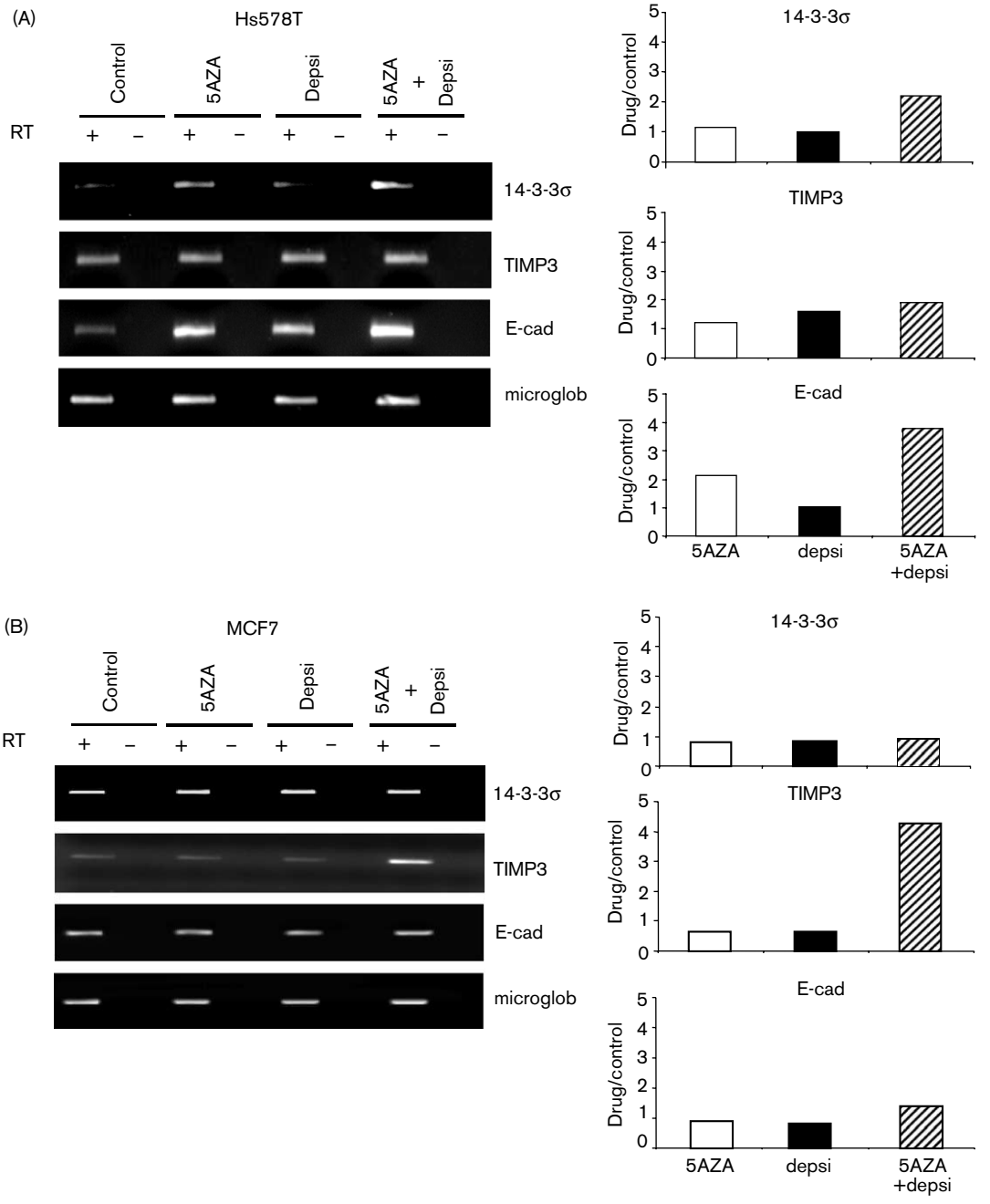
Fig. 2



Standard curves for the amplification of different number of cDNA molecules of β_2-microglobulin (A), TIMP3 (B), 14-3-3σ (C) and E-cadherin (D) by PCR. The amount of amplified DNA was quantitated by an Agilent 2100 Bioanalyzer. The curves show an excellent correlation coefficient ($r>0.9$) between the amount of amplified DNA and the number of cDNA molecules for each of the target genes.

control cells for both of these cell lines. For TIMP3, the drug combination produced a remarkable synergistic activation of its expression in the MCF-7 cells accompanied by a slight expression of this gene in control cells (Fig. 3B). The work with E-cadherin indicated its very synergistic activation of its expression by the co-treatment of the Hs578T, MDA-MB-231 and MDA-MB-435 cells (Figs 3A, and 4A and B). Particularly for the

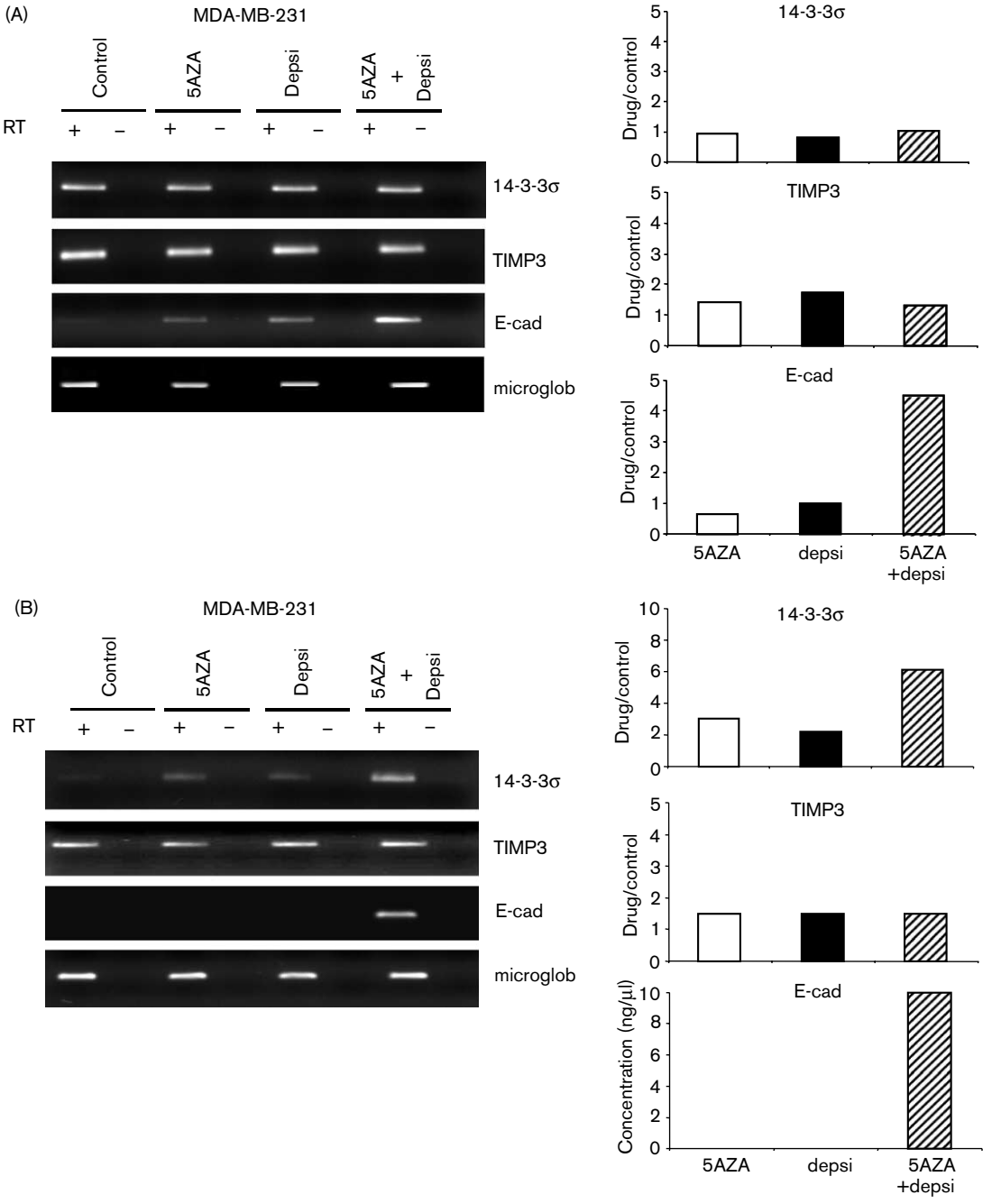
Fig. 3



Effect of 5-AZA-CdR (20 ng/ml) and depsi (1 ng/ml), alone or in combination, on mRNA expression of 14-3-3σ, TIMP3 and E-cadherin in Hs578T (A) and MCF-7 (B) human breast carcinoma cells. The cells were exposed to drug for 48 h and RNA was isolated at 72 h. RT-PCR was used to quantitate mRNA expression by staining with ethidium bromide (left panel) and by measurement of amount of the DNA amplified by an Agilent 2100 Bioanalyzer (right panel). The ratios of DNA amplified in drug-treated cells relative to control cells are shown. Statistical analysis: (A) Hs578T cells: for the E-cadherin and 14-3-3σ genes, 5-AZA-CdR or depsi alone versus combination: $p < 0.01$; for the TIMP3 gene, 5-AZA-CdR alone, but not depsi, versus combination: $p < 0.001$. (B) MCF-7 cells: for TIMP-3, 5-AZA-CdR or depsi alone versus combination: $p < 0.001$, but not significant for 14-3-3σ and E-cadherin. RT+/–, presence or absence of reverse transcriptase; 5AZA, 5-AZA-CdR; microglob, β2-microglobulin; E-cad, E-cadherin.

MDA-MB-435 cells, we could not detect any activation of expression of E-cadherin by each agent alone, whereas the combination produced an exceptional reactivation of this gene. Constitutive expression of the target genes was observed in some cell lines: E-cadherin in MCF-7 cells, TIMP3 in MDA-MB-231 and MDA-MB-435 cells, and 14-3-3σ in MCF-7 and MDA-MB-231 cells.

Fig. 4



Effect of 5-AZA-CdR (20 ng/ml) and depsi (1 ng/ml), alone or in combination, on mRNA expression of 14-3-3σ, TIMP3 and E-cadherin in MDA-MB-231 (A) and MDA-MB-435 (B) human breast carcinoma cells. The cells were exposed to drug for 48 h and RNA was isolated at 72 h. RT-PCR was used to quantitate mRNA expression by staining with ethidium bromide (left panel) and by measurement of amount of the DNA amplified by an Agilent 2100 Bioanalyzer (right panel). The ratios of DNA amplified in drug-treated cells relative to control cells are shown. Statistical analysis: (A) MDA-MB-231 cells: for the E-cadherin gene, 5-AZA-CdR or depsi alone versus combination: $p<0.001$, but not significant for the 14-3-3σ and TIMP3 genes. (B) MDA-MB-435 cells: for the 14-3-3σ and E-cadherin genes, 5-AZA-CdR or depsi alone versus combination: $p<0.001$, but not significant for the TIMP3 gene. RT + / –, presence or absence of reverse transcriptase; 5AZA, 5-AZA-CdR; microglob, β2-microglobulin; E-cad, E-cadherin.

Discussion

Women with advanced metastatic breast cancer that is resistant to hormonal therapy usually respond poorly to conventional chemotherapy and their life expectancy is limited. There is an urgent need to develop more effective therapy for these patients. In addition to

mutations and chromosomal abnormalities, two major epigenetic events play important roles in tumorigenesis. These latter events are aberrant methylation and chromatin compaction, which can lead to the silencing of tumor suppressor genes [3,18]. An interesting rationale is to design a form of epigenetic therapy for breast cancer which involves the use of a combination of inhibitors that target DNA methylation and chromatin structure. The objective of this approach was to reactivate the genes that suppress tumorigenesis. In support of this hypothesis were the reports that the inhibitor of methylation, 5-AZA-CdR, in combination with an HDAC inhibitor produced a synergistic activation of cancer-related genes [19,30]. In addition, our laboratory and others reported that the combination of 5-AZA-CdR and TSA produced an enhanced reactivation of the tumor suppressor gene, retinoic acid receptor (RAR) β2 [29,33] and of several genes silenced in different human cancers [34].

These reports led us to propose the hypothesis that 5-AZA-CdR in combination with HDAC inhibitors should produce an additive or synergistic antineoplastic effect on tumor cells. We confirmed previously this positive interaction using 5-AZA-CdR plus TSA or depsi against MDA-MB-231 and MDA-MB-435 human breast carcinoma cells [29,30]. In this study, we have investigated the antineoplastic activity of the combination of 5-AZA-CdR with depsi on MCF-7 and Hs578T breast carcinoma cell lines. In addition, we investigated their capacity to reactivate some cancer-related genes in all these four cell lines. We chose depsi for this investigation because it was a much more potent inhibitor of HDAC than TSA [35] and it was currently in clinical trials in patients with cancer [14].

The antineoplastic activity of 5-AZA-CdR and depsi as single agents against the MCF-7 and Hs578T tumor cells was studied using a clonogenic assay (Table 1). A summary of the inhibitory action of 5-AZA-CdR and depsi with respect to inhibition of DNA synthesis and loss of clonogenicity for different human breast carcinoma cell lines is shown in Table 2. Both agents were potent inhibitors as indicated by their IC_{50} values. The Hs578T cells were less sensitive to the inhibitory effects of 5-AZA-CdR than the other cell lines. The reason for this finding is unknown, but possibly due to a higher level of cytidine deaminase in the Hs578T cells as compared to the other cell lines. We have reported previously that tumor cells with enhanced levels of cytidine deaminase showed signs of drug resistance to 5-AZA-CdR [36]. On the other hand, since 5-AZA-CdR is a prodrug that must be activated by deoxycytidine kinase [37], it is possible that the low sensitivity to this analog by Hs578T cells was due to low level of this enzyme.

When used in combination, 5-AZA-CdR and depsi produced a significantly greater antineoplastic effect on the MCF-7 and Hs578T tumor cells than either drug alone (Fig. 1). The results obtained with the clonogenic assays indicated a synergistic antineoplastic effect by the drug combination against the breast carcinoma cells as defined by Valeriote and Lin [31,32] These results confirmed the observations of Primeau *et al.* [30] for the MDA-MB-231 and MDA-MB-435 tumor cells. The presence of a synergy between these agents in four different human breast carcinoma cell lines suggests that a similar type of interaction may occur in patients with breast cancer where tumor heterogeneity is a common occurrence. The synergistic interaction between 5-AZA-CdR and depsi with respect to the *in vitro* loss of clonogenicity by the tumor cells should be confirmed in animal tumor models in order to provide a solid rationale for clinical investigation of this form of combinational therapy in women with advanced breast cancer.

In order to understand the molecular mechanism involved in the positive interaction between 5-AZA-CdR and depsi, we investigated their capacity, alone and in combination, to activate the expression of different cancer-related genes silenced by aberrant methylation in breast carcinoma cell lines. To analyze if the combination produced an enhancement of gene expression, we used concentrations for each of these agents that do not produce a maximal response. In addition, for translational research, we selected concentrations that are in the range of the plasma levels observed in clinical trials on these agents in patients with cancer [14,38]. As target genes, we chose 14-3-3σ whose expression was reported to be reduced by hypermethylation in MDA-MB-435 and Hs578T cells [22], and E-cadherin whose expression was reported to be diminished by hypermethylation in MDA-MB-231, MDA-MB-435 and Hs578T cells [24]. TIMP3 was selected because methylation of its promoter has been observed in human breast carcinoma cell lines and in primary breast tumors [28]. 14-3-3σ is a cell cycle check point gene [21], whereas E-cadherin and TIMP3 function as suppressors of tumor metastasis [23,26].

In this report, we detected in different cell lines low expression of the following genes in the absence of inhibitors: E-cadherin in MDA-MB-231 and Hs578T cells (Fig. 3A and 4A), 14-3-3σ in MDA-MB-435 and Hs578T cells (Figs 3A and 4B), and TIMP3 in MCF-7 cells (Fig. 3B). We do not know the reason for this observation. It may be due to tumor cell heterogeneity with variable, partial or complete methylation of their promoter region [18] as it has been reported for E-cadherin [39]. 5-AZA-CdR alone has been reported to activate many different types of cancer-related genes [3]. HDAC inhibitors showed more variation with respect to which genes they can activate. For example, TSA did not activate the expression of different methylated genes, but can activate the expression of certain unmethylated genes

[14,34]. However, in certain situations, some methylated genes that are related to nuclear receptors can be activated by TSA. For example, this HDAC inhibitor was shown to activate methylated RARβ2 gene in breast carcinoma cells [29], especially in the presence of retinoic acid, the ligand for this nuclear receptor gene [33]. Similar results were obtained with the estrogen receptor-α gene which was activated by TSA in a dose- and time-dependent manner [40]. Using microarray analysis, Suzuki *et al.* found different groups of silenced genes in colorectal carcinoma cells that were reactivated by 5-AZA-CdR or TSA alone [34]. In this regard, methylated genes may have two different chromatin configurations: one that can be activated by a HDAC inhibitor and the other that cannot be activated. In accord with this hypothesis, we observed that depsi (1 ng/ml) alone produced a significant activation of E-cadherin in MDA-MB-231 cells (Fig. 4A) and of TIMP3 in Hs578T cells (Fig. 3A). It is interesting to note that this activation of TIMP3 by depsi was of the same order of magnitude as the combination. It is possible that TIMP3 in Hs578T cells contains CpG islands that are not methylated and can be activated by a HDAC inhibitor, such as depsi, as described by Suzuki *et al.* [34].

We observed a remarkable synergistic activation of E-cadherin in the MDA-MB-435 cells where 5-AZA-CdR or depsi alone produced no detectable activation of this gene (Fig. 4B). A potent synergistic activation of TIMP3 was found in the MCF-7 cells but in this case, the control cells and the drug treated cells showed a similar weak expression of this gene (Fig. 3B). In accord with our findings, co-administration of 5-AZA-CdR and depsi was reported to produce greater expression of NY-ESO-1 and MAGE-3 cancer testis antigen than either agent alone in several tumor cell lines [41,42]. These findings support our hypothesis that combination of inhibitors of DNA methylation and histone deacetylation can produce an enhanced activation of specific genes silenced by epigenetic events. The molecular mechanisms involved in these interactions may be related to the removal of the methyl binding proteins from the demethylated promoters and the opening of the chromatin structure, which allows the accessibility of the transcription factors to initiate gene expression. Our results also indicated that the type of interaction between methylation and chromatin remodeling could show variations for different genes and different tumor cell lines.

We do not know if the enhanced activation of these three genes by 5-AZA-CdR and depsi was responsible for the synergistic antineoplastic action of this combination on the breast carcinoma cells. In addition to the genes that we have investigated in our study, it is possible that other tumor suppressor genes have to also be reactivated to produce an irreversible loss of proliferative potential by the tumor cells. Future investigations are required to fully understand these events.

In conclusion, our data suggest that the combination of DNA methylation and HDAC inhibitors is a very interesting form of epigenetic therapy that merits investigation for breast cancer.

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