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ORIGINAL ARTICLE

Sequence-dependent antitumor effects of differentiation agents in combination with cell cycle-dependent cytotoxic drugs

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Abstract
Purpose Combination of two differentiation agents such as phenylbutyrate (PB) and 13-*cis*-retinoic acid (CRA) has been shown to have an additive inhibitory effect on tumor growth in preclinical studies. In this report we explored the hypotheses that these "cytostatic" agents may have a greater antitumor activity in combination with "cytotoxic" compounds and their biological effect may be sequence-dependent.
Methods The antitumor activity of combination of PB and CRA with paclitaxel (TX) and doxorubicin (DOXO) on human prostate and colon carcinoma cell lines was assessed both in vitro and in vivo. The effect on cell cycle, apoptotic rate, cycline expression and induction of p21 expression was also determined.
Results Following treatment of tumor cells with PB + CRA + TX or DOXO, inhibition of tumor cell growth was greatly enhanced as compared to PB + CRA, TX or DOXO alone, with >90% growth inhibition. However, when the cells were pretreated with PB + CRA followed by TX or DOXO, the enhanced inhibition was abolished suggesting a protective effect to this sequence. Interestingly treatment with PB + CRA restored sensitivity to DOXO in PC-3 human prostate cancer cell line. PB + CRA induced p21 expression and cell-cycle arrest in G_1 phase, while TX and DOXO induced G_2/M arrest. p21 and p53-deficient colon carcinoma cell lines were more sensitive to the effect of PB + CRA and TX as single agents and in combination, as compared to the wild type cells. When p21-deficient cells were pretreated with PB + CRA followed by TX the protective effect was still observed. Treatment of tumor cells with combination of these drugs induced cell cycle delay at multiple mitotic checkpoints before undergoing apoptosis. Tumor growth was significantly inhibited and delayed in animals treated with either TX or concomitantly with TX and PB + CRA as compared to control. Animals treated with all three agents demonstrated further growth inhibition or delay than the TX alone or PB + CRA arm.
Conclusions These results suggest a rational therapeutic approach for combination of differentiation-inducing agents with cytotoxic drugs given concomitantly, but not sequentially.

Keywords Differentiation agents · Cytotoxic drugs · Cell cycle · Prostate cancer

Introduction

The use of differentiation inducing compounds is gaining an increased interest as a therapeutic strategy in cancer treatment [1]. Specific gene expression modulation in tumor cells that reinstates a more differentiated phenotype may represent the main stem of the so called "differentiation therapy". Several natural products and synthetic compounds continue to be tested in preclinical models and in clinical trials. Our group has

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been involved in the preclinical and clinical development of the small fatty acid chain Phenylbutyrate (PB), a differentiation-inducing agent with histone deacetylase (HDAC) inhibition properties [5, 6, 16, 17, 21]. This compound inhibits tumor cell proliferation in several tumor types by induction of G_1 cell cycle arrest and specific cell cycle—related proteins such as p21. We also reported that PB in combination with 13-*cis*-retinoic acid (CRA) has an additive inhibitory effect in human prostate carcinoma cell lines in vitro and in vivo [17]. Treatment with the combination of CRA and chemotherapy (paclitaxel, vinblastine) was well tolerated in phase-I/II studies. Objective responses including complete remissions were observed in patients with squamous cell carcinomas, while minimal activity in patients with advanced renal cell cancer was observed in a phase II study [2, 26, 27, 30].

Cell-cycle checkpoints represent novel potential targets for cancer therapy [22, 28]. By activating checkpoint-mediated apoptosis pathways or by exploiting drug sensitivity due to loss of checkpoint function, novel molecular targeted therapeutic strategies are currently under clinical testing. Several studies support the notion that the cell cycle plays also a critical role in chemosensitivity for combination chemotherapy. Commonly used chemotherapy agents induce cell arrest in G_2/M. Microtubule inhibitors (MTI) and antracyclins such as paclitaxel and doxorubicin (DOXO), respectively, induce a P53-independent G_2/M arrest and subsequent apoptosis. However, "cytostatic" agents such as the differentiation-inducing agents small chains fatty acids (i.e. PB) and retinoids, induce G_1 arrest or G_1/S checkpoint delay.

The observation that cytostatic agents such as PB and CRA can induce G_1 arrest raises the possibility that this class of agents might inhibit the effects of proliferation-dependent agents. In fact, combination therapy of the cyclin dependent kinase inhibitor flavopiridol induced chemoresistance when CPT-11, paclitaxel and docetaxel were administered subsequently in in-vivo cancer models. When flavopiridol was given after the chemotherapeutic agents an enhanced antitumor effect was observed [13, 14]. Similar results have been observed in combination with gemcitabine [7]. Flavopiridol in combination with DOXO induced a synergistic antitumor effect when doxorubicin treatment followed flavopiridol [3]. Other combination strategies that sensitized tumor cells to chemotherapeutic drugs include the combination of all-*trans*-retinoic acid (ATRA) and CRA with gemcitabine and cisplatin, the combination of vitamin D analogues with either cisplatin or carboplatin, and the combination of cyclooxygenase inhibitors or the synthetic retinoid fenretinide with carboplatin and cisplatin in different tumor models [8, 11, 15, 24]. Furthermore, the orally active EGFR tyrosine kinase inhibitor gefitinib, which causes G_1 cell cycle arrest, has been shown to enhance the antitumor effect of paclitaxel when given intermittently but not when given continuously [23].

In a recent report the HDAC inihibitor depsipeptide (FK228) induced P-glycoprotein expression and DOXO resistance in promyelocytic leukemia, when cells were treated first with the combination of ATRA and FK228 followed by DOXO. In contrast, when ATRA/FK228 was administered after DOX exposure, the apoptotic rate was increased [25]. The HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) has been shown to antagonize DOXO cytotoxicity, but when given at least 48 h prior to DOXO exposure, the combination treatment resulted in synergistic antitumor activity against breast cancer cells [10].

In this report we explored the hypothesis that the "cytostatic" differentiation-inducing agents PB and CRA may have a greater inhibitory effect in combination with "cytotoxic" compounds paclitaxel (TX) and DOXO. The combination is active in different tumor types both in vitro and in vivo, but its efficacy is schedule-dependent. Our findings confirm that the interaction between differentiation-inducing agents and cell cycle dependent chemotherapy agents is of great interest in the development of novel antitumor therapies and highlights the importance of preclinical studies for a rational clinical design of combination strategies.

Materials and methods

Cell lines and reagents

Human prostate carcinoma cell lines PC3, DU-145 (ATCC), and colon carcinoma cell lines HCT116, HCT116 $p21^{-/-}$, HCT116 $p53^{-/-}$ (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center) were maintained in RPMI 1640 and McCoy media (Gibco BRL), respectively. Murine renal cell carcinoma cell line (RENCA) was maintained in DMEM. Media were supplemented with 10% fetal bovine serum (FBS)(Sigma Chemical Co., St Louis, MO, USA) and 2 mm L-glutamine (Gibco, BRL). Stock solutions of 10^{-3} M 13-*cis*-RA (CRA) (Sigma Chemical Co., St Louis, MO, USA), 250 mM PB (Triple Crown America, Perkorie, PA, USA) were prepared in DMSO and PBS, respectively, and stored at 4°C. Paclitaxel (TX) was purchased as stock solution in cremophor/ETOH at concentration of 6 mg/ml (7 mM) (Bristol-Myers-Squibb) or in powder form (Sigma).

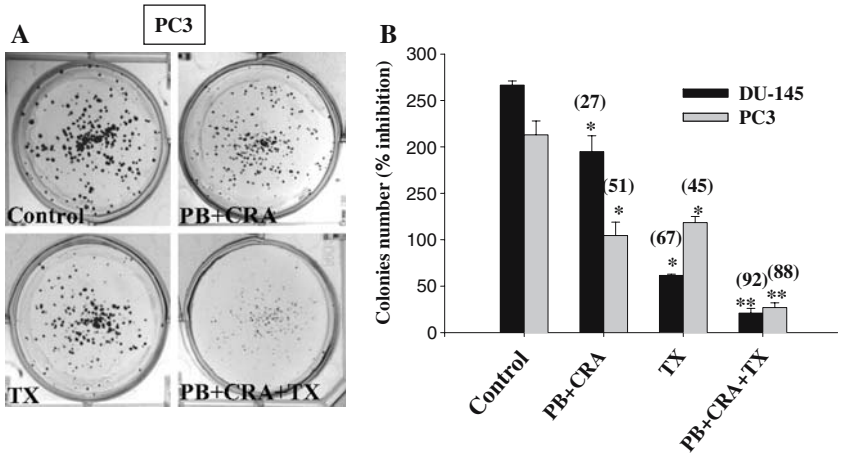

Fig. 1 Combination of PB, CRA, and TX has an inhibitory effect on tumor cell growth in vitro. PC3 and DU-145 cells were plated in six well plates (5×10^2/well) and cultured in the presence of 2.5 mM PB/10 μM CRA and 10 nM TX for 72 and 4 h, respectively. After 10 days tumor cells colonies were counted in duplicates. **a** Representative example of colony formation assay. **b** TX or PB + CRA treatment alone showed an inhibitory effect on hormone-independent human prostate carcinoma cell lines PC3 and DU-145. Combination of PB + CRA + TX resulted in an additive inhibitory effect. Results are expressed as mean of colonies (% inhibition) ± SEM. The experiments were repeated twice with similar results. *PB + CRA or TX versus control = $P < 0.05$; **PB + CRA + TX versus TX or PB + CRA = $P < 0.01$

Doxorubicin (DOXO) was kindly provided by the pharmacy at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins. For the in vivo studies the PB, CRA, and TX were either dissolved or diluted in PBS (PB), propileneglicole (CRA) or DMSO (TX and DOXO). For the in vitro studies the compounds were dissolved in PBS (PB) or DMSO (CRA, TX, and DOXO).

Colony formation assay

Exponentially growing tumor cells were seeded at 500 cells per well in six-well plates and allowed to attach for 48 h. Cells were treated with 2.5 mM PB + 10 μM CRA with either 10–20 nM TX, 50 nM DOXO, or combination in complete medium containing DMSO less than 0.001 or 0.1%, respectively. After 4 h (TX or DOXO) or 72 h (PB + CRA) cells were rinsed, and fresh medium was added. Cultures were observed for 7–10 days and then were fixed and stained with crystal violet. Colonies of greater than 30 cells were scored as survivors. Each condition was counted in duplicate (10 fields/well) on an inverted microscope. Results are expressed as mean colonies number ± SEM. The experiments were repeated two times with similar results.

Apoptosis assay

Exponentially DU-145 tumor cells were seeded in 60 mm wells in duplicates and allowed to attach for 48 h. Then, cells were treated with TX (1 nM) for 4 h or/and PB + CRA (2.5 mM and 10 μM) for 24 h. Cells were rinsed, total cells were harvested, fixed in methanol and stained with DAPI. Stained nuclei were viewed using fluorescence microscopy and scored. A minimum of 200 cells were counted for each determination.

FACS analysis for cell cycle and apoptosis

The cell cycle analysis was performed under similar experimental conditions as described above with either DU145 or PC3 cells. Cells were treated with DOXO (50 nM) or TX (1 nM) for 4 h with or without PB + CRA (2.5 mM and 10 μM) for 24 h. Cell cycle analysis was performed using the Cellular DNA Flowcytometric Analysis Kit (Roche, Indianapolis, IN, USA). Cells treated with agents were harvested by trypsinization, washed, and fixed in 70% ethanol on ice, followed by incubation with RNase. The cells were then stained with propidium iodide (PI) and subjected to a flow cytometry analysis of cell cycle by FACSCalibur (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA) and CellQuest software (BD). To determine the apoptosis rate, aliquots of 10^5 cells were washed three times in PBS and resuspended in binding buffer. A total of 5 μl of annexin V-FITC and/or 5 μl of a PI were added, followed by incubation at room temperature in the dark for 15 min using Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience). Cells were immediately analyzed by FACSCalibur as described above. PI negative (−) and annexin V (+) positive cells

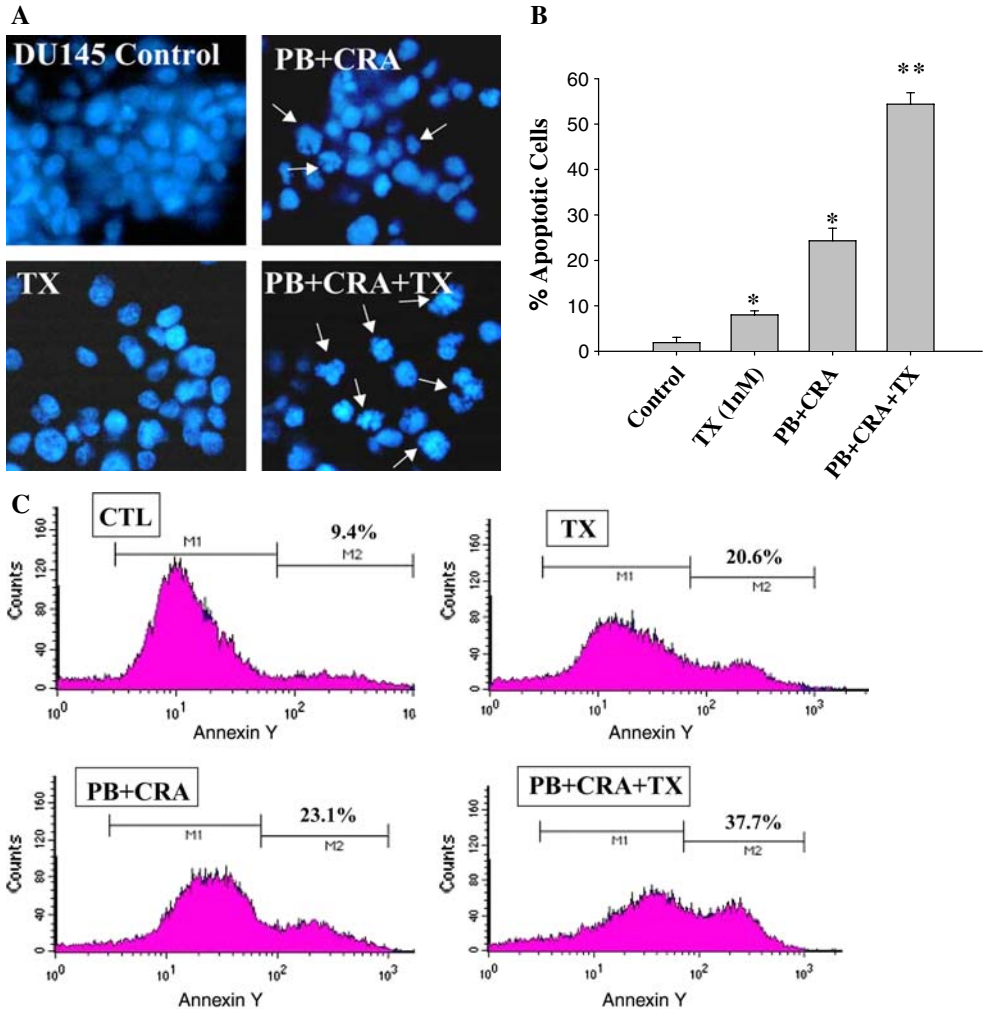

Fig. 2 Combination of PB, CRA, and TX induces tumor cell apoptosis. **a** DU-145 prostate carcinoma cells were treated with PB + CRA (2.5 mM, 10 μM) or TX (1 nM) for 4 and 24 h, respectively, and apoptosis was determined by nuclear fragmentation with DAPI. Combination of PB + CRA with TX induced a significant increase in the apoptotic rate as compared to single agents. In **b** results are expressed as mean % apoptotic cells ± SEM. *PB + CRA or TX versus control = $P < 0.004$; **PB + CRA + TX versus CRA + PB or TX = $P < 0.0001$. **c** DU145 cell annexin-V staining showed a significant increase of the % apoptotic cells assessed by FACS-analysis

were considered apoptotic, and PI (+) positive and annexin V (+) positive cells were considered dead cells.

Western blot analysis of cyclins

After the same treatment conditions as above for 24, 48, and 72 h, the cells were washed twice with PBS and homogenized with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Proteins (10 μg/lane) from the cell lysates were applied to 4–15% Tris–HCl gel (Bio-Rad, Hercules, CA, USA) and blotted with primary antibodies anti-Cyclin A, Cyclin B, Cyclin E or ß-actin. The antibody binding was revealed using horseradish peroxidase-conjugate secondary antibodies and an enhanced chemiluminescence blot detection system (Amersham Biosciences, Piscataway, NJ, USA). All antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for the anti-ß-actin antibody (Sigma, St, Louis, MO, USA). Results were reproducible in repeated experiments. Enhanced chemiluminescence (Perkin Elmer Life sciences, Boston, MA, USA) was used for detection.

Reverse transcription-PCR for p21

The semi-quantitative PCR for p21 performed using the same primer sequences for p21 (5′-GCCGAAGTC AGTTCCTT-3′ and 5′-TCATGCTGGTCTGCCGC-3′) as described previously [19]. In short, total mRNA from cells was isolated with Trizol reagent (Invitrogen). One thousand nanogram of (total) mRNA was

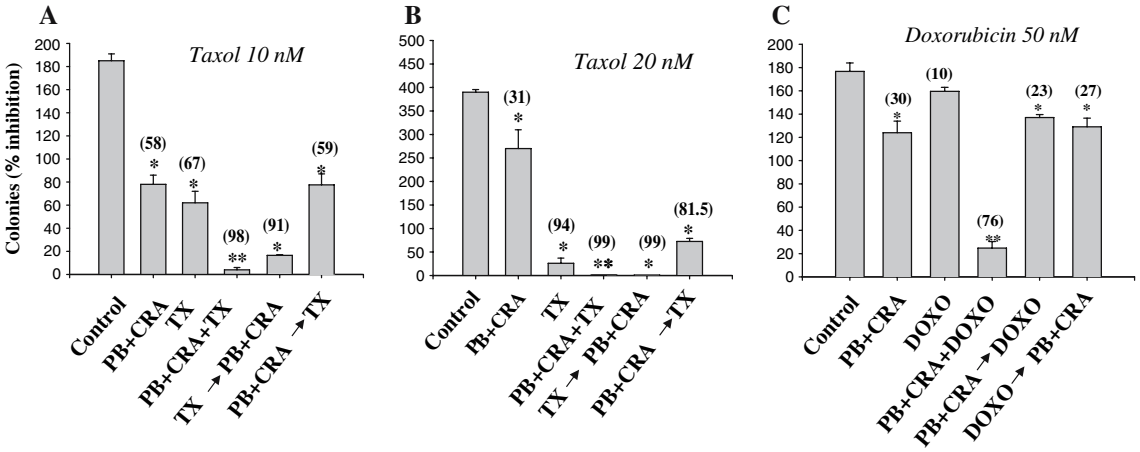

Fig. 3 The inhibitory effect induced by combination of differentiation agents (PB and CRA) with cytotoxic drugs (TX and DOXO) on tumor cell growth is schedule dependent. Colony formation assay showed that when tumor cells (PC3) were treated concomitantly with PB + CRA (2.5 mM + 10 μM, respectively) and either 10 nM TX (**a**), 20 nM TX (**b**) or 50 nM DOXO (**c**), an additive inhibitory effect was achieved. However, as tumor cells were pretreated with PB + CRA for 68 h and then with TX or DOXO the additive effect was abolished (similar effect when DOXO or TX preceded PB + CRA). Results are expressed as mean of colonies (% inhibition) ± SEM. **a**, **b** *PB + CRA or TX or PB + CRA followed by TX versus control, or TX followed by PB + CRA veruss PB + CRA followed by TX = $P < 0.01$. **PB + CRA + TX versus PB + CRA and TX or DOXO = $P < 0.03$. **c** *PB + CRA or PB + CRA followed by DOXO or DOXO followed by PB + CRA versus control = $P < 0.01$. **PB + CRA + DOXO versus PB + CRA or DOXO = $P < 0.03$

subjected to semiquantitative reverse transcription-PCR for p21 using a Mastercycler (Eppendorf, Westbury, NY, USA). Similar results were observed in repeated experiments.

Tumor growth in vivo

Male athymic nude or female BALBc mice (Taconic) 4–6 weeks old were kept in a temperature-controlled room on a 12/12 h light/dark schedule with food and water ad libitum. Animals were injected subcutaneously in the flank region bilaterally with 2×10^6 tumor cells (PC3, HCT116 or RENCA) resuspended in HANKS solution and mixed with Matrigel (1:1) (Collaborative Biomedical Products, Bedfors, MA, USA) in a final volume of 0.2 ml. In one set of experiments ("intervention" model) as the tumor volume reached a measurable size (50–100 mm^3) 20 animals for each tumor were randomly placed in four groups (five animals/group): control, PB + CRA, TX, and PB + CRA + TX. Animals in the control group were treated with daily administration of vehicle (polyethylenglicole and/or cremophor) by 20′′ gauge gavage needle and with intraperitoneal (i.p.) injections, respectively. PB (600 mg/kg/day) was administered concomitantly by i.p. injections (300 mg/kg/day, 9 a.m. and 5 p.m., for 4 weeks). CRA was administered by gavage needle (30 mg/kg/day, 9 a.m., for 4 weeks). TX was administered i.p. (5 mg/kg/day 9 a.m., 5 days a week for 2 weeks). In a separate set of experiments in the PC3 model ("prevention" model) drugs were administered starting the day after tumor implantation. Tumor volume was measured with a caliper twice a week and calculated according to the formula = A (length) × B (width) × C (height) × 0.5236 and reported as mean ± SEM. The animals were treated for approximately 4–8 weeks, then were euthanized. Animals treated with PB + CRA sometimes showed a transient weight loss during the second week of treatment with spontaneous recovery.

Statistical analysis

Differences between means of unpaired samples were evaluated by Student's t-test using the Sigmaplot program. $P < 0.05$ was taken to indicate statistical significance.

Results

Combination of PB+CRA and paclitaxel has a greater antitumor effect in vitro

To determine the antiproliferative effects of PB, CRA, and TX either individually or in combination, colony formation assays were performed. Treatment with combination of PB and CRA induced a 27 and 51% growth inhibition in PC3 and DU-145 prostate carcinoma cell lines, respectively (Fig. 1a, b). PC3 and DU-145 growth

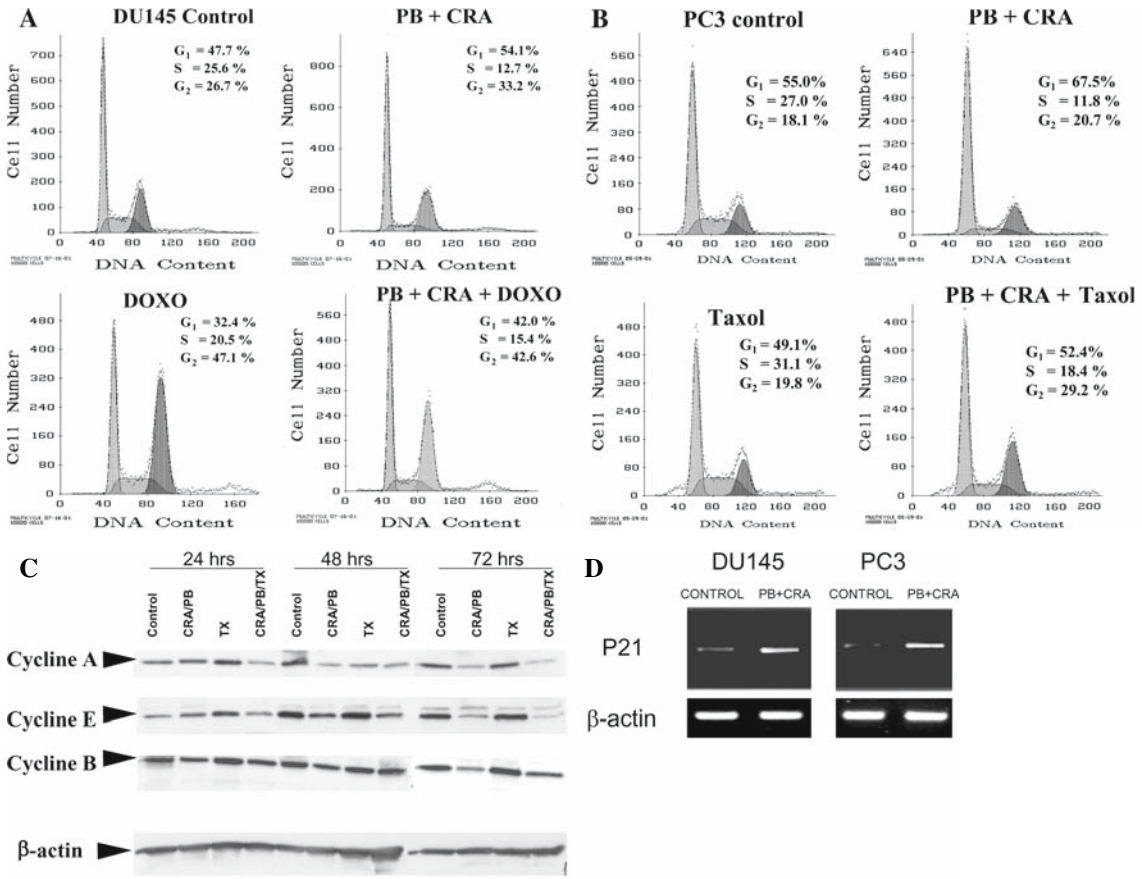

Fig. 4 Combination of PB + CRA with DOXO induces both G_1 and G_2 cell cycle arrest. **a** DU-145 tumor cells were treated as above and harvested for cell cycle analysis. DOXO (50 nM) induced increased G_2/M arrest, while PB + CRA (2.5 mM + 10 μM, respectively) induced G_1/S arrest. Combination of these cytotoxic agents induced both G_1 and G_2 arrest and decreased cells in S phase compared to untreated control cells. **b** PC3 cells were treated with TX (1 nM) with or withour PB + CRA (2.5 mM + 10 μM, respectively) and harvested for cell cycle analysis as described above. TX in combination with PB + CRA also induced a G_2 arrest. **c** Treatment of PC3 cells for respectively 24, 48, and 72 h resulted in a significant reduction of protein expression of Cyclin A and E. **d** Treatment of PC3 and DU-145cells with PB + CRA induced expression of p21 at mRNA level

were inhibited 45 and 67% by TX, respectively. However, the addition of TX to PB and CRA resulted in a greater effect of 90% growth inhibition of these human prostate carcinoma cell lines. To address the question whether combination of PB and CRA is required for a greater antitumor effect of TX, we performed single agent treatment with CRA and PB and in combination with TX. Treatment with CRA alone, PB alone or the combination of PB and TX or CRA and TX resulted in a growth inhibition of 29, 34, 39, and 49%, respectively, in the PC3 cell line. These results clearly showed that a combination of CRA and PB is required to increase the growth inhibitory activity of TX.

The antitumor effect of PB + CRA and TX was associated with induction of apoptosis assessed by DAPI staining (Fig. 2a, b). PB + CRA and TX induced 25 and 10% apoptosis as compared to 2% in control, respectively. The combination of the differentiation agents and cytotoxix agent had an additive effect with a ~55% induction of apoptosis. Comparable effect on apoptosis was confirmed by Annexin V staining studied by FACS (Fig. 2c).

Sequence-dependent effect of combination of PB + CRA and TX or DOXO

To determine whether the order of drug addition affects the observed inhibitory effect, we varied the treatment schedule. When human prostate PC3 cells were treated with TX and PB + CRA simultaneously an additive dose-dependent inhibitory effect in the colony formation assay was observed (up to 98–99% inhibition of proliferation Fig. 3a, b). However, when the cells were treated in the PB + CRA → TX sequence, the additive inhibitory effect was abrogated and a significant antagonism was observed since the inhibition of proliferation dropped to 59% versus 67% with paclitaxel only. Thus, pretreatment of tumor cells with PB + CRA partially antagonized the action of TX. This sequence-dependent

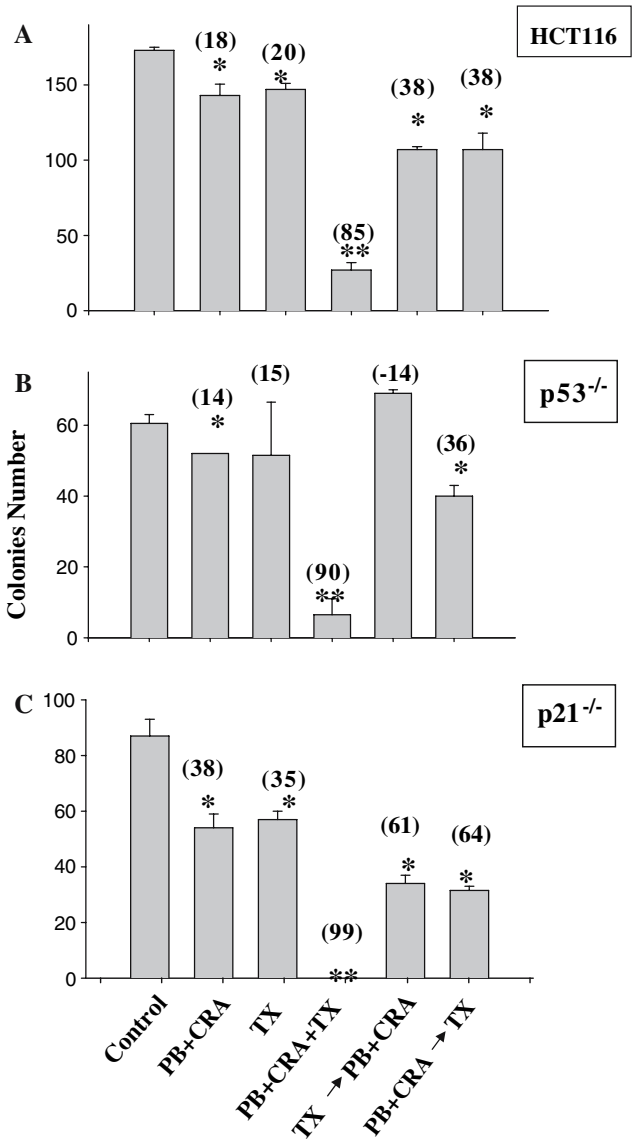

Fig. 5 The inhibitory effect induced by combination of PB, CRA, and TX on tumor growth is p21/p53 independent. **a** Concomitant treatment with PB + CRA (2.5 mM + 10 μM, respectively) + TX (10 nM) showed additive/synergistic inhibitory effect in colonies formation in human colon carcinoma HCT116 cells. However, as described in PC3 cells, pretreatment of HCT116 with PB + CRA for 68 h abolished the additive effect. HCT116 $p21^{-/-}$ and HCT116 $p53^{-/-}$ are equally sensitive to this combination, suggesting a p21/p53 independent mechanism. Results are expressed as mean of colonies (% inhibition) ± SEM. **b**, **c** *PB + CRA or TX or PB + CRA followed by TX versus control, or TX followed by PB + CRA versus PB + CRA followed by TX = $P < 0.01$. **PB + CRA + TX versus PB + CRA and TX or DOXO = $P < 0.03$

interaction was not limited to the combination of PB + CRA with paclitaxel. In a similar experiment PC3 cells were treated with DOXO, PB + CRA and combination. PC3 cells were resistant to DOXO as single agent, but simultaneous treatment with PB + CRA and DOXO a potent inhibitory effect was observed (up to 76% inhibition; Fig. 3c). However, when the cells were treated in the PB + CRA → DOXO or DOXO → PB + CRA sequence, this inhibitory effect was abolished (23 and 27% inhibition, respectively, similar to PB + CRA only with 30% inhibition).

Simultaneous treatment with PB + CRA and cell cycle-dependent cytotoxic agents imposes multiple artificial checkpoints

Cell cycle checkpoints and apoptosis are regulated by cdc2 kinases and their inhibitors [22]. To investigate the potential mechanism of the sequence-dependent additive inhibitory effect, first we examined the effect of PB + CRA and either DOXO or TX on cell cycle distribution of treated tumor cells. Twenty-four hour PB + CRA treatment of DU145 (or PC3, data not shown) tumor cell line resulted in a decreased percentage of S phase cells and a decreased number of cells going through mitosis as cell accumulated in G_1, while DOXO treatment induced G_2 arrest (Fig. 4a). Similar results were observed with TX in PC3 cells (Fig. 4b). Combination treatment revealed a combined G_1 and G_2 arrest as compared to control and single agents. We also assessed the modulation of cyclins involved in cell cycle checkpoints including cyclin A, B, and E. Cyclin A and E, and to a lesser extent cyclin B, were downregulated at the protein levels following treatment with PB + CRA with a maximum effect at 72 h (Fig. 4c). Then, we assessed the role of p21, which controls G_1 and S phase checkpoints and is also involved in the regulation of the G_2/M checkpoint. P21 has been shown to be upregulated by differentiation agents such as PB and retinoids. Treatment of PB + CRA induced expression of p21 at mRNA level (Fig. 4d). The induction of p21 was independent of wild-type p53 since PC3 cell line is p53-null. We tested this combination also in a human colon carcinoma cell line, HCT116, and p21- and p53-deficient derived cell lines. In both cell lines as well as in the parental cell line combination of PB + CRA with TX had an additive inhibitory effect on proliferation (85–99% inhibition as compared to control) (Fig. 5). Again, when PB + CRA treatment either preceded or followed TX treatment in the p21-, p53-deficient and parental cell lines the additive inhibitory effect was abolished.

Combination of PB + CRA with TX has an additive inhibitory effect in tumor models in vivo

To determine the antitumor effect of PB + CRA and the cytotoxic agent TX we tested this combination in three different tumor models. In the PC3 tumor model, growth

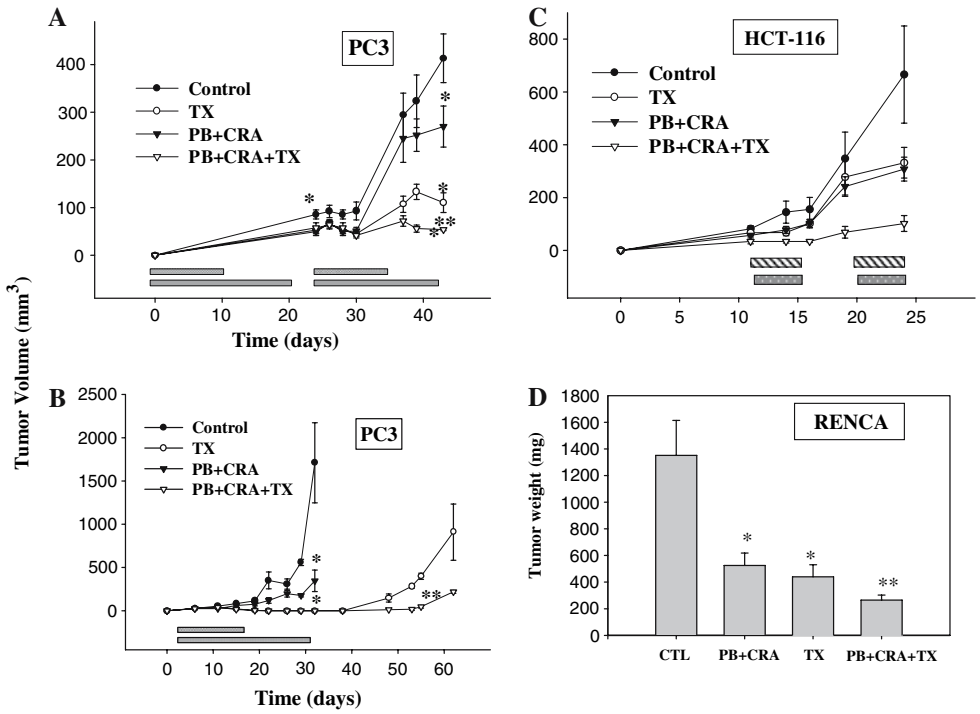

Fig. 6 Combination of PB, CRA, and TX has an inhibitory effect on tumor cell growth in vivo. **a**, **c** Animals bearing established HCT116 human colon and PC3 human prostate tumors (50–100 mm^3) were treated with PB + CRA (600, 30 mg/kg/day), TX (5 mg/kg/day) or PB + CRA + TX. Single agents PB + CRA and TX showed tumor growth inhibition as compared to control. A greater inhibitory effect upon combination of PB + CRA + TX was observed. Results are expressed as mean of tumor volume ± SEM. $*P < 0.05$ versus control; $**P < 0.02$ versus PB + CRA and TX. **b** Tumor growth delay experiment. In this experiment, treatment as described for **a**, **c** was started 1 day after tumor implantation before tumors were established. PC3 tumor growth was significantly delayed in animals treated with either TX or concomitantly with TX and PB + CRA as compared to control. However, animals treated with all three agents demonstrated further growth delay than the TX alone arm $*P < 0.003$ versus control $**P < 0.012$ versus TX. **d** Treatment in animals injected with murine RENCA cells were treated when tumors were established as described for **a**, **c**. Combination treatment with PB + CRA + TX as compared to single agents showed a greater inhibition as determined by primary tumor weights. Results are expressed as means of tumor weights (mg) from two separate experiments ±SEM. $*P < 0.038$ versus control; $**P < 0.024$ versus PB + CRA and TX. *Dashed bar* Duration of treatment with TX. *Filled bar* Duration of treatment with PB + CRA

was significantly inhibited with either TX or PB + CRA as compare to control (73 and 35% inhibition, respectively). However animals treated with concomitant PB, CRA and paclitaxel showed a greater tumor growth inhibition as compared to TX alone or PB + CRA (87% inhibition) (Fig. 6a). In the "prevention" model tumor growth was significantly delayed by TX treatment as compared to either control or PB + CRA alone. However, further tumor growth delay was observed with the PB + CRA + TX treatment (Fig. 6b). Similar antitumor effect of the PB + CRA + TX combination was obtained in the HCTT116 human colon carcinoma model and the murine RENCA orthotopic model (Fig. 6c, d).

Discussion

In this report we investigated the antitumor effect of combination of differentiation agents with cell-cycle dependent cytotoxic compounds both in vitro and in vivo. The combination of the G_1 arrest-inducing PB and CRA with the G_2 arrest-inducing cytotoxic agents paclitaxel and doxorubicin resulted in a greater inhibition of cell proliferation and increased tumor cell apotosis as compared to PB + CRA or cytotoxic agent alone. Our results revealed that combination of PB and CRA with pretreatment of tumor cells with G_1 arrest-inducing agents could significantly repress the cell-killing activity of cell cycle-dependent agents such as paclitaxel and doxorubicin. This combination of differentiation agents and cell-cycle dependent cytotoxic drugs resulted in simultaneous cell-cycle checkpoint delays at the G_1/S and G_2/M transitions, setting up for "apoptosis-prone collisions" [9] (Fig. 7). We hypothesize that when cells are treated simultaneously with drugs activating more than one different cell-cycle checkpoint, the cells are unable to escape, and consequently undergo apoptosis. These results suggest that a

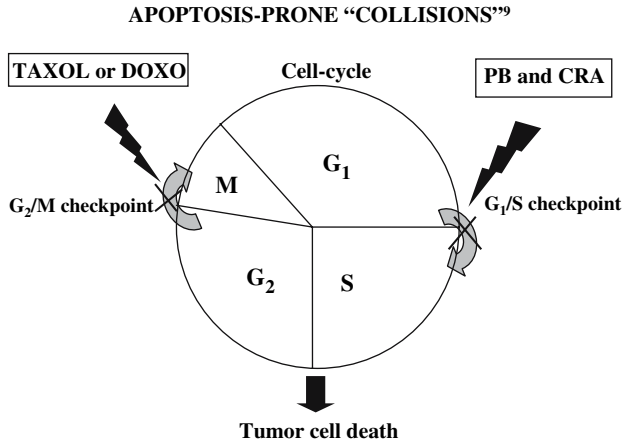

Fig. 7 Model of apoptosis-prone "collisions" induced by differentiation agents in combination with cell cycle-dependent cytotoxic drugs

decrease in the number of tumor cells traversing the cell cycle during PB + CRA treatment, as represented by increased G_1 arrest, presumably contributes to the antagonism between PB + CRA and paclitaxel or doxorubicin during the sequential exposure.

The antagonism described upon combination of cytotoxic drugs with cytostatic agents has been reported with other agents. For example, when flavopiridol, a kinase inhibitor which induces G_1 arrest, was administered concomitantly with or preceding paclitaxel, docetaxel and gemcitibine or SN38 (the metabolite of irinotecan), similar antagonistic effects were reported [13]. Similarly, following treatment with the EGFR tyrosine kinase inhibitor, gefitinib, that induces a G_1 arrest, resistance against paclitaxel was observed in murine tumor models [23]. The sequence of drug exposure for enhanced antitumor activity was also important in the combination of DOXO with either flavopiridol or with ATRA and FK228 [3, 25]. These reports and our results indicate that the sequence-dependent effect observed with PB + CRA in combination with either paclitaxel or doxurubicin may be related to the differentiation agent induced G_1 arrest. The difference between DOXO and TX may be due to the fact that DOXO has topoisomerase inhibitory activity while TX is a microtubule inhibitor. Following treatment with DOXO DNA transcription may be disrupted but not with TX. Differentiation agents such as CRA and PB may interfere in DNA function and transcription. We speculate that DOXO specifically interferes with this activity of the differentiation agents and thereby abolishes the activity of PB + CRA when given following the differentiation agents.

Other agents with more specific histone deacethylase inhibition activity are currently in early clinical development. Several preclinical studies suggest that HDAC inhibitors may induce primarily tumor cell growth inhibition at lower doses and tumor cell apoptosis at higher concentrations. The benzamide MS-275 has been shown to induce primarily G_1 arrest in epithelial and leukemia cells. Interestingly, the HDAC inhibitor SAHA has been shown to induce G_1 arrest at <0.5 μm concentration and G_2 arrest at higher in vitro drug concentrations [10]. Thus, the drug concentration-dependent effect on cell cycle may have direct impact on the optimal clinical trial design of these compounds when tested in combination with cytotoxic agents. Clinical studies with the combination of CRA and chemotherapy (TX and vinblastine) showed that this combination is well tolerated but has minimal clinical benefit in renal cell cancer [2, 26, 27]. PB has been clinically studied as monotherapy. PB is also well tolerated and caused stable diseases and one complete remission in a group of patients with recurrent malignant gliomas [16]. Treatment strategies of CRA plus HDAC inhibitors have been clinically tested in patients with hematologic malignancies. A recent study with the combination of valproic acid plus CRA revealed that this combination is well tolerated, but only showed some benefit in a subgroup of patients with AML [4, 20]. Based on our recent preclinical data a phase I clinical trial with the HDAC inhibitor MS-275 in combination with CRA has been initiated at our institution [29]. To date no combination strategies with of CRA, HDAC inhibitors and chemotherapy have been clinically tested. Our results provide a strong rationale for such a combination strategy.

Preliminary results with the novel HDAC inhibitors are showing potential antagonism with different schedules. Thus, the sequence dependent phenomenon described in our study suggests that careful pharmacokinetic studies should guide the clinical testing of the novel HDAC inhibitors in combination with cell-cycle dependent cytotoxic agents. The sequence dependent additive effect may be affected by the HDACI plasma/tumor concentration reached in the treated patients and whether G_1 or G_2 tumor cell arrest is the result of the treatment.

As previously reported with differentiation inducing agents the treatment of PB + CRA induced p21 gene expression and reduced cyclin A and E expression at the protein level, which correlates with a G_1 cell cycle arrest. P21 prevents cell-cycle progression by inhibiting the activity of cyclin dependent kinase inhibitors such as cyclin E-associated CDK2. Cyclin E is primarily involved in G_1–S phase transition, while cyclin A determines S phase progression and cyclin B is involved in G_2 [22]. The experiments with p21 null cell lines suggest that p21

induction is not required for the sequence dependent effect of PB + CRA + TX combination. Previous reports suggest the absence of p21 may sensitize tumor cell to MTI induced apoptosis, but the exact role of p21 in HDAC inhibitor induced apoptosis remains unclear [12].

Another very interesting and potential clinical important finding is that the DOXO resistant PC3 cell line can be sensitized by combination of CRA + PB. To date no clinical agent has been found to effectively circumvent multidrug resistance-1 (DOXO) in cancer patients. Several clinical attempts to circumvent drug resistance against DOXO in solid malignancies have been explored including cyclosporin and verapamil, agents that were very potent in preclinical models [18]. However, no clinical benefit from these agents has been observed in the clinic, partly because circumvention of drug resistance required very high doses of these drugs causing severe toxicity.

In conclusion, our study demonstrates that combination of differentiation agents (PB and retinoids) with cell-cycle dependent cytotoxic drugs (TX and DOXO) produces a greater schedule-dependent inhibitory effect as compared to single agents in different tumor cell lines both in vitro and in vivo. Because of the current clinical testing of more suitable differentiation agents and specific HDAC inhibitors, this combination therapy is of great interest, and confirms a potential avenue for developing novel antitumor combination therapies. These results suggest that concomitant disruption of the cell cycle at different checkpoints may force tumor cell to undergo apoptosis. Thus, combinations of drugs that impose different artificial checkpoints may represent a novel therapeutic strategy to be tested in clinical trials. Further insight into the mechanisms involved in the interaction among these agents will optimize treatment design and will expand the potential role of differentiation inducing agents in the treatment of cancer.

References

1. Altucci L, Gronemeyer H (2001) The promise of retinoids to fight against cancer. Nat Rev Cancer 1:181–193
2. Bacoyiannis C, Dimopoulos MA, Kalofonos HP, Nicolaides C, Aravantinos G, Bafaloukos D, Samelis G, Onyenadum A, Kiamouris C, Skarlos D et al (2002) Vinblastine and interferon-gamma combination with and without 13-cis retinoic acid for patients with advanced renal cell carcinoma. Results of two phase II clinical trials. Oncology 63:130–138
3. Bible KC, Kaufmann SH (1997) Cytotoxic synergy between flavopiridol (NSC 649890, L86–8275) and various antineoplastic agents: the importance of sequence of administration. Cancer Res 57:3375–3380
4. Bug G, Ritter M, Wassmann B, Schoch C, Heinzel T, Schwarz K, Romanski A, Kramer OH, Kampfmann M, Hoelzer D et al (2005) Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer 104:2717–2725
5. Carducci MA, Nelson JB, Chan-Tack KM, Ayyagari SR, Sweatt WH, Campbell PA, Nelson WG, Simons JW (1996) Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. Clin Cancer Res 2:379–387
6. Carducci MA, Gilbert J, Bowling MK, Noe D, Eisenberger MA, Sinibaldi V, Zabelina Y, Chen TL, Grochow LB, Donehower RC (2001) A Phase I clinical and pharmacological evaluation of sodium phenylbutyrate on an 120-h infusion schedule. Clin Cancer Res 7:3047–3055
7. Jung CP, Motwani MV, Schwartz GK (2001) Flavopiridol increases sensitization to gemcitabine in human gastrointestinal cancer cell lines and correlates with down-regulation of ribonucleotide reductase M2 subunit. Clin Cancer Res 7:2527–2536
8. Kalemkerian GP, Ou X (1999) Activity of fenretinide plus chemotherapeutic agents in small-cell lung cancer cell lines. Cancer Chemother Pharmacol 43:145–150
9. Li CJ, Li YZ, Pinto AV, Pardee AB (1999) Potent inhibition of tumor survival in vivo by beta-lapachone plus taxol: combining drugs imposes different artificial checkpoints. Proc Natl Acad Sci USA 96(23):13369–13374
10. Marchion DC, Bicaku E, Daud AI, Richon V, Sullivan DM, Munster PN (2004) Sequence-specific potentiation of topoisomerase II inhibitors by the histone deacetylase inhibitor suberoylanilide hydroxamic acid. J Cell Biochem 92:223–237
11. Moffatt KA, Johannes WU, Hedlund TE, Miller GJ (2001) Growth inhibitory effects of 1alpha, 25-dihydroxyvitamin D(3) are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. Cancer Res 61:7122–7129
12. Mork CN, Faller DV, Spanjaard RA (2005) A mechanistic approach to anticancer therapy: targeting the cell cycle with histone deacetylase inhibitors. Curr Pharm Des 11:1091–1104
13. Motwani M, Jung C, Sirotnak FM, She Y, Shah MA, Gonen M, Schwartz GK (2001) Augmentation of apoptosis and tumor regression by flavopiridol in the presence of CPT-11 in Hct116 colon cancer monolayers and xenografts. Clin Cancer Res 7:4209–4219
14. Motwani M, Rizzo C, Sirotnak F, She Y, Schwartz GK (2003) Flavopiridol enhances the effect of docetaxel in vitro and in vivo in human gastric cancer cells. Mol Cancer Ther 2:549–555
15. Pettersson F, Colston KW, Dalgleish AG (2001) Retinoic acid enhances the cytotoxic effects of gemcitabine and cisplatin in pancreatic adenocarcinoma cells. Pancreas 23:273–279
16. Phuphanich S, Baker SD, Grossman SA, Carson KA, Gilbert MR, Fisher JD, Carducci MA (2005) Oral sodium phenylbutyrate in patients with recurrent malignant gliomas: a dose escalation and pharmacologic study. Neuro-oncol 7:177–182
17. Pili R, Kruszewski MP, Hager BW, Lantz J, Carducci MA (2001) Combination of phenylbutyrate and 13-cis retinoic acid inhibits prostate tumor growth and angiogenesis. Cancer Res 61:1477–1485
18. Pinedo HM, Giaccone G (1997) Chemotherapy. Lancet 349(Suppl 2):SII7–SII9
19. Qian DZ, Wang X, Kachhap SK, Kato Y, Wei Y, Zhang L, Atadja P, Pili R (2004) The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. Cancer Res 64:6626–6634
20. Raffoux E, Chaibi P, Dombret H, Degos L (2005) Valproic acid and all-trans retinoic acid for the treatment of elderly patients with acute myeloid leukemia. Haematologica 90:986–988

21. Rudek MA, Zhao M, He P, Hartke C, Gilbert J, Gore SD, Carducci MA, Baker SD (2005) Pharmacokinetics of 5-azacitidine administered with phenylbutyrate in patients with refractory solid tumors or hematologic malignancies. J Clin Oncol 23:3906–3911
22. Schwartz GK, Shah MA (2005) Targeting the cell cycle: a new approach to cancer therapy. J Clin Oncol 23:9408–9421
23. Solit DB, She Y, Lobo J, Kris MG, Scher HI, Rosen N, Sirotnak FM (2005) Pulsatile administration of the epidermal growth factor receptor inhibitor gefitinib is significantly more effective than continuous dosing for sensitizing tumors to paclitaxel. Clin Cancer Res 11:1983–1989
24. Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr, Chou TC (1999) Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. Cancer Res 59:6178–6184
25. Tabe Y, Konopleva M, Contractor R, Munsell M, Schober WD, Jin L, Tsutsumi-Ishii Y, Nagaoka I, Igari J, Andreeff M (2006) Up-regulation of MDR1 and induction of doxorubicin resistance by histone deacetylase inhibitor depsipeptide (FK228) and ATRA in acute promyelocytic leukemia cells. Blood 107:1546–1554
26. Thalasila A, Poplin E, Shih J, Dvorzhinski D, Capanna T, Doyle-Lindrud S, Beers S, Goodin S, Rubin E, DiPaola RS (2003) A phase I trial of weekly paclitaxel, 13- cis-retinoic acid, and interferon alpha in patients with prostate cancer and other advanced malignancies. Cancer Chemother Pharmacol 52:119–124
27. Vaishampayan U, Flaherty L, Du W, Hussain M (2001) Phase II evaluation of paclitaxel, alpha-interferon, and cis-retinoic acid in advanced renal cell carcinoma. Cancer 92:519–523
28. van Vugt MA, Bras A, Medema RH (2005) Restarting the cell cycle when the checkpoint comes to a halt. Cancer Res 65:7037–7040
29. Wang XF, Qian DZ, Ren M, Kato Y, Wei Y, Zhang L, Fansler Z, Clark D, Nakanishi O, Pili R (2005) Epigenetic modulation of retinoic acid receptor beta2 by the histone deacetylase inhibitor MS-275 in human renal cell carcinoma. Clin Cancer Res 11:3535–3542
30. Wieder R, Pavlick AC, Bryan M, Hameed M, Baredes S, Pliner L, Saunders T, Korah R (2002) Phase I/II trial of accutane as a potentiator of carboplatin and paclitaxel in squamous cell carcinomas. Am J Clin Oncol 25:447–450