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Preclinical report

Antineoplastic action of 5-aza-2′-deoxycytidine and phenylbutyrate on human lung carcinoma cells

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Current chemotherapy of advanced non-small cell lung cancer produces only a modest increase in survival time. New approaches are needed to improve its effectiveness. During tumorigenesis, silencing of tumor suppressor genes can occur by aberrant methylation. The DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-AZA-CdR), can reactivate the expression of these genes. Nucleosomes containing unacetylated positively charged histones bind tightly to DNA producing a compact configuration, which inhibits transcription. Phenylbutyrate (PB), an inhibitor of histone deacetylase (HDAC), increases histone acetylation, neutralizing its positive charge and resulting in DNA with a more open structure, which favors transcription. It has been reported that 5-AZA-CdR in combination with HDAC inhibitor can increase the expression of silent tumor suppressor genes. The objective of our study was to determine if these agents, in combination, produce an enhancement of their antitumor activity. We evaluated the antineoplastic activity of 5-AZA-CdR and PB alone or in combination on human A549 and Calu-6 lung carcinoma cell lines by inhibition of DNA synthesis and clonogenic assays. 5-AZA-CdR and PB in combination produced a greater inhibition of DNA synthesis than either agent alone. Also, in a clonogenic assay the combination of these drugs showed a significant synergistic antitumor effect. These results provide a rationale to investigate the combination of 5-AZA-CdR and PB in patients with advanced lung cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: 5-Aza-2′-deoxycytidine, antitumor effect, DNA methylation, histone deacetylation, phenylbutyrate.

Introduction

Although recent developments in the chemotherapy of patients with metastatic non-small cell lung cancer have increased their response rate, the prolongation of their survival is still very limited.[1] There is an urgent need to develop more effective chemotherapeutic regimens for this disease. Recent progress in the understanding of the molecular events that occur during tumorigenesis can be used to design novel therapies for lung cancer. The inactivation of tumor suppressor genes is an important event in tumorigenesis. These genes can be inactivated by mutations, deletions and, as demonstrated more recently, epigenetic events, such as aberrant methylation of DNA[2–4] and changes in chromatin structure.[5]

The aberrant methylation of CpG islands in the promoter region is an epigenetic mechanism involved in the silencing of many tumor suppressor genes.[2–4] This aberrant methylation can be reversed by 5-aza-2′-deoxycytidine (5-AZA-CdR), a potent inhibitor of DNA methylation. This inhibition produced by 5-AZA-CdR results in the reactivation of tumor silenced suppressor genes and other cancer-related genes.[3,6–8] 5-AZA-CdR was reported to be an active agent in patients with leukemia.[9] In a pilot clinical trial, 5-AZA-CdR also showed interesting activity in patients with advanced lung cancer,[10] suggesting that this analog should be investigated further for its potential in the therapy of this disease.

Acetylation of histones is another epigenetic event, which affects the transcription of genes by modification of nucleosome structure. The process is mediated by histone acetylases (HAT) and histone deacetylases (HDAC). The chromatin is transcriptionally active when the histones are acetylated, whereas when the histones are deacetylated, the chromatin is more compact and transcriptionally inactive. Inhibitors of HDAC have been shown to produce gene reactivation and induction of differentiation of neoplastic cells.[5] HDAC inhibitors also have been demonstrated to inhibit tumor growth *in vitro* and in mouse models, indicating that they have potential as chemotherapeutic agents for cancer.[11]

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Phenylbutyrate (PB) is a HDAC inhibitor that recently showed promising antineoplastic activity in a patient with acute promyelocytic leukemia that was resistant to retinoic acid.[12] Since PB shows significant *in vitro* antineoplastic activity against human prostate and neuroblastoma cell lines,[13,14] it may be an interesting agent to test against human lung carcinoma cell lines.

Several observations indicate that acetylation and methylation act jointly to regulate gene expression.[15–18] For example, in tumor cells 5-AZA-CdR, in combination with the HDAC inhibitor, trichostatin A (TSA), produced a synergistic activation of the expression of different tumor suppressor genes.[18,19] These studies provided us with a rationale to investigate the antineoplastic activity of 5-AZA-CdR and PB on lung carcinoma cells. In this report, we show that 5-AZA-CdR and PB in combination have an enhanced antineoplastic effect on the human lung carcinoma cell lines, A549 and Calu-6.

Materials and Methods

Cell lines

Calu-6 (human lung adenocarcinoma cells) and A549 (human lung carcinoma cells) were obtained from the ATCC (Manassas, VA). The cells were cultivated at

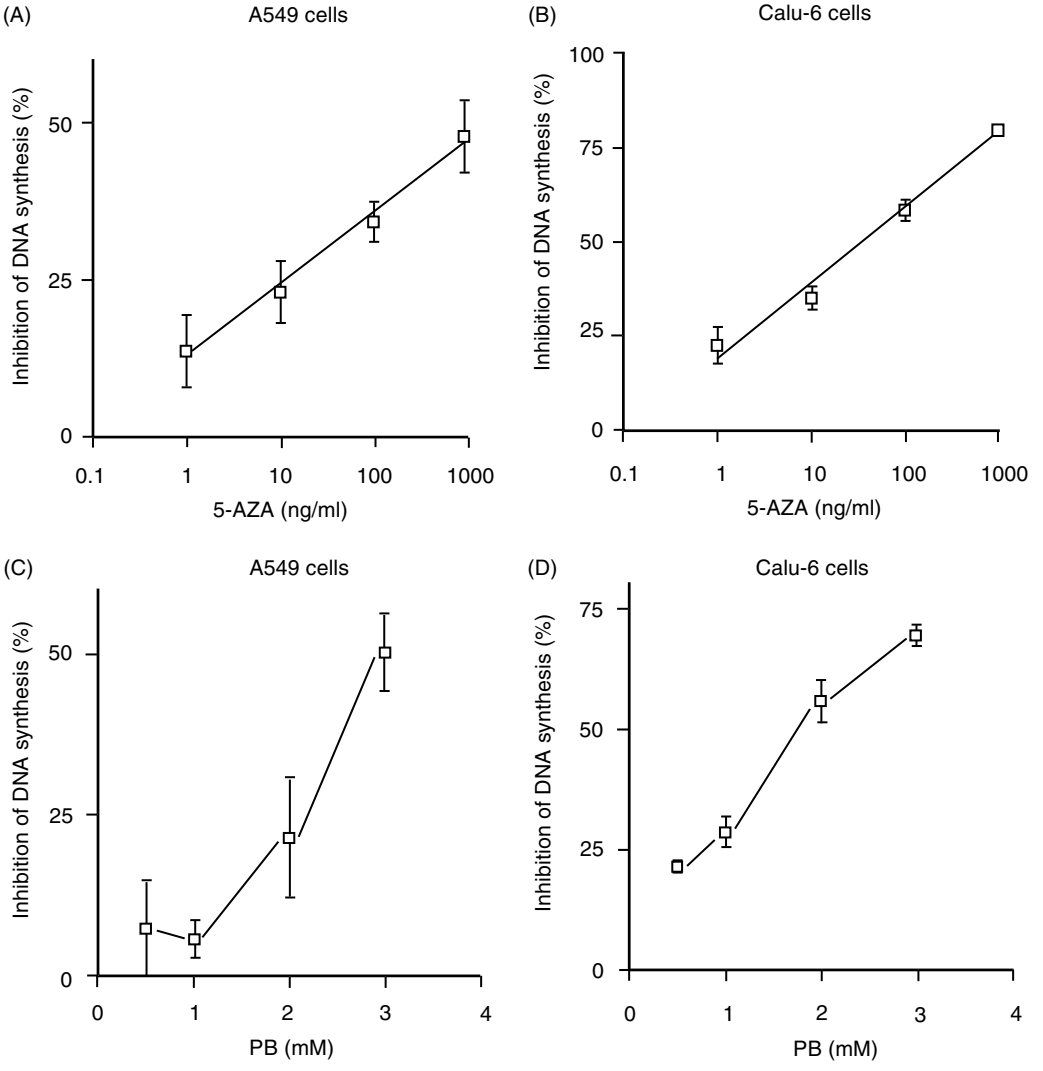

Figure 1. Effect of different concentrations of 5-AZA-CdR (A and B) and PB (C and D) on inhibition of DNA synthesis on A549 (left) and on Calu-6 cells (right). The cells were incubated in the presence of the indicated concentration of drug for 72 h. The values are mean ± SD ($n=4$) relative to control cells without treatment.

37°C with 5% CO_2 as monolayers in RPMI 1640 medium (Life Technologies, Burlington, Ontario, Canada) with 10% heat-inactivated fetal calf serum (Wisent, St Bruno, Quebec, Canada).

Inhibition of DNA synthesis assay

The inhibition of DNA synthesis by the A549 and Calu-6 cells by drugs was determined by the incorporation of radioactive thymidine into DNA. Aliquots of 10 000 cells in 2 ml of medium were placed in each well of a six-well 3-mm dish and then exposed to 5-AZA-CdR (Pharmachemie, Haarlem, Netherlands) and/or PB (Fluka, Oakville, Ontario) at the indicated concentrations. Then, 0.5 μCi ^{3}H-labeled thymidine (0.25 μCi/μl) was added to the medium for an additional 24 h. The cells were then trypsinized, suspended in a 0.9% NaCl, placed on a GF/C 25-mm glass fiber filter disk, and washed with cold 5% trichloroacetic acid and ethanol. The disks were dried, placed in scintillation fluid and assayed for radioactivity.

Clonogenic assay

The clonogenic potential of the cell lines A549 and Calu-6 was assessed after drug exposure by placing 100 cells in each well of a six-well 35-mm dish. The following day, the drugs 5-AZA-CdR and PB were added alone or in combination at the indicated concentrations for 72 h. The cells were washed with drug-free medium, and were incubated for 11–12 days and stained with 0.5% methylene blue in 50% methanol. The colonies (more than 500 cells) were counted.

Data analysis

We determined the significance of the results with its p value using the one-way ANOVA method, which compared the results of each drug alone with the results of the combination. To determine whether the interaction of the drugs was additive, antagonistic or synergic, in the clonogenic assays we compared the survival fraction of the combination of drugs to the product of the survival fraction of the two drugs added alone.[20,21] If the product of survival fraction of each drug alone is superior to that of the survival fraction of the combination, the drug interaction is synergistic. If the product of the survival fraction of each drug alone is equal to that of the survival fraction of the combination, the interaction is additive. If the product of the survival fraction of each drug alone is less than the combination, the interaction is antagonistic.

Results

The effects of different concentrations of 5-AZA-CdR or PB alone on DNA synthesis in the cell lines A549 and Calu-6 are shown in Figure 1. For a 72-h exposure, the concentration that produced 50% of

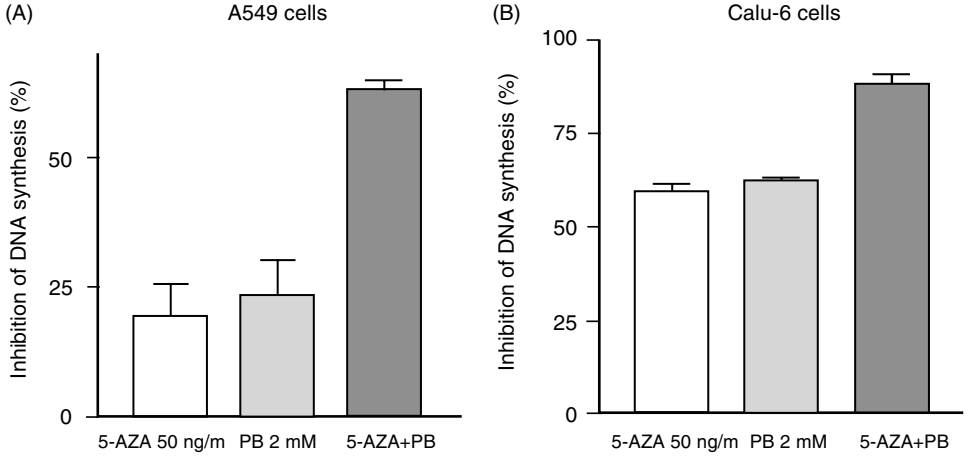

Figure 2. Effect of 5-AZA-CdR and/or PB on inhibition of DNA synthesis in A549 cells (A) and Calu-6 cells (B). The cells were incubated in the presence of the indicated concentration of drugs for 72 h. The values are mean ± SD (n=3) relative to control cells without treatment. Statistical analysis: PB versus 5-AZA-CdR + PB, $p < 0.001$ and 5-AZA-CdR versus 5-AZA-CdR + PB, $p < 0.001$.

inhibition (IC_{50}) for the A549 cells was in the range of 100 ng/ml (Figure 1A) and in the range of 20 ng/ml for the Calu-6 cells (Figure 1B). For PB, the IC_{50} was in the range of 3 mM for the A549 cells (Figure 1C) and in the range of 2 mM for the Calu-6 cells (Figure 1D).

The effects of 5-AZA-CdR or PB alone and in combination on DNA synthesis in the cell lines A549 and Calu-6 are shown in Figure 2. The combination produced a significant greater inhibition of DNA synthesis than either agent alone ($p < 0.001$).

The effects of different concentrations of 5-AZA-CdR or PB alone on colony formation for the cell lines A549 and Calu-6 are illustrated in Figure 3. For a 72-h exposure, the IC_{50} for 5-AZA-CdR was in the range of 50 ng/ml for the A549 cells (Figure 3A) and in the range of 100 ng/ml for the Calu-6 cells (Figure 3B). For PB, the IC_{50} was in the range of 3–4 mM for the A549 cells (Figure 3C) and in the range of 4 mM for the Calu-6 cells (Figure 3D).

The effects of 5-AZA-CdR or PB alone and in combination on the loss of clonogenicity for the cell lines A549 and Calu-6 are shown in Figure 4. For the two cell lines, the combination produced a significantly greater loss of clonogenicity than either agent alone ($p < 0.001$). The interaction between 5-AZA-CdR and PB as defined by Valeriote and Lin[20,21] was clearly synergistic.

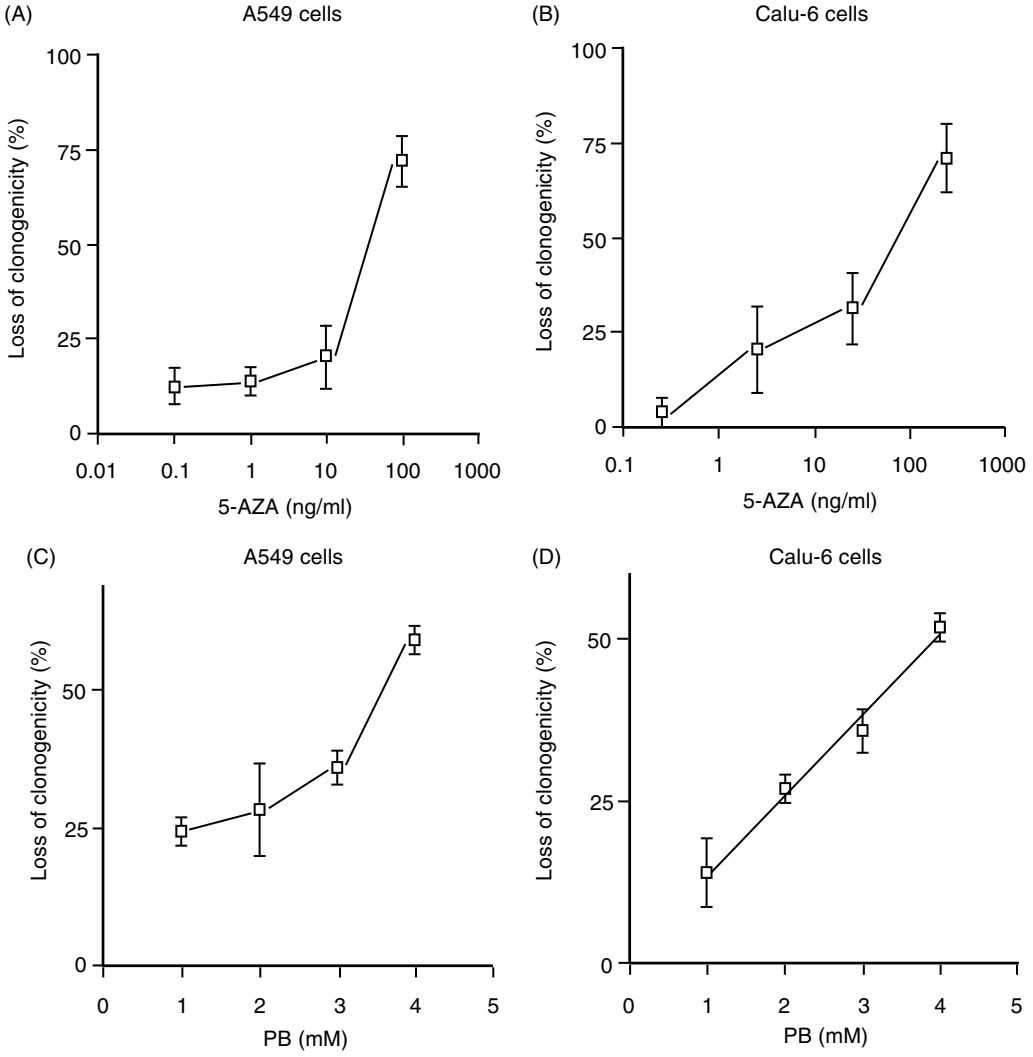

Figure 3. Effect of different concentrations of 5-AZA-CdR (A and B) and PB (C and D) on loss of clonogenicity of A549 cells (left) and Calu-6 cells (right). The cells were incubated in the presence of the indicated concentration of drug for 72 h. The values are mean ± SD ($n = 3$) relative to control cells without treatment.

Discussion

Current chemotherapy of advanced lung cancer is not very effective. New approaches should be sought to treat this disease. The combination of an inhibitor of DNA methylation with an inhibitor of HDAC merits investigation from the recent reports on the interesting interaction between these classes of agents. 5-AZA-CdR, in combination with the HDAC inhibitor TSA, produces a synergistic activation of the p16 tumor suppressor gene in tumor cells.[18] We reported previously that this drug combination also produces a synergistic antineoplastic effect on breast carcinoma cells.[19] In this report we investigated the antitumor activity of 5-AZA-CdR and the HDAC inhibitor, PB, alone and in combination on lung carcinoma cells.

The inhibition of DNA synthesis assay used in our study measured the ability of the tumor cells to replicate DNA after a 72 h exposure to antineoplastic drugs, 5-AZA-CdR and PB. Each drug alone showed the ability to inhibit DNA replication (Figure 1). PB was less potent than 5-AZA-CdR with respect to its inhibition of DNA synthesis. The combination of these agents produced significantly a greater inhibition of DNA synthesis than either agent alone (Figure 2) indicating a positive drug interaction between these classes of agents.

5-AZA-CdR alone showed potent activity to induce a loss in clonogenicity for A549 and Calu-6 lung carcinoma cells (Figure 3A and B). It should be noted that the concentrations used in the clonogenic assay are in the range of the plasma levels of 5-AZA-CdR in clinical studies.[9,10] PB was much less potent to induce a loss of clonogenicity for the lung carcinoma cells. It required concentrations in the millimolar range as compared to 5-AZA-CdR which was in the micromolar range. Pharmacokinetic studies on PB in phase I clinical trials showed that it is possible to obtain plasma concentration of this agent in the millimolar range.[11] It is interesting to note that PB, when administered in combination with retinoic acid, produced a complete remission in a patient with acute promyelocytic leukemia that was resistant to retinoid therapy.[12]

The combination of 5-AZA-CdR and PB produced a synergistic loss of clonogenicity for both the A549 and Calu-6 lung carcinoma cell lines (Figure 4). The clonogenic assay measures the long-term proliferative potential of the tumor cells after drug treatment and the data obtained has the potential to be translated in clinical trials. However, investigations in animal models with human lung tumor xenografts should be performed to confirm the synergistic activity of this combination *in vivo* and to evaluate its host toxicity.

The probable molecular mechanism responsible for the interaction between 5-AZA-CdR and PB is the activation of silent tumor suppressor genes, which induced the lung carcinoma cells to undergo irreversible terminal differentiation and lose their proliferative potential. More experimental work is required to fully understand these events. A tumor suppressor gene that is a potential target for this type of therapy is the retinoic acid receptor (RAR)-β. We have demonstrated previously that 5-AZA-CdR, in combination with the HDAC inhibitor TSA, activated RAR-β in breast carcinoma cells.[19] RAR-β was

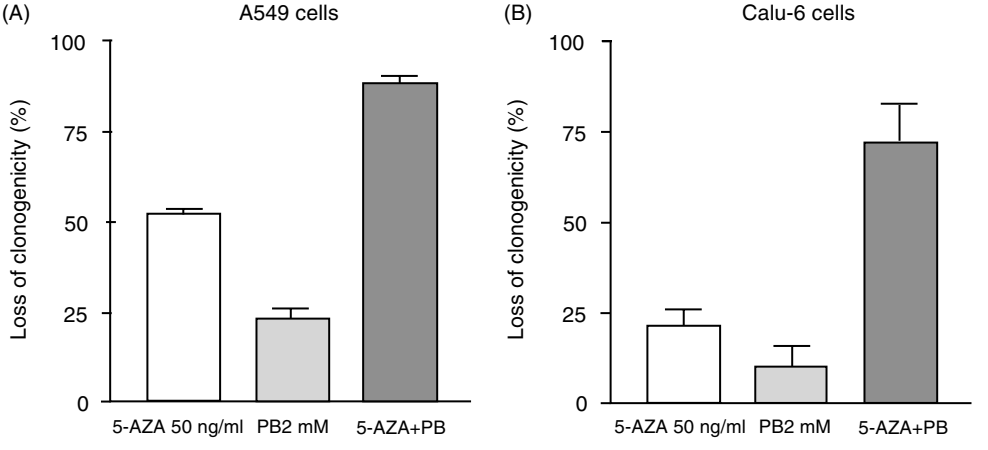

Figure 4. Effect of 5-AZA-CdR and/or PB on loss of clonogenicity of A549 cells (A) and Calu-6 cells (B). The cells were incubated in the presence of the indicated concentration of drug for 72 h. The values are mean $\pm$ SD ($n=4$) relative to control cells without treatment. Statistical analysis: 5-AZA-CdR versus 5-AZA-CdR + PB, $p<0.001$ and PB versus 5-AZA-CdR + PB, $p<0.001$.

reported to be silenced frequently by aberrant methylation in primary lung cancer.[22] It is probable that other tumor suppressor and cancer-related genes that are silenced by epigenetic events will be identified in lung cancer, and may also be targets for a combination of 5-AZA-CdR and a HDAC inhibitor.

In conclusion, our data indicate that 5-AZA-CdR in combination with the HDAC inhibitor, PB, produce a synergistic antineoplastic effect on human lung carcinoma cells. Since both these agents are available for clinical investigation, it may be a very interesting drug combination to study for the clinical treatment of advanced lung cancer. The DNA methylation inhibitor, 5-AZA-CdR, has already shown some potential for the therapy of lung cancer in patients with advanced disease.[10,23]

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