Evaluation of antineoplastic action of 5-aza-2′-deoxycytidine (Dacogen) and docetaxel (Taxotere) on human breast, lung and prostate carcinoma cell lines

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The antineoplastic activity of 5-aza-2′-deoxycytidine (5-AZA-CdR) and docetaxel (Taxotere, Taxo) alone or in combination against human MDA-MB-231 breast, Calu-6 lung and DU-145 prostate carcinoma cell lines was evaluated by clonogenic assay. We also investigated by RT-PCR the capacity of these agents to re-activate the expression of E-cadherin and maspin, two tumor suppressor genes that were silenced by DNA methylation. 5-AZA-CdR and Taxo in combination produced a greater loss of clonogenicity than either agent alone. In MDA-MB-231 breast carcinoma cells, Taxo did not interfere with the re-activation of E-cadherin and maspin genes by 5-AZA-CdR. These results provide a rationale for clinical trials on the combination of 5-AZA-CdR and Taxo in patients with advanced cancer. *Anti-Cancer Drugs* 15:161–167 © 2004 Lippincott Williams & Wilkins.

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Introduction

The discovery that many genes that suppress tumorigenesis can be silenced by aberrant methylation of CpG islands in different types of tumors [1,2] has led to an increased interest in the chemotherapeutic potential of demethylating agents, such as 5-aza-2′-deoxycytidine (Dacogen, Decitabine, 5-AZA-CdR). 5-AZA-CdR has demonstrated the capacity to re-activate many of these silent tumor suppressor genes (TSGs) in human tumor cell lines [3]. In clinical trials, 5-AZA-CdR has produced remissions in patients with hematological malignancies [4–6] and partial responses in patients with lung cancer [7].

The future clinical use of 5-AZA-CdR in cancer therapy will most likely involve its use in combination with other antineoplastic agents to overcome the problem of drug resistance and to increase the efficacy of the treatment. Analysis of the mechanism of action of 5-AZA-CdR suggests that it should not be used in combination with genotoxic antineoplastic drugs, which have the potential to mutate the TSGs that are potential targets for re-activation by this cytosine analog.

Docetaxel (Taxotere, Taxo) is an interesting drug to consider for combination chemotherapy with 5-AZA-CdR since it shows significant preclinical activity against different types of tumors [8] and it is a non-mutagenic agent [9]. Taxo inhibits microtubule depolymerization and enhances microtubule polymerization, resulting in the arrest of cell cycle in G_2/M phase and induction of apoptosis [10]. Taxo also inhibits tumor cell invasion and motility [11].

The objective of this study was to investigate the *in vitro* antineoplastic activity of 5-AZA-CdR in combination with Taxo against human breast, lung and prostate carcinoma cell lines. We observed that the combination produced a greater antineoplastic effect than either agent alone. We also noticed in MDA-MB-231 breast carcinoma cells that the silent E-cadherin and maspin genes were re-activated by 5-AZA-CdR. Taxo did not interfere with gene re-activation by 5-AZA-CdR.

Material and methods

Material

5-AZA-CdR was procured from Pharmachemie (Haarlem, Netherlands). Taxo was obtained from Aventis (Collegeville, PA). The human MDA-MB-231 breast, Calu-6 lung and DU-145 prostate carcinoma cell lines were obtained from the ATCC (Manassas, VA). The cells were cultivated as monolayer, for MDA-MB-231 and Calu-6 in RPMI 1640 medium (Life Technologies, Burlington, Ontario, Canada) and for DU-145 in DMEM (Life Technologies) with 10% heat-inactivated fetal calf serum (Wisent, St-Bruno, Quebec) at 37°C with 5% CO_2 atmosphere.

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Inhibition of DNA synthesis assay

The inhibition of DNA synthesis by 5-AZA-CdR and/or Taxo was measured by the incorporation of radioactive thymidine into DNA. Aliquots of 10^4 cells in 2 ml of medium were placed in each well of a six-well 35-mm dish. The next day, the cells were exposed to the indicated concentrations of 5-AZA-CdR and/or Taxo simultaneously. Then, at 48 h, 0.5 μCi of [^{3}H]thymidine (6.7 Ci/mmol; ICN Biomedicals, Irvine, CA) was added to the medium for an additional 24 h. The cells were then trypsinized, suspended in 0.9% NaCl, placed on a GF/C 2-mm glass fiber filter disk, and washed with cold 0.9% NaCl, 5% trichloroacetic acid and ethanol. The filters were dried, placed in EcoLite scintillation fluid (ICN Biomedicals) and the radioactivity was measured with a scintillation counter.

Clonogenic assay

The loss of clonogenicity of MDA-MB-231, Calu-6 and DU-145 cell lines was assessed after drug exposure by placing 100–150 cells in each well of a six-well 35-mm dish. The next day, 5-AZA-CdR and Taxo were added simultaneously alone or in combination at indicated concentrations for 48 h. The cells were washed with drug-free medium, and were incubated for an additional 7–11 days and then stained with 0.5% methylene blue in 50% methanol. The colonies (more than 500 cells) were counted.

Isolation of RNA and RT-PCR analysis

In order to study the re-activation of maspin and E-cadherin genes in MDA-MB-231 breast carcinoma cell line, we treated cells with 5-AZA-CdR (100 ng/ml) and/or Taxo (0.5 ng/ml), simultaneously, for 48 h. Cells were harvested 24 h after the removal of the drugs. Total RNA was isolated at 24 h after drug removal using the RNeasy Mini-Kit (Qiagen, Mississauga, Ontario, Canada). For cDNA synthesis, total RNA was reverse-transcribed in 20 μl of reaction mixture using the OmniScript RT Kit (Qiagen). 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 spanning different exons for maspin, E-cadherin and 18S ribosomal RNA. For maspin (GenBank accession no. NM_002639), the primers were sense 5′-GCT TTT GCC GTT GAT CTG TTC-3′ and antisense 5′-GAT CTG ACC TTT CGT TTC TTC CA-3′. The length of the PCR product of maspin was 369 bp. For E-cadherin (GenBank accession no. NM_004360), the primers were sense 5′-CAA TCC CAC CAC GTA CAA G-3′ and antisense 5′-CTG GGC AGT GTA GGA TGT GA-3′. The length of the PCR product of E-cadherin was 410 bp. The human 18S ribosomal RNA gene (GenBank accession no. X03205) was amplified as an internal control using as sense primer 5′-TCG ATG GTA GTC GCC GTG CCT A-3′ and antisense 5′-CTG CTG CCT TCC TTG GAT GTG GTA-3′. The length of the PCR product of 18S ribosomal RNA was 110 bp. Samples were amplified in a thermocycler under the following conditions. For maspin, the PCR conditions were 15 min at 95°C to activate Taq polymerase, denaturing at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min for 5 cycles and then the annealing temperature was lowered at 57°C for 32 more cycles. For E-cadherin, the PCR conditions were 15 min at 95°C, 40 s at 94°C, 30 s at 58°C and 40 s at 72°C for 5 cycles. Then, the annealing temperature was lowered at 56°C for 31 more cycles. For 18S ribosomal RNA, the PCR conditions were 15 min at 95°C, 45 s at 94°C, 30 s at 60°C and 30 s at 72°C for 5 cycles. Then, the annealing temperature was lowered at 58°C for 11 more cycles. For each gene, the number of cycle was determined during the exponential phase of DNA amplification. The PCR products were electrophoresed on 2% agarose gel and detected by ethidium bromide staining. The measurement of the absolute concentration of amplified DNA was obtained with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) as described previously [12,13]. This latter method, which is very sensitive, uses capillary electrophoresis and fluorescent detection to measure both the size and quantity of DNA (ng/μl).

Data analysis

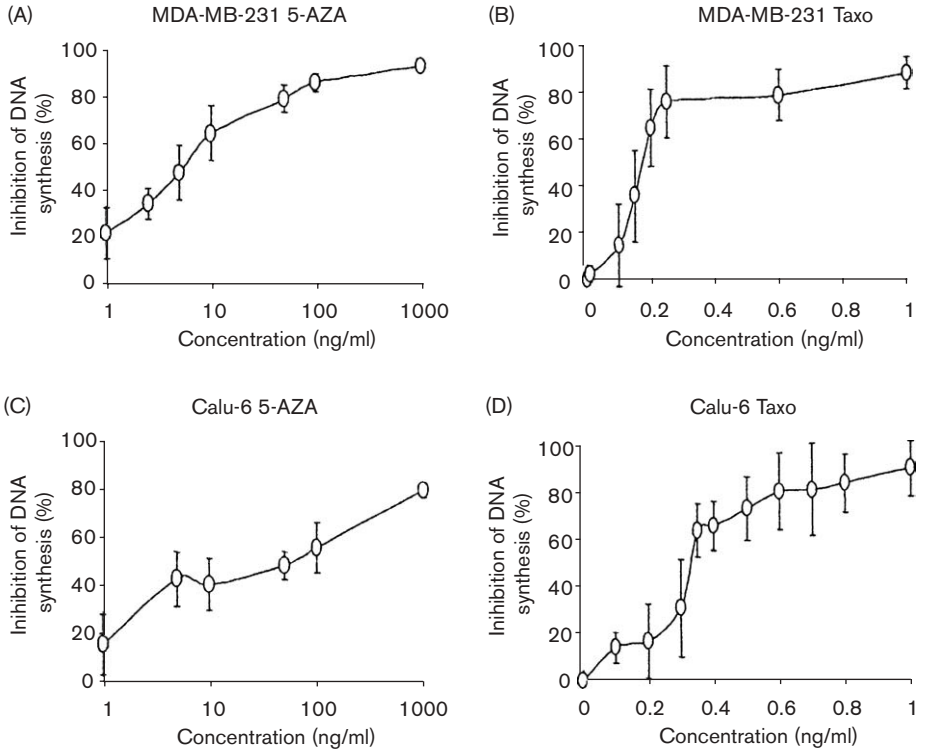
The data are the mean values ± SD for $n \geq 3$. Differences between groups were analyzed using one-way ANOVA test, by comparing the result of each drug alone with the results of the combination of both agents. The critical level of significance was set at $p \leq 0.05$.

Results

To assess the antineoplastic action of 5-AZA-CdR or Taxo, the effects of different concentrations of these agents on DNA synthesis in the MDA-MB-231 breast and Calu-6 lung carcinoma cell lines are shown in Figure 1. The concentration of 5-AZA-CdR that produced 50% of inhibition (IC_{50}) for 72 h exposure was in the range of 5 ng/ml for MDA-MB-231 cells and 50 ng/ml for Calu-6 cells (Fig. 1A and C). The IC_{50} values of Taxo were in the range of 0.2 ng/ml for MDA-MB-231 and 0.35 ng/ml for Calu-6 (Fig. 1B and D). The effect of 5-AZA-CdR in combination with Taxo on DNA synthesis was investigated for both cell lines (Fig. 2). The cells were exposed to concentrations in the range of IC_{50}. The combination of both drugs at these concentrations produced a greater inhibition of DNA synthesis than either agent alone.

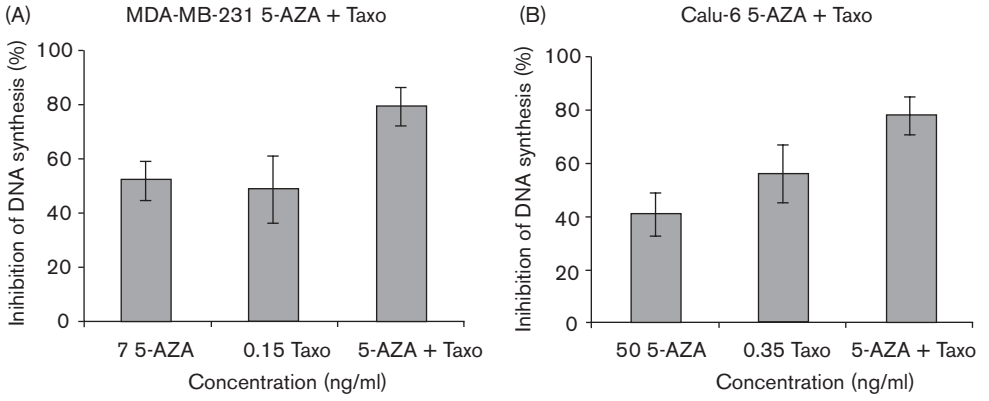
The effects of a 48-h exposure with different concentrations of 5-AZA-CdR and Taxo alone on colony formation in the cell lines MDA-MB-231, Calu-6 and DU-145 are shown in Figure 3. The IC_{50} values of 5-AZA-CdR for MDA-MB-231, Calu-6 and DU-145 were in range of 50 ng/ml (Fig. 3A, C and E). The IC_{50} values of Taxo were in

Fig. 1



Effect of different concentrations of 5-AZA-CdR (A and C) and Taxo (B and D) on inhibition of DNA synthesis on MDA-MB-231 and Calu-6 cell lines. The cells were incubated in the presence of the indicated concentration of drug for 72 h. Data shown are mean values ± SD, $n \geq 3$

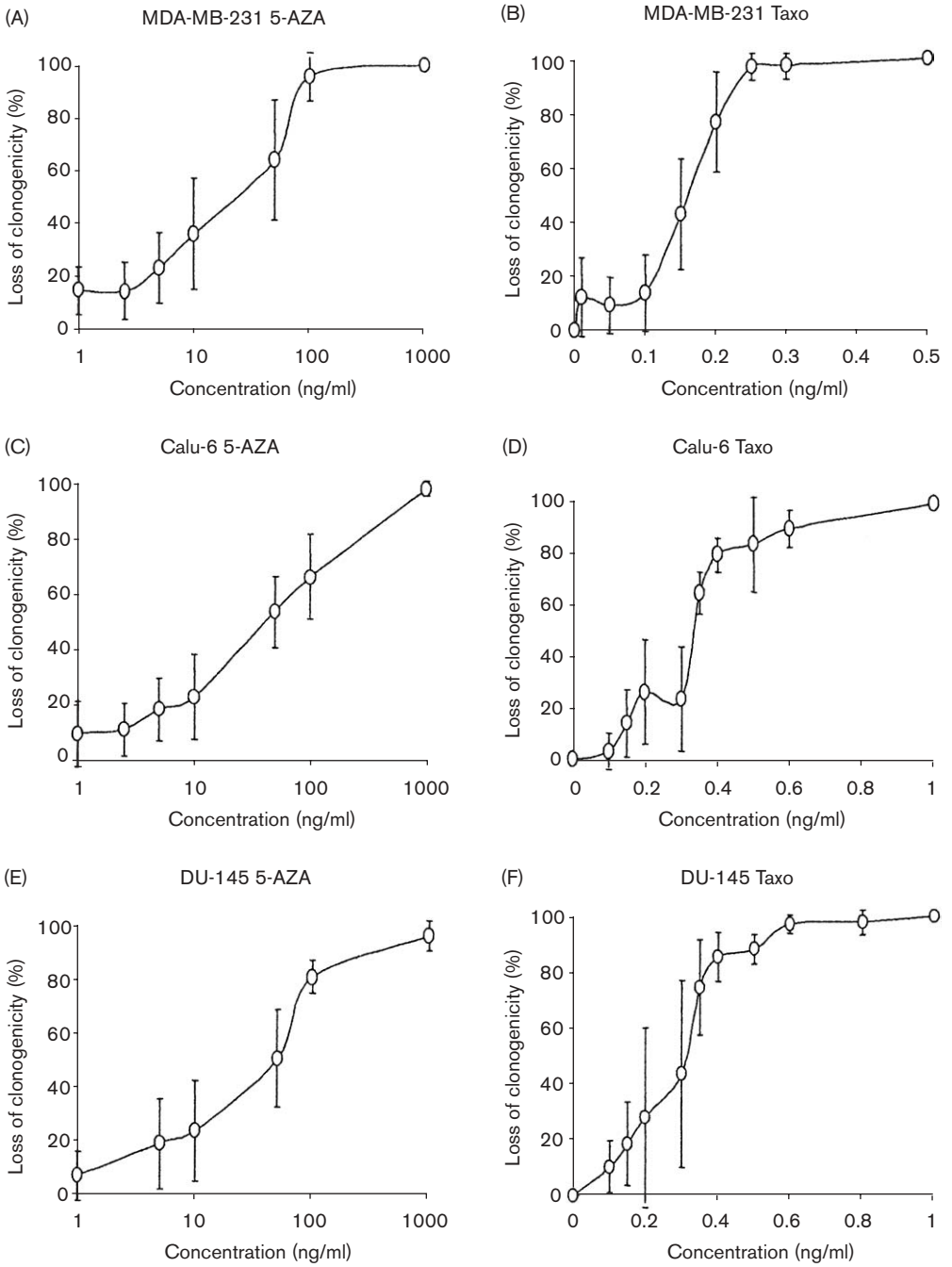
Fig. 2



Effect of 5-AZA-CdR and/or docetaxel on inhibition of DNA synthesis determined by incorporation of [^{3}H]thymidine of MDA-MB-231 (A) and Calu-6 (B) cell lines. The cells were incubated in the presence of the indicated concentration of drug for 48 h. Data shown are mean values ± SD, $n \geq 3$. Statistical analysis: $p < 0.001$.

range of 0.15 ng/ml for MDA-MB-231, 0.35 ng/ml for Calu-6 and 0.3 ng/ml for DU-145 (Fig. 3B, D and F). The effect of 5-AZA-CdR in combination with Taxo was investigated on these cell lines (Fig. 4). The cells were exposed for 48 h to concentrations in the range of IC_{50}. The combination of both drugs at these concentrations produced a greater loss of clonogenicity than either agent alone.

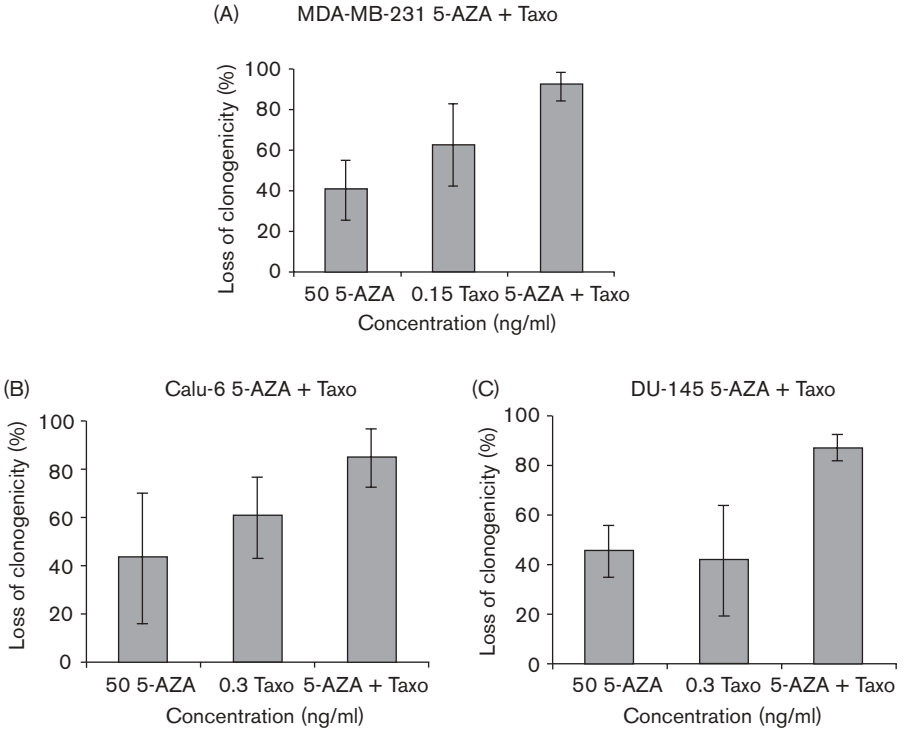
Fig. 3



Effect of different concentrations of 5-AZA-CdR (A, C and E) and Taxo (B, D and F) on loss of clonogenicity of MDA-MB-231, Calu-6 and DU-145 cell lines. The cells were incubated in the presence of the indicated concentration of drug for 48 h. Data shown are mean values ± SD, $n \geq 3$.

The antineoplastic activity of 5-AZA-CdR is related to its activation of TSGs silenced by aberrant methylation. E-cadherin and maspin are genes involved in the suppression of tumor metastasis [14,15]. Since these genes are silenced by epigenetic events in MDA-MB-231 breast carcinoma cell line, we investigated their activation by 5-AZA-CdR and/or Taxo, alone or in combination. The gene expression was evaluated by RT-PCR and the amount of DNA amplified after PCR quantitated by using the Agilent 2100 Bioanalyzer (Fig. 5). 5-AZA-CdR alone produced a significant re-activation of maspin. Taxo has no effect on the expression of this gene. The combination

Fig. 4



Effect of 5-AZA-CdR and/or docetaxel on loss of clonogenicity determined by colony assay of MDA-MB-231 (A), Calu-6 (B) and DU-145 (C) cell lines. The cells were incubated in the presence of the indicated concentration of drug for 48 h. Data shown are mean values ± SD, $n \geq 3$. Statistical analysis: (A) $p < 0.001$, (B) 5-AZA versus 5-AZA + Taxo: $p < 0.001$, Taxo versus 5-AZA + Taxo: $p < 0.01$, (C) $p < 0.001$.

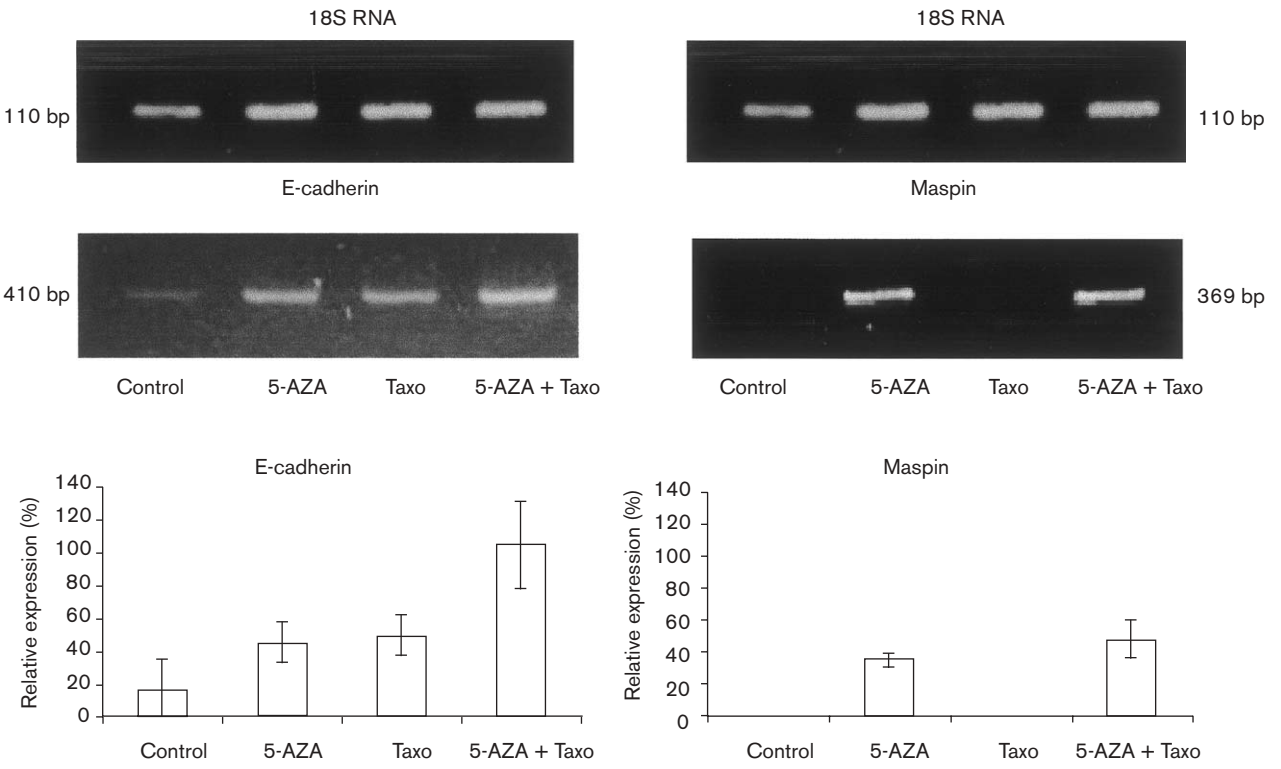
induced the same level of gene expression as 5-AZA-CdR alone. Therefore, Taxo did not interfere with 5-AZA-CdR gene-re-activation activity. The expression of E-cadherin was increased after treatment with 5-AZA-CdR and Taxo alone by 3-fold as compared to the basal expression level. The combination of these two agents produced over 7-fold increase in expression of E-cadherin.

Discussion

Most chemotherapeutic regimens for advanced metastatic breast, lung and prostate cancer produced few responses. There is urgent need to develop new approaches for the chemotherapy of these diseases. The inhibitor of DNA methylation, 5-AZA-CdR, is an interesting agent to investigate for tumor therapy. This cytosine analog has a novel mechanism of action, and shows promising antineoplastic activity in patients with hematological malignancies [4–6] and lung cancer [7]. Since the standard approach to tumor therapy is to use drug combinations to overcome the problem of drug resistance, it is of interest to find a complementary antineoplastic agent to use in combination with 5-AZA-CdR. Genotoxic antineoplastic drugs have the potential to inactivate genes by mutations. The ideal agent should not interfere with the gene re-activation produced by 5-AZA-CdR. Taxo is one of the most active antineoplastic drugs for advanced cancer [16–18]. In addition, Taxo is not a mutagenic drug [9]. For these reasons we chose to investigate the antineoplastic action of Taxo in combination with 5-AZA-CdR.

5-AZA-CdR or Taxo alone produced a significant inhibition of DNA replication for human MDA-MB-231 breast and Calu-6 lung carcinomas cells (Fig. 1). For both tumor cell lines, Taxo was a more potent inhibitor than 5-AZA-CdR. When these two agents were combined, they produced a greater inhibition of DNA synthesis than either agent alone (Fig. 2). Using a clonogenic assay, we also demonstrated that 5-AZA-CdR and Taxo alone are both potent cytotoxic agents. They produced a significant loss of clonogenicity for human MDA-MB-231 breast, Calu-6 lung and DU-145 prostate carcinoma cell lines (Fig. 3). The concentrations used in the clonogenic assay are in the range of the plasma level observed in clinical trials on 5-AZA-CdR [6,19] and Taxo [20]. Since the clonogenic assay measures the long-term proliferation potential of tumor cells after drug treatment, the data obtained may be more relevant to clinical therapy than the data on DNA synthesis. The combination of 5-AZA-CdR and Taxo showed a significant enhancement of loss

Fig. 5



Effect of 5-AZA-CdR (100 ng/ml), Taxo (0.5 ng/ml) and in combination after 48 h exposure on the induction of expression of E-cadherin and maspin gene in MDA-MB-231 breast carcinoma cells as determined by RT-PCR. The amplified cDNA was analyzed by electrophoresis on agarose (top) and quantitated by the Agilent Bioanalyzer 2100 (bottom). The cDNA of 18S ribosomal RNA was used as an internal standard. Data shown are mean values ± SD. Statistical analysis: for E-cadherin: $n=9$; control versus 5-AZA, control versus Taxo: $p<0.01$; 5-AZA versus 5-AZA + Taxo, Taxo versus 5-AZA + Taxo, control versus 5-AZA + Taxo: $p<0.001$. For maspin: $n=4$; control versus 5AZA, control versus 5-AZA + Taxo, Taxo versus 5-AZA + Taxo: $p<0.001$; 5-AZA versus 5-AZA + Taxo: $p<0.01$.

of clonogenicity as compared to either agent alone for the three tumor cell lines (Fig. 4). The data from both DNA synthesis and clonogenic assays support the hypothesis that Taxo does not interfere with the antineoplastic action produced by 5-AZA-CdR.

Since the chemotherapeutic action of 5-AZA-CdR is related to its re-activation of silent TSGs [3], our second objective was to determine if Taxo interferes with the activation of the E-cadherin and maspin genes by this cytosine analog. The expression of the tumor metastasis suppressor genes, E-cadherin and maspin, have been reported to be silenced by aberrant DNA methylation in different tumor cell lines, including MDA-MB-231 breast carcinoma cells [12,13,21,22]. We observed in this latter cell line that 5-AZA-CdR alone re-activated the expression of both E-cadherin and maspin (Fig. 5). Taxo alone also re-activated the expression of E-cadherin, but not maspin. It has been reported that Taxo can influence the expression of genes implicated in apoptosis, cell-cycle progression and angiogenesis [23]. The molecular mechanisms on how Taxo modulates gene expression have not been established. Taxo in combination with 5-AZA-CdR produced an enhanced expression of E-cadherin that was greater than either agent alone. This marked enhancement produced by the combination was not observed with maspin. Of major importance, Taxo did not interfere with the re-activation of both these genes by 5-AZA-CdR.

Conclusion

In conclusion, our data showed that 5-AZA-CdR in combination with Taxo produced a greater antineoplastic effect on human breast, lung and prostate carcinomas cells than either drug alone. Also, this taxoid did not interfere with re-activation by this cytosine analog of two genes that suppress tumorigenesis. Our data support the rationale to investigate the combination of 5-AZA-CdR and Taxo in patients with advanced breast, lung and prostate cancer.

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