Enhanced Growth Inhibition and Differentiation of Fluorodeoxyuridine-treated Human Colon Carcinoma Cells by Phenylbutyrate

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ABSTRACT

The effect of phenylbutyrate (PB), a nontoxic differentiation inducer, in human colon carcinoma cell lines treated with 5-fluorodeoxyuridine (FUDR) was evaluated. Two HT-29 human colon carcinoma subclones (U4 well differentiated and U9 poorly differentiated) were equally growth inhibited by 16 h of FUDR (0.2 μM) treatment but recovered cell growth in 3–6 days after the removal of FUDR. PB as a single agent had minimal effect on cell growth, but after FUDR treatment, PB inhibited cell growth for 12 days. The inhibition of cell growth in FUDR-treated cells by PB was more sustained in U4 than U9 cells and was associated with an increased and sustained expression of p21<sup>WAF1</sup> protein, secretion of transforming growth factor β1, mediators of p53-dependent or -independent G<sub>1</sub> cell cycle arrest, and an increase in the alkaline phosphatase activity as well, considered a marker of differentiation in colon carcinoma cells. These effects of PB were seen only in FUDR-pretreated cells because PB alone had minimal effect on the expression of these genes. The sequential use of FUDR followed by PB in patients with colon carcinoma should be explored because two subclones of HT29, irrespective of their state of differentiation, respond to this clinically achievable regimen.

INTRODUCTION

Advanced colorectal cancer is refractory to the vast majority of chemotherapeutic agents that have been tested (1). The fluoropyrimidines, in particular 5-fluorouracil, are the most widely used drugs for patients with metastatic disease, although responses of only 20% are generally achieved (2). The use of fluoropyrimidines in various schedules or in combination with other antiproliferative agents, such as leucovorin to enhance binding to the target enzyme (3, 4), appears to have improved therapeutic response and may improve survival (5). IFN-α has also been used to potentiate fluoropyrimidine antitumor activity with controversial clinical results in colon cancer (6). Several explanations have been offered for <i>de novo</i> resistance to fluoropyrimidines, including mutant p53 and overexpression of cyclin D1, which diminish G<sub>1</sub> cell cycle arrest and apoptosis in response to DNA-damaging agents (7). Moreover, colon carcinoma cells acquire drug resistance by multiple mechanisms that diminish fluoropyrimidine incorporation into DNA or RNA (8), allowing for emergence of resistant cells during the rest periods between fluoropyrimidine treatments. Therefore, nontoxic agents used between cycles of fluoropyrimidines to prevent tumor cell recovery may enhance tumor cell reduction and as a consequence also diminish emergence of drug resistance.

Several investigators have studied the effects of differentiation agents in human colon carcinoma cell lines (9–11). These studies demonstrate that differentiation agents, i.e., HMBA, DMSO, and sodium butyrate, could induce morphological, antigenic, and functional changes in many human colon carcinoma cell lines including loss of anchorage-independent cell growth in vitro and decreased in vivo tumorigenicity in the nude mouse model (11). NaPA and PB, which proved to be three to four times as potent as NaPA and tributyrin (12), have been identified as new nontoxic differentiation inducers in several cell malignant lines including leukemia, breast, prostate, and brain (13, 14). These agents are pleiotropic and enhance the activity of other differentiation inducers, such as retinoids (15), and hormonal antagonists, such as tamoxifen in breast cancer cells (16). The combination of PB and retinoids synergizes differentiation and the transcription of p21<sup>WAF1</sup>, a cyclin-dependent kinase inhibitor, which induces p53-independent G<sub>1</sub> cell cycle arrest (15). NaPA and PB are in clinical trials as single agents, demonstrate tumor cell (glioblastoma) growth inhibition, and appear to be nontoxic on normal proliferating cells.

The combination of fluoropyrimidines with nontoxic agents, such as leucovorin to enhance binding to the target enzyme (3, 4), appears to have improved therapeutic response and may improve survival (5). IFN-α has also been used to potentiate fluoropyrimidine antitumor activity with controversial clinical results in colon cancer (6). Several explanations have been offered for <i>de novo</i> resistance to fluoropyrimidines, including mutant p53 and overexpression of cyclin D1, which diminish G<sub>1</sub> cell cycle arrest and apoptosis in response to DNA-damaging agents (7). Moreover, colon carcinoma cells acquire drug resistance by multiple mechanisms that diminish fluoropyrimidine incorporation into DNA or RNA (8), allowing for emergence of resistant cells during the rest periods between fluoropyrimidine treatments. Therefore, nontoxic agents used between cycles of fluoropyrimidines to prevent tumor cell recovery may enhance tumor cell reduction and as a consequence also diminish emergence of drug resistance.

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The abbreviations used are: HMBA, hexamethylene bisacetamide; NaPA, sodium phenylacetate; PB, phenylbutyrate; FUDR, fluorodeoxyuridine; ALP, alkaline phosphatase; TGF, transforming growth factor.
Pretreatment with fluoropyrimidines arrests mouse erythroleukemia cells in G1-S, and when followed by differentiation agents, cells are committed to terminal cell division with a decrease in repair mechanisms such as DNA ligase activity (22) and salvage pathways (17), resulting in apoptosis (18, 23). This synergism is p53 independent, associated with increased G1 cell cycle arrest, and synergism does not occur in normal bone marrow cells as measured by colony assay (17). Moreover, the synergism is schedule dependent, requiring fluoropyrimidines prior to the differentiation inducers (18).

To design an effective combination cytotoxic-differentiation therapy, we treated U4 (well differentiated) and U9 (poorly differentiated) subclones of the HT29 human colon carcinoma cell line with FUdR followed by PB. We show here that PB used at clinically achievable nontoxic doses in the two subclones substantially increases the efficacy of FUdR by inducing differentiation and decreasing clonogenicity.

Parameters to demonstrate G1 cell cycle arrest and differentiation induction may identify molecular mechanisms for this combination therapy. ALP activity is a biochemical marker for differentiation in HT29 cells (10, 24), and p21wafl and TGFβ1 are mediators involved in cell growth arrest and differentiation (15, 25–28). In this study, it was found that ALP activity is induced only by sequential treatment of FUdR and PB, and expression of p21wafl and secretion of TGFβ1 were enhanced by combination therapy in both U4 and U9 cells.

**MATERIALS AND METHODS**

Subclones U4 and U9 of HT29 human colon carcinoma cells were generous gifts from Dr. Eileen Friedman of Memorial Sloan-Kettering Cancer Center (New York, NY). U4 can be grown to confluence (25), and U9 cells are set up in a T25 flask containing 5 ml of complete medium [MEM (Gibco, Grand Island, NY)] supplemented with 30 mM HEPES (pH 7.4), 40 μg/ml of gentamicin, 1.2 mg/ml of sodium bicarbonate, 10 μM leucovorin, used to enhance the anti-DNA effect of FUdR (3, 4), and 7% heat-inactivated fetal bovine serum, and placed in a humidified 5% CO₂/air incubator at 37°C for 1 day. FUdR (0.2 μM) was added for 16 h; then cells were washed with complete medium once and replaced with fresh medium with and without PB. At indicated times, attached cells were counted with a Coulter counter.

Soft agar assay was performed as described (29). Plates containing 3 ml of 1% SeaPlaque agarose underlayer in complete medium were prepared in 60-mm Corning Petri dishes with 2-mm grids. One thousand cells (>90% trypan blue negative) were mixed with 2 ml of 0.4% agarose in complete medium, placed over 1% agarose, and incubated at 37°C. Colonies >0.1 mm (~50 cells) were counted after 13 days of growth by microscope. The cloning efficiency of U4 cells without any drug treatment was 24% ± 3%, and U9 cells was 30% ± 3%.

**Measure of ALP Activity.** Harvested cells were lysed in a buffer containing 25 mM Tris-HCl (pH 8), 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP40, 50 μg/ml of aprotinin, 1 mM PMSF, and 50 μM leupeptin at 4°C for 10 min. Lysates were centrifuged, the amount of protein in the supernatants was measured by Bradford’s method (30). Lysates were stored in liquid nitrogen. Measure of ALP activity was performed at 30°C according to the manufacturer’s instruction.

**Analysis of p21wafl and TGFβ1 Protein.** The amount of p21wafl was determined by Western blot analysis (31). At indicated times, cells were lysed as described above. One hundred or 50 μg of protein of each lysate was mixed with one-half volume of 3X protein sample buffer containing 15% β-mercaptoethanol and heated at 95°C for 2 min before loading onto a 10% SDS polyacrylamide gel. Protein transfer was carried out by a GENIE (Idea Scientific Co., Minneapolis, MN). p21wafl was detected by wafl monoclonal antibody, and TGFβ1 was detected by anti-TGFβ1 neutralizing antibody as primary antibody, followed by the ECL method as described in Amersham’s instruction. Intensities of protein bands were measured by a LKB enhanced laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Samples for TGFβ1 analysis were prepared as described (32). Cells harvested at the indicated times were washed twice with serum-free medium and incubated in 5 ml of serum-free medium at 37°C overnight. This conditioned medium was collected and centrifuged at 10,000 rpm for 20 min. The supernatant was concentrated by Micron and Centricron concentrators (Amicon, Beverly, MA) to ~25 μl/10⁶ cells. One-half volume of 3X protein sample buffer without reducing agents (30% glycerol, 9% SDS, and 0.188 mM Tris-HCl, pH 6.8) was added to each sample and heated at 95°C for 2 min before loading onto a 10% SDS gel. Protein transfer was carried out by GENIE, and TGFβ1 neutralizing antibody was followed by the ECL method as described by the manufacturer’s instructions (Amersham). Intensities of the protein bands were measured by a LKB enhanced laser densitometer (Pharmacia LKB Biotechnology).

**RESULTS**

**Effect of PB on the Cytotoxic Effect of FUdR.** FUdR (0.2 μM) treatment produced 50% growth inhibition of U4 and U9 cells 24 h after treatment. The inhibition of growth by FUdR persisted for 3 days in drug-free medium, but thereafter the rate of cell growth recovered (Fig. 1) and was similar to untreated cells. In contrast, cell growth was inhibited 90% in U4 and 77% in U9 when FUdR treatment was followed by PB (1 mM) for up...
to 12 days. PB (1 mM) as a single agent inhibited cell growth by no more than 25% during 12 days of exposure (data not shown).

Prevention of recovery from FUdR treatment by PB is dramatically demonstrated by the clonogenic assay (Fig. 2). In these experiments, U4 or U9 cells were treated with FUdR for 16 h, washed and placed in drug-free medium or medium containing PB (1 mM) for the indicated times (Fig. 2). Thereafter, one thousand adherent cells were placed into soft agar as
Fig. 3  Induction of ALP activity. Cells were treated with FUdR then incubated in the presence (■) or absence (□) of PB. ALP activity was assayed as described in "Materials and Methods." One hundred or 50 μg of protein of each sample were used. Absorbance at 595 nm was measured at 30°C at 0, 10, and 20 min. Data are from at least three experiments, and other data are averages of two experiments; bars, SD. A, U4 cells; B, U9 cells. ALP activity for U4 cells treated with PB only for 3 days was 2.0 ± 0.4 and for U9 cells, 2.2 ± 0.5.

Fig. 4  Commitment assay. U4 cells were treated with FUdR (0.2 μM) and followed by incubation with (■) or without (□) 1 mM PB for 3 days. Cells were harvested and reseeded at 1 × 10⁶ cells/flask and placed in fresh drug-free medium for 3 and 5 days. Cells were counted (A) and collected for measure of ALP activity (B) as described in "Materials and Methods."

described in “Materials and Methods,” and colonies were counted after 13 days. The clonogenicity of U4 and U9 cells treated with FUdR and placed in drug-free medium was reduced for 3 days but longer incubation in drug-free medium resulted in recovery of clonogenicity. In contrast, if FUdR-treated U4 and U9 cells were maintained in the presence of 1 mM PB, the clonogenicity of the cells remained reduced, even after 12 days of exposure, and this effect was more pronounced in U4 (Fig.
4A. U4 cells placed in drug-free medium remained significantly growth suppressed after combination FUdR+PB treatment followed by PB treatment and trypsinized, and 0.5 X 10^6 viable cells were treated with FUdR for 16 h, washed, and placed in fresh drug-free medium for the period of time noted in Fig. 3A. Thereafter the cells were exposed to PB. The peak activity in U4 cells treated with PB and followed by FUdR (data not shown).

The duration of the induction of ALP activity was longer in U4 cells than in U9 cells when treated by the combination. FUdR or PB as single agents did not induce ALP activity in either U4 or U9 cells (data not shown).

The Effect of Sequential Treatment with FUdR and PB on Commitment to Terminal Differentiation. A modified liquid commitment assay was used to determine whether FUdR cells were committed to differentiation and terminal cell division when released from PB treatment. In these studies, cells were treated with FUdR for 16 h, washed, and placed in fresh medium with or without PB for 3 days. Thereafter the cells were washed and trypsinized, and 0.5 x 10^6 viable cells were placed in fresh drug-free medium for the period of time noted in Fig. 4A. U4 cells placed in drug-free medium remained significantly growth suppressed after combination FUdR/PB treatment compared with FUdR treatment alone. Commitment to differentiation in drug-free medium was evident as measured by persistent elevation of ALP activity in FUdR/PB-treated cells but not FUdR alone (Fig. 4B). In contrast, U9 cells were less committed to terminal cell division because FUdR/PB treatment caused only 40% greater growth inhibition and 2-fold induction of ALP activity compared with FUdR alone after 3 days of incubation in drug-free medium. Enhanced commitment to differentiation and terminal cell division was not observed when cells were first treated with PB and followed by FUdR (data not shown).

p21^{waf1} and TGFβ1 Is Induced in FUdR-treated Cells followed by PB. Both U4 and U9 cells constitutively express mutant p53 (26). FUdR treatment in a p53-independent manner increases the level of p21^{waf1}, a protein involved in G1 cell cycle arrest (Fig. 5). In agreement with the experimental results described above in FUdR-treated U4 cells exposed to PB for 3 days, the level of p21^{waf1} protein correlated well with the inhibition of cell growth; it remained elevated when the FUdR-treated cells were exposed to PB and diminished as FUdR cells recovered from growth inhibition in the absence of PB. Similar observations were obtained in U9 cells (data not shown).

TGFβ secretion in the medium was three times higher from FUdR-treated U4 cells than from control cells, and addi-
tional increases in TGFβ₁ secretion was observed by the sequentially treated cells (Fig. 6). The highest level of TGFβ₁ protein was found in medium from FUdR-treated cells 72 h after PB treatment (FUdR, 0.279 ± 0.134 versus FUdR + PB, 0.718 ± 0.16; P < 0.05) and correlated with the maximal induction of p21<sup>wafl</sup> and induction of ALP activity.

**DISCUSSION**

Fluoropyrimidines today represent the most effective and most widely used chemotherapeutic agents against colon carcinoma, although the response is limited and short lived (2). We describe here experimental conditions that increase the efficacy of FUdR on colon carcinoma cell lines. Because studies demonstrated that in leukemia cell lines the combination of chemotherapeutic agents with differentiation inducers are more effective in inducing differentiation, terminal cell division, and apoptosis (17–19), we treated colon carcinoma cell lines with FUdR followed by PB. To assess the effect of these compounds on colon carcinoma cells in different stages of differentiation, we used two subclones of the HT29 line, one of which is well differentiated (U4) and one which is poorly differentiated (U9; Ref. 25). They also differ in their response to the differentiation inducer HMBA, which causes antiproliferation, differentiation, and TGFβ₁ induction in U4 but fails to inhibit growth despite induction of TGFβ₁ in U9 cells (33).

Initially, we found that independently of the state of differentiation the two colon carcinoma cell lines react similarly to sequential treatment of FUdR and PB. A 16-h treatment with 0.2 µM FUdR had a pronounced antiproliferative effect on both cell lines. The effect was, however, reversible, and at 3–6 days after the removal of FUdR, both cell lines multiplied with approximately the same rate as untreated cells. In contrast, the growth inhibition persisted for 12 days if after the removal of FUdR the cells were grown in the presence of 1 mM PB, whereas the latter alone caused minimal growth inhibition.

In agreement with these data, PB substantially reduced the clonogenicity of FUdR-treated U4 cells, but interestingly, it proved to be somewhat less effective in the less differentiated U9 cells. This is consistent with the observation that U4 and U9 cells have different responses to the differentiation inducer HMBA (25).

p21<sup>wafl</sup> and TGFβ₁, two molecular indices of G<sub>1</sub> cell cycle arrest, are also modulated by the combined treatment of FUdR and PB. In both cell lines, the sequential treatment caused a more pronounced expression of these proteins than either of the single agents. Besides affecting cell growth, clonogenicity, and reversibility, the combined treatment is also more effective in inducing differentiation as measured by the activity of ALP.

Thus, in both cell lines the efficacy of the cytotoxic agent FUdR is increased by the differentiation agent PB. These effects of PB manifested itself by prolonging the effect of FUdR on cell growth inhibition, on the clonogenicity of the cells, on increasing the time FUdR-treated cells recover, on p21<sup>wafl</sup> production, on secretion of TGFβ₁, and commitment to differentiation. The conventional treatment of colon carcinomas in vivo with fluoropyrimidines requires periods of rest. Thus, based on these data, it seemed feasible to design a protocol for sequential fluoropyrimidines and PB for the treatment of colon carcinoma. To that end, 24-h infusion of 5–2000 mg/m<sup>2</sup> fluorouracil, followed by 5 days of continuous infusion of 410 mg/kg/24 h PB with 1 day rest for 6 week cycles, was initiated in patients with advanced refractory colon carcinoma. Thus far, six patients have received at least 1 full 6-week cycle, including 1 patient on treatment for 6 months. The treatment has been well tolerated except for minor lethargy, and disease stability was noted in three patients.

The finding that U9 cells treated with FUdR and PB are less committed to terminal differentiation than U4 cells suggests that additional drugs will be necessary to prevent colon carcinoma cell recovery from fluoropyrimidine treatment. Human colon carcinoma cell lines have a wide range of growth inhibitory response to FUdR and PB (34, 35). IFN-γ but not IFN-α, alone or in combination with PB, significantly diminishes recovery of several fluoropyrimidine-treated colon carcinoma cell lines (34, 35). IFN-γ is known to induce p21<sup>wafl</sup> expression through a STAT-1 response element and when given with PB produces synergistic growth inhibition and apoptosis in HT29 cell lines (34, 35). These data are consistent with the need to use a combination of agents with different mechanisms of action to enhance terminal differentiation and prevent recovery of colon carcinoma cells between cycles of fluoropyrimidine treatment.

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