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ORIGINAL ARTICLE

Annie Hurtubise · Richard L. Momparler

Effect of histone deacetylase inhibitor LAQ824 on antineoplastic action of 5-Aza-2′-deoxycytidine (decitabine) on human breast carcinoma cells

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Abstract *Purpose*: Epigenetic silencing of tumor suppressor genes (TSGs) by aberrant DNA methylation and chromatin deacetylation provides interesting targets for chemotherapeutic intervention by inhibitors of these events. 5-Aza-2′-deoxycytidine (decitabine, 5AZA-CdR) is a potent demethylating agent, which can reactivate TSGs silenced by aberrant DNA methylation. LAQ824 (LAQ) is a novel inhibitor of histone deacetylase (HDAC) that shows antineoplastic activity and can activate genes that produce cell cycle arrest. Both 5AZA-CdR and LAQ as single agents are currently under clinical investigation in patients with cancer. Previous reports indicate that the "cross-talk" between inhibitors of DNA methylation and HDAC can result in a synergistic activation of silent TSGs. These observations suggest that combination of these inhibitors may be an effective form of epigenetic therapy for breast cancer. The objective of our study was to determine if the combination of 5AZA-CdR and LAQ would show additive or synergistic antineoplastic activity on human MDA-MB-231 and MCF-7 breast carcinoma cells. The antineoplastic activity of these agents was evaluated by clonogenic assay and inhibition of DNA synthesis. *Results*: The combination produced greater antineoplastic activity for the MDA-MB-231 tumor cells than either agent alone. For the MCF-7 tumor cells, there were signs of antagonism between 5AZA-CdR and LAQ when administered simultaneously. When a sequential schedule (first 5AZA-CdR followed by LAQ) was used, there were no signs of antagonism of the antineoplastic action for the MCF-7 tumor cells. The mechanism of this interaction is probably due to the reduction of progression of MCF-7 tumor cells into S phase by LAQ. This would interfere with the antineoplastic action of 5AZA-CdR, since it is an S phase specific agent. *Conclusions*: These studies demonstrated the importance of the schedule of administration of 5AZA-CdR and LAQ and may have application for future clinical trials on the treatment of breast cancer with these agents.

Keywords 5-Aza-2′-deoxycytidine · LAQ824 · Histone deacetylase inhibitor · DNA methylation · Dose-schedule · Breast cancer

A. Hurtubise · R. L. Momparler (✉)
Département de pharmacologie, Centre de recherche pédiatrique, Service d'hématologie-oncologie,
Hôpital Sainte-Justine, Université de Montréal,
3175 Côte Sainte-Catherine, H3T 1C5 Montréal, QC, Canada
E-mail: richard.l.momparler@umontreal.ca

Introduction

Women with advanced metastatic breast cancer following treatment with conventional chemotherapy have a very limited survival. There is an urgent need to develop more effective treatment for this stage of disease. The discovery that many genes that suppress tumorigenesis can be silenced by aberrant methylation of CpG islands in tumors [3, 11, 15] has lead to an interest chemotherapeutic potential of demethylating agents, such as 5-Aza-2′-deoxycytidine (decitabine, 5AZA-CdR). 5AZA-CdR is a potent inhibitor of DNA methyltransferase with demonstrated capacity to reactivate silent tumor suppressor genes (TSGs) in human tumor cell lines [17]. In clinical trials, 5AZA-CdR has produced remissions in patients with hematological malignancies [10, 20, 21] and interesting responses in patients with lung cancer [16].

Some genes that inhibit cellular growth can be silenced by the deacetylation of histones resulting in a closed configuration of chromatin, which is unfavorable for transcription [1, 25]. Histone deacetylase (HDAC) inhibitors can reverse this process to produce an inhibition of tumor growth [7, 12]. LAQ824 (LAQ) is a potent HDAC inhibitor with antineoplastic activity and is currently in clinical trial in patients with cancer. Studies on human tumor and leukemic cell lines have shown that LAQ can inhibit cellular growth, induce apoptosis, produce cell cycle arrest and activate $p21^{WAF1}$ [2]. LAQ has

also shown significant activity in nude mice with different human tumor xenografts [19].

Our initial hypothesis was that the use of 5AZA-CdR and LAQ in combination would result in an enhancement of their antineoplastic activity. This assumption is based on the "cross-talked" between DNA methylation and histone modifications of chromatin that produces a marked suppression of transcription. This interaction can be reversed by the use of 5AZA-CdR in combination with HDAC inhibitors to produce a synergistic reactivation of TSGs [6]. Our objective was to investigate the antineoplastic action of 5AZA-CdR in combination with LAQ on MDA-MB-231 and MCF-7 breast carcinoma cell lines. Our laboratory had previously report synergistic antineoplastic action by the combination of 5AZA-CdR with the HDAC inhibitors trichostatin A or depsipeptide on breast carcinoma cell lines [5, 18].

In the present study, we observed a greater antineoplastic effect of 5AZA-CdR in combination with LAQ administered simultaneously on MDA-MB-231 breast carcinoma cells than either agent alone. However, for the MCF-7 breast carcinoma cells, we observed an antagonism between these two agents when use simultaneously, but not when used sequentially. These data illustrate the importance of the schedule of administration for these epigenetic agents.

Materials and methods

Material

The 5AZA-CdR was procured from Pharmachemie (Haarlem, Netherlands). LAQ was kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ, USA). The human MDA-MB-231 and MCF-7 breast carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultivated as monolayer in RPMI 1640 medium (Life Technologies, Burlington, ON, Canada) with 10% heat-inactivated fetal calf serum (Wisent, St-Bruno, QC, Canada) at 37°C with 5% CO_2 atmosphere.

Clonogenic assay

The loss of clonogenicity of MDA-MB-231 and MCF-7 cell lines was assessed after drug exposure by placing 100–250 cells in each well of a six-well 35 mm dish. The next day, 5AZA-CdR (1–100 ng/ml) and LAQ (1–100 ng/ml) were added simultaneously alone or in combination at indicated concentrations for 48 h. 5AZA-CdR was added at 0 and 24 h. The cells were washed with drug-free medium and were incubated for an additional 7–11 days and then stained with 0.5%

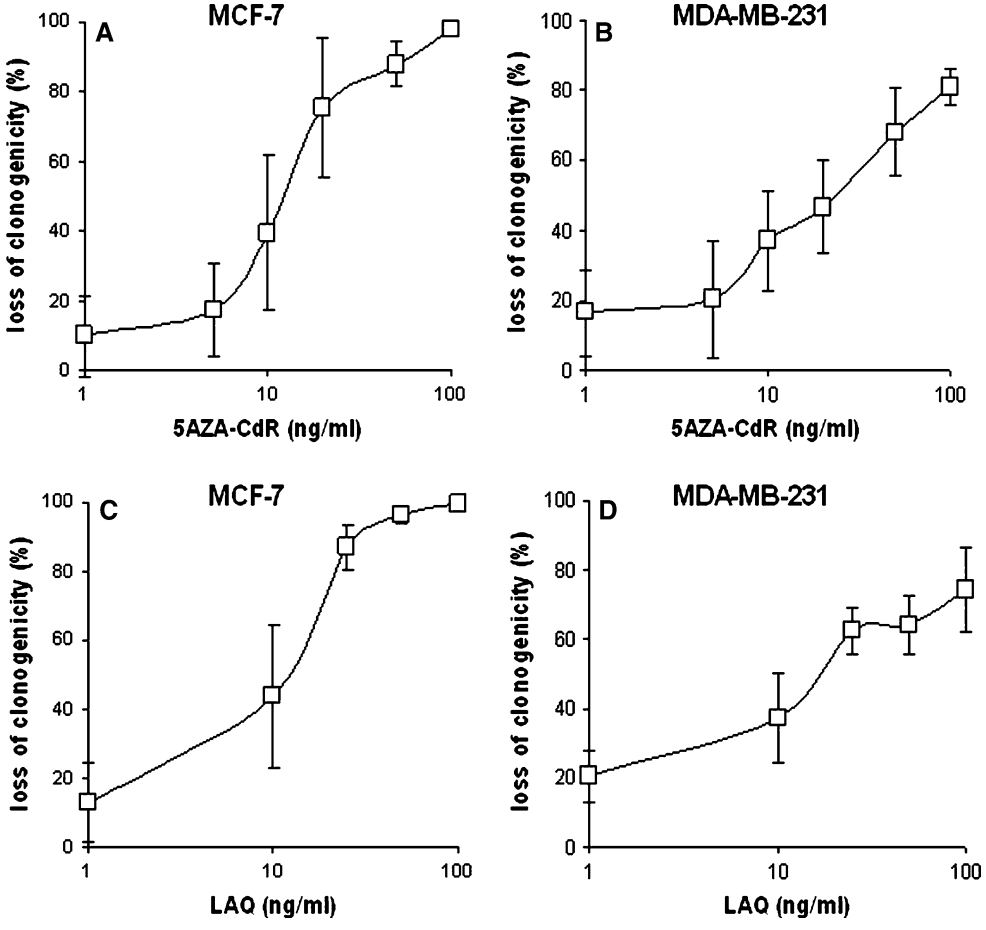

Fig. 1 Effect of different concentrations of 5AZA-CdR (**a, b**) and LAQ (**c, d**) on loss of clonogenicity on MCF-7 and MDA-MB-231 breast carcinoma cells. The cells were incubated in the presence of the indicated concentration of drug for 48 h. Data shown are mean values ± SD, $n \geq 3$

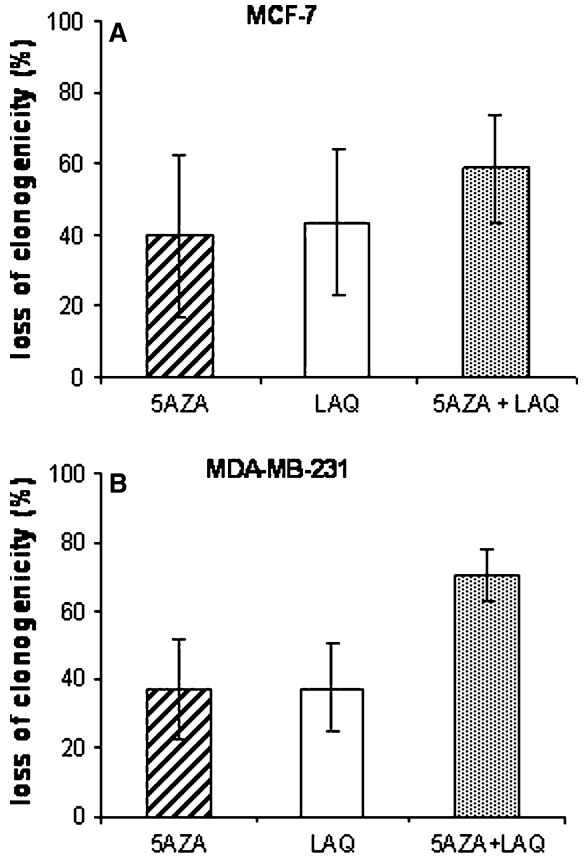

Fig. 2 Effect of simultaneous exposure of 48 h of 5AZA-CdR (5AZA) (10 ng/ml) and/or LAQ (10 ng/ml) on loss of clonogenicity of (**a**) MCF-7 and (**b**) MDA-MB-231 breast carcinoma cells. Data shown are mean values ± SD, $n \geq 3$. Statistical analysis: **a** 5AZA versus 5AZA+LAQ: ns; LAQ versus 5AZA+LAQ: ns. **b** 5AZA versus 5AZA+LAQ: $P < 0.01$; LAQ versus 5AZA+LAQ: $P < 0.01$

methylene blue in 50% methanol. The colonies (>500 cells) were counted. We also evaluated the possible antagonistic effects of 5AZA-CdR in combination with LAQ on MCF-7 and MDA-MB-231 cell lines. This was assessed by placing 25,000 cells in a 25 cm^2 flasks. 96 h later, for the simultaneous experiment: cells were treated with 5AZA-CdR (200 ng/ml) and/or LAQ (20 ng/ml) for 48 h treatment and for the sequential experiments: 5AZA-CdR (200 ng/ml) was had for 48 h, the flasks were then washed with drug free medium and LAQ (20 ng/ml) was added for another 24 h. 5AZA-CdR was added at 0 and 24 h. The cells were then trypsinised, counted and plated in a six-well 35 mm dish with 200 or 2,000 cells. The cells were incubated for an additional 7–11 days and then stained with 0.5% methylene blue in 50% methanol. The colonies (>500 cells) were counted.

Inhibition of DNA synthesis assay

The inhibition of DNA synthesis by 5AZA-CdR and/or LAQ 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 at the indicated concentrations of 5AZA-CdR (1–100 ng/ml) and/or LAQ (1–100 ng/ml) simultaneously. 5AZA-CdR was added at 0 and 24 h. Then, at 48 h, 0.5 μCi of radioactive tritium-labeled thymidine (6.7 Ci/mmol, ICN Biomedicals, Irvine, CA, USA) was added to the medium for an additional 24 h. The cells were then trypsinised, suspended in 0.9% NaCl, placed on a GF/C 25 mm glass fiber filter disc, 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.

Cell cycle analysis

The cell cycle analysis assay was performed on MCF-7 breast carcinoma cells by plating 1.5×10^6 cells in a 150 cm^2 flasks. The cells were treated with LAQ (100 ng/ml) for 48 h treatment. The cells were then trypsinised and wash three times with RPMI+10% FCS. Krishan buffer [0.1% sodium citrate, 0.02 mg/ml RNase A, 0.3% Igepal (Sigma) and 50 μg/ml propidium iodide, solution at pH 7.4] was added to 3×10^6 cells and incubated for 1 h at 4°C. After centrifugation for 10 m, the supernatant was removed and 1 ml of Krishan buffer added to the cell pellet. The cells were suspended and passed through Nitex 40 μ filter. The samples were then analyzed by flow cytometry using FACScan (Becton Dickinson).

Data analysis

The data are the mean values ± SD for $n \geq 3$. Differences between groups were analyzed using one-way ANOVA test coupled with a Tukey-Kramer 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

The antineoplastic action of different concentrations of 5AZA-CdR or LAQ as single agents was evaluated by a clonogenic assay on MCF-7 and MDA-MB-231 breast carcinoma cells as shown in Fig. 1. The concentration of 5AZA-CdR for 48 h exposure that produced 50% reduction in colony formation (IC_{50}) was in the range of 15 ng/ml for MCF-7 cells and 20 ng/ml for MDA-MB-231 cells (Fig. 1a, b). The IC_{50} values for 48 h exposure of LAQ were in the range of 15 ng/ml for MCF-7 and for MDA-MB-231 cells it was in the range of 20 ng/ml (Fig. 1c, d).

The effect of the combination of 5AZA-CdR with LAQ on colony formation of MCF-7 and MDA-MB-231 breast carcinoma cells is shown in Fig. 2. The tumor cells were exposed for 48 h at concentrations in the range of IC_{40} for each agent. For MDA-MB-231 cells, the

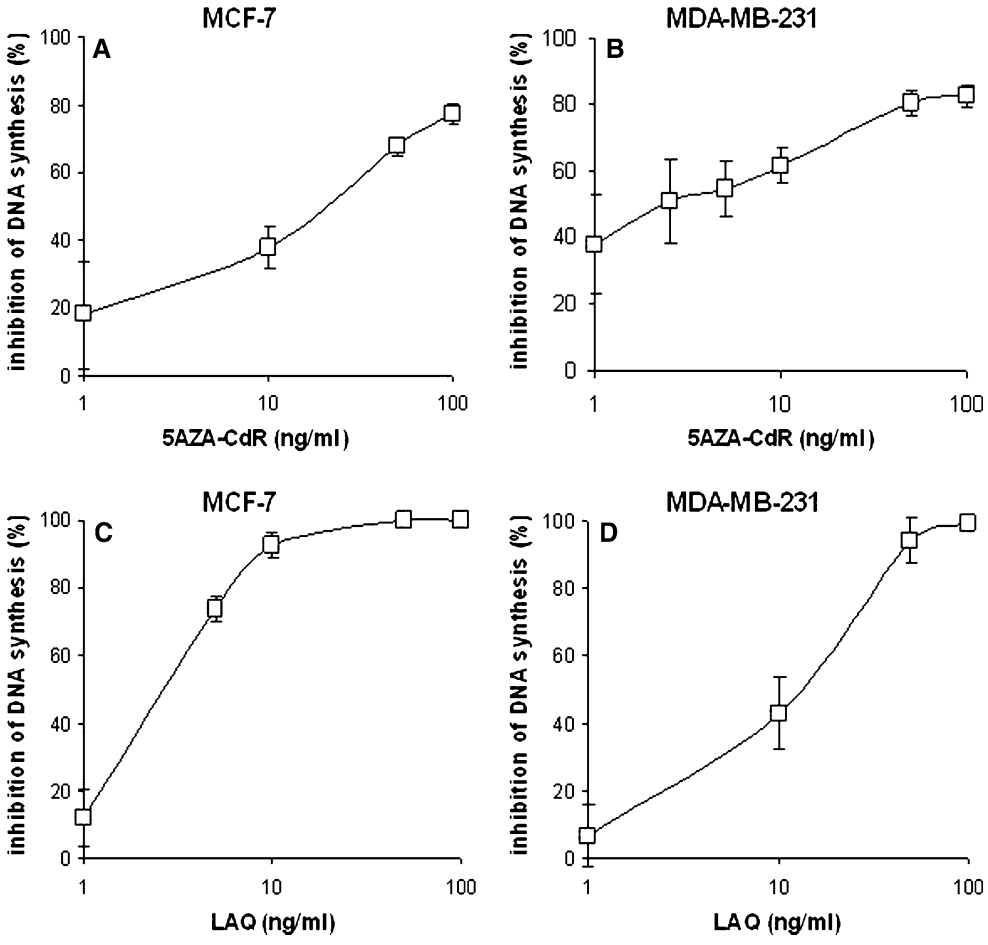

Fig. 3 Effect of different concentrations of 5AZA-CdR (**a, b**) and LAQ (**c, d**) on inhibition of DNA synthesis on MCF-7 and MDA-MB-231 breast carcinoma cells. The cells were incubated in the presence of the indicated concentration of drug for 72 h. Data shown are mean values ± SD, $n \geq 3$

combination of these drugs produced a significant greater loss of clonogenicity than either agent alone. However for MCF-7 cells, the difference between the loss of clonogenicity produced by the combination as compared to the drugs alone was not significant.

The effect of a 72 h exposure with different concentrations of 5AZA-CdR or LAQ alone on inhibition of DNA synthesis in MCF-7 and MDA-MB-231 breast carcinoma cells are shown in Fig. 3. The IC_{50} values of 5AZA-CdR were in the range of 25 ng/ml and 2.5 ng/ml for MCF-7 and MDA-MB-231 cells, respectively (Fig. 3a, b). The IC_{50} values of LAQ were in the range of 2 ng/ml for MCF-7 cells and 15 ng/ml for MDA-MB-231 cells (Fig. 3c, d). The effect of the combination of 5AZA-CdR with LAQ on DNA synthesis was also investigated on these tumor cell lines (Fig. 4). The cells were exposed for 72 h to concentrations, for MCF-7 in the range of IC_{40} for 5AZA-CdR and IC_{10} for LAQ; for MDA-MB-231 in the range of IC_{60} for 5AZA-CdR and IC_{40} for LAQ. The combination of these drugs at these concentrations produced a significant greater inhibition of DNA synthesis than either agent alone for the MDA-MB-231 cells, but not for the MCF-7 cells.

In order to investigate the interaction between these agents when used simultaneously, we used a concentration of 5AZA-CdR (200 ng/ml) that produced a >95% loss of clonogenicity for the breast carcinoma cell lines. We added a concentration of LAQ (20 ng/ml) that cause 15% of loss of clonogenicity. For the MCF-7 cells, we observed that the combination showed a 20% loss of antineoplastic activity as compared to 5AZA-CdR alone (Fig. 5a, c). For the MDA-MB-231 cells, there was no significant difference between the number of cells killed by 5AZA-CdR alone and the combination (Fig. 5d).

In order to evaluate the importance of the schedule of administration, we exposed the MCF-7 tumor cells to a sequential administration of 5AZA-CdR and LAQ. These cells were treated first with 5AZA-CdR for 48 h and after drug removal the cells were treated with LAQ for an additional 48 h (Fig. 5b, e). The antagonism of the antineoplastic activity of 5AZA-CdR by LAQ was eliminated by the sequential treatment of these agents on the MCF-7 cells. There was no significant difference between the number of cells killed by 5AZA-CdR alone and the combination.

It is possible that the antagonism produced by LAQ on MCF-7 tumor cells was due to its action on the cell cycle. In order to evaluate this, we analyzed the cell cycle effects of LAQ on MCF-7 cells by flow cytometry (Fig. 6). When we treated the MCF-7 cells with LAQ, we observed a significant reduction in the percentage of cells in S phase as compared to the control untreated cells.

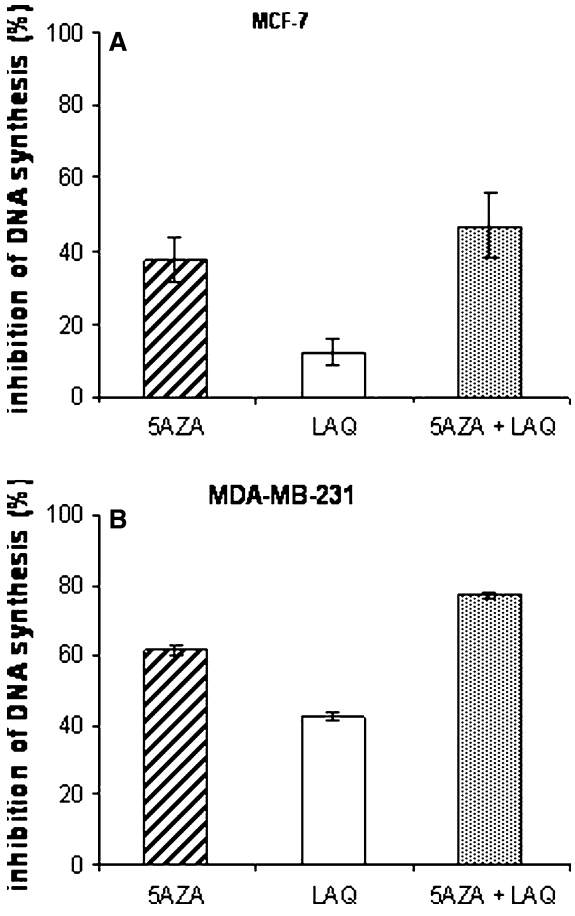

Fig. 4 Effect of simultaneous 72 h exposure 5AZA-CdR (5AZA) and/or LAQ on inhibition of DNA synthesis in breast carcinoma cells. In (**a**) MCF-7 cells were exposed to 10 ng/ml 5AZA and/or 1 ng/ml LAQ. In (**b**) MDA-MB-231 cells were exposed to 10 ng/ml 5AZA and/or 10 ng/ml LAQ. Data shown are mean values ± SD, $n \geq 3$. Statistical analysis: **a** 5AZA versus 5AZA+LAQ: ns; LAQ versus 5AZA+LAQ: $P < 0.001$. **b** 5AZA versus 5AZA+LAQ: $P < 0.05$; LAQ versus 5AZA+LAQ: $P < 0.001$

Under the experimental conditions used in this report, we did not detect apoptosis after treating both tumor cell lines with 5AZA-CdR and or LAQ using a sub-G1 assay (data not shown).

Discussion

The silencing of genes that regulate tumor growth by aberrant DNA methylation and histone modifications in chromatin provides interesting targets for chemotherapeutic intervention [9]. There exists a "cross-talk" between these two epigenetic events that silence the expression of genes that suppress tumorigenesis. The molecular mechanism of this interaction is the result of the attachment of methylated CpG binding proteins (MBDs) to methylated promoter region of the target gene, which prevents transcriptions factors to initiate transcription. In addition, MBDs by the recruitment of HDAC, the open chromatin is converted to a compact configuration that is unfavorable for gene expression. The importance of this "cross-talk" is illustrated by studies on both leukemic and tumor cell lines in which 5AZA-CdR in combination with different HDAC inhibitors was shown to produce a synergistic activation of silent tumor genes [5, 6, 18]. For some tumor cell lines, the in vitro combination of these epigenetic agents also produced an additive or synergistic antineoplastic action [5, 18].

These results have lead to the initiation of clinical trials on patients with hematological malignancies and tumors with combinations of inhibitors of DNA methylation and different HDAC inhibitors [13, 22–24, 26]. The objective of our study was to compare the antineoplastic action of two different schedules of administration of 5AZA-CdR in combination with the potent HDAC inhibitor, LAQ on MDA-MB-231 and MCF-7 human breast carcinoma cell lines.

For MDA-MB-231 tumor cells, we observed greater antineoplastic activity by using combination than either agent alone (Figs. 2, 4). However, this was not the case for the MCF-7 cells. For this latter tumor cell line, we observed that there was an antagonistic interaction between these agents when used simultaneously (Fig 5). When we used a concentration of 5AZA-CdR that produced >95% loss of clonogenicity for the MCF-7 tumor cells, the simultaneous addition of LAQ resulted in a significant loss of antineoplastic activity for the combination. The sequential administration of 5AZA-CdR and LAQ removed the antagonistic activity of this HDAC inhibitor.

As an explanation for this antagonistic effect between these two drugs, we propose that LAQ blocked the progression of MCF-7 tumor cells into the S phase. Since 5AZA-CdR is an S phase specific agent [14], this cell cycle arrest produce by LAQ can interfere with the antineoplastic action of this inhibitor of DNA methylation. This blocking action of cell cycle progression by LAQ was demonstrated by the reduction of the fraction of cells in S phase for the MCF-7 tumor cells following treatment with this HDAC inhibitor (Fig. 6). Other investigators have reported that LAQ produces cell cycle arrest on specific tumor cell lines [2]. We observed that LAQ did not produce a significant block in cell cycle progression for the MDA-MB-231 tumor cells (data not shown), which is the explanation for lack of antagonism between 5AZA-CdR and LAQ during a simultaneous exposure. An important question to ask is our in vitro data on these epigenetic agents translatable to an animal model? In this regard it is interesting to note the report that the sequential treatment with 5AZA-CdR followed by the HDAC inhibitor, phenylbutyrate, prevented carcinogen-induced lung cancer in mice [4].

Translation of our preclinical data into a clinical trial should be done with caution. Continuous exposure of tumor cells in vitro to 5AZA-CdR translates more readily to a continuous infusion of this analog in patients. Co-administration of a HDAC inhibitor that produces

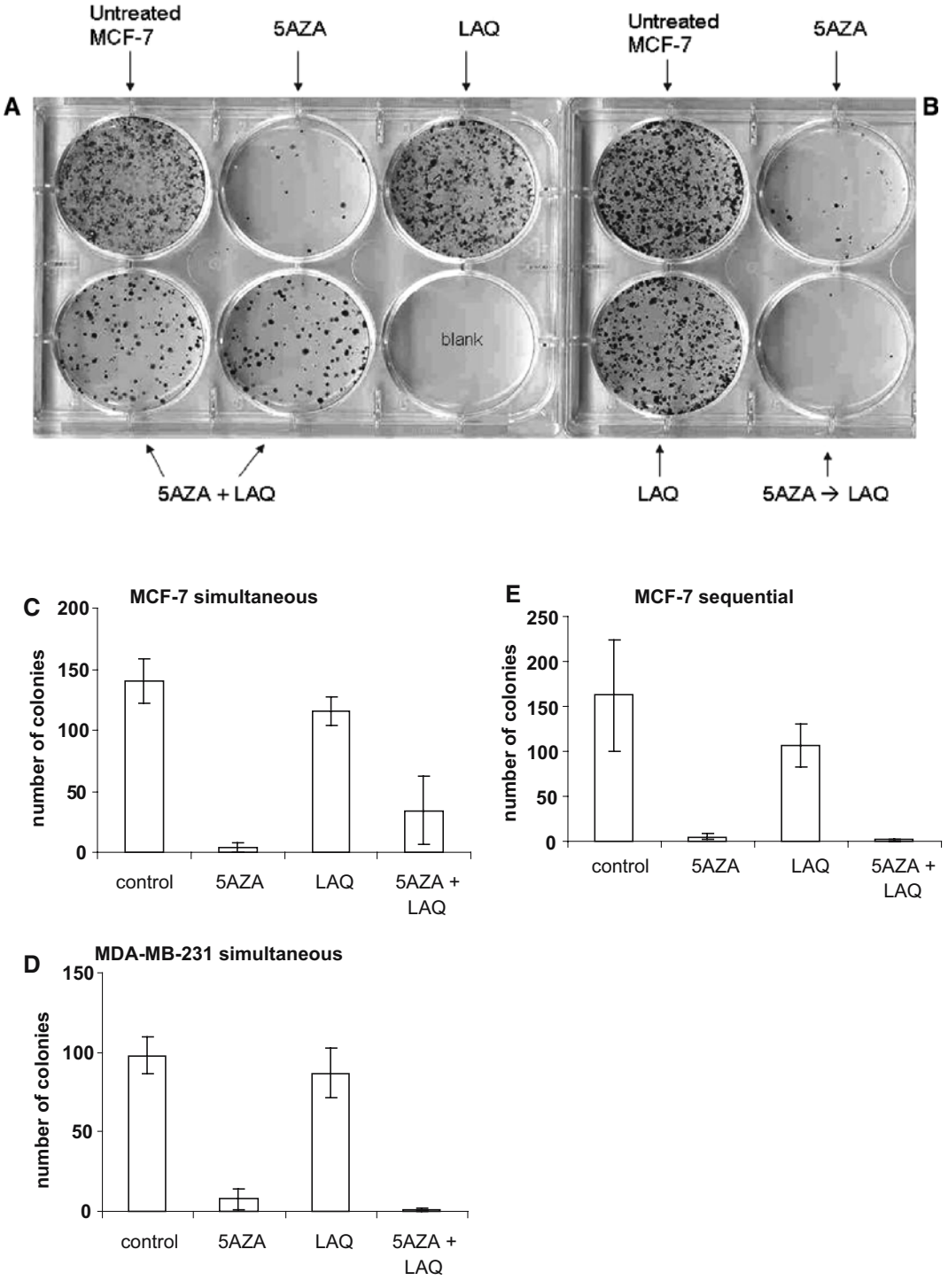

Fig. 5 Effect of simultaneous 48 h exposure to 200 ng/ml of 5AZA-CdR (5AZA) and/or 20 ng/ml of LAQ on loss of clonogenicity of MCF-7 (**a**, **c**) and MDA-231 (**d**) breast carcinoma cells or sequential exposure (**b**, **e**) on colony formation by MCF-7 breast carcinoma cells. The cells were plated at 1,000 cells per 35 mm well. Data shown are mean values ± SD, $n \geq 3$. Statistical analysis: **c** LAQ versus 5AZA+LAQ: $P < 0.001$; 5AZA versus 5AZA+LAQ: $P < 0.01$. **d** LAQ versus 5AZA+LAQ: $P < 0.001$; 5AZA versus 5AZA+LAQ: ns. **e** LAQ versus 5AZA+LAQ: $P < 0.001$; 5AZA versus 5AZA+LAQ: ns

cell cycle arrest in the target tumors during the infusion of 5AZA-CdR has the potential to antagonize the antineoplastic action of this nucleoside analogue. If this type of dose-schedule is chosen for clinical investigation, it may be advantageous, if possible, to perform cell cycle analysis on the tumor cells during the treatment with the HDAC inhibitor. If an intermittent pulse dose-schedule is selected for these epigenetic agents, the pharmacodynamics becomes more complex and it is possible that little or no antagonism will occur between these agents.

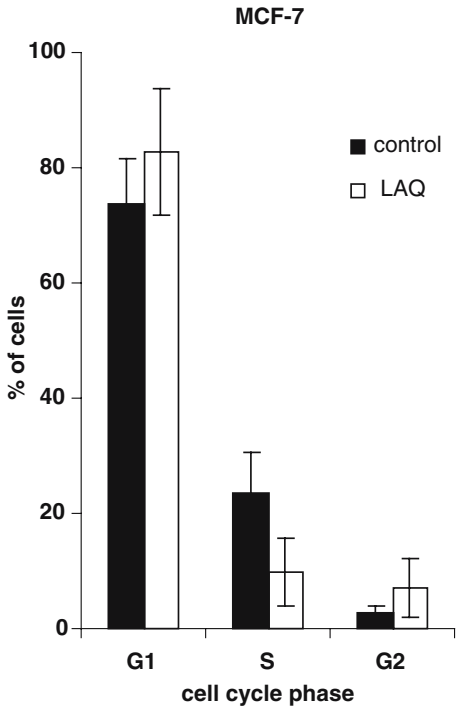

Fig. 6 Effect of LAQ on cell cycle determined by flow cytometry on MCF-7 breast carcinoma cell line. The cells were incubated in the presence of LAQ (100 ng/ml) for 48 h. Data shown are mean values ± SD, $n \geq 3$. Statistical analysis: control versus LAQ: S-phase $P < 0.05$

However, this should be verified by experimentation in preclinical models.

In conclusion, our results illustrate the importance of finding the optimal schedule for epigenetic agents and may have application in the design of clinical trials to test their efficacy for the treatment of patients with cancer [9].

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