Cancer Chemother Pharmacol (1999) 44: 81–87 © Springer-Verlag 1999

ORIGINAL ARTICLE

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In vitro evaluation of flavopiridol, a novel cell cycle inhibitor, in bladder cancer

Received: 7 July 1998 / Accepted: 28 October 1998

Abstract *Purpose*: To determine the in vitro effects of flavopiridol on bladder cancer cell lines, immortalized urothelial cell lines, and normal urothelial cells well characterized for defects in p53, pRb, and p16. *Methods*: Growth inhibition was assessed via an MTT assay and apoptosis via DAPI nuclear staining. Cell cycle analysis was performed via propidium iodide staining and fluorescent activated cell sorting (FACS). Multidrug-resistant cells were generated by continuous exposure to doxorubicin. *Results*: Growth inhibition was not correlated with inactivation of p53, pRb, or p16. All cells experienced G2/M arrest within 24 h of flavopiridol exposure. Modest apoptosis was observed but required 72 h of continuous drug exposure to become evident. There was no obvious synergistic or antagonistic toxicity when flavopiridol was combined with radiotherapy or cisplatin dosed at the IC_{50} despite the observation that radiotherapy and flavopiridol led to more profound G2/M arrest than either agent alone. Doxorubicin-resistant cells, demonstrated to overexpress the MDR1 multidrug-resistance protein were equally as sensitive to flavopiridol as the parental cells. *Conclusions*: Flavopiridol is a novel cell cycle inhibitor that may be a useful agent in bladder cancers with tumor suppressor gene alterations and/or multidrug resistance.

Supported in part by: Cancer Research Foundation Young Investigator Award, American Cancer Society Research Project Grant RPG-97-171-01-CNE, and the Sections of Hematology/Oncology and Urology of the University of Chicago

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Key words Cyclin-dependent kinase · Cytotoxicity · Apoptosis · Multidrug resistance

Introduction

One of the fundamental abnormalities in human cancer is dysregulated cell cycle control [19]. In fact, the three most commonly altered tumor suppressor proteins in human cancer, p53, pRb, and p16, are all critical to normal cell cycle regulation. Progression through the mammalian cell cycle is dependent on the sequential activation of a series of cyclin-dependent kinases. Inhibition of these kinases leads to cell cycle arrest, and in the correct context, apoptosis [19]. Drugs that directly interact and inhibit these kinases are thus attractive putative chemotherapeutic agents, especially in cells with demonstrated alterations of p53, pRb, and/or p16.

One such agent, flavopiridol, is already in early clinical trials. Initial in vitro studies have revealed that it induces cell cycle arrest, preferentially in the G2/M phase, and leads to growth inhibition of and cytotoxicity in exponentially growing but not stationary breast carcinoma cells [7]. Further studies have shown that flavopiridol alters the phosphorylation status of the cdc2 cyclin-dependent kinase and directly inhibits this critical cell cycle protein [10, 21]. More recently it has been demonstrated that flavopiridol is also a potent inhibitor of the CDK4 and CDK2 cyclin-dependent kinases suggesting that it is a more general inhibitor of this class of enzymes [4]. The structural basis of flavopiridol's inhibitory activity has been solved by cocrystallization with CDK2, and it is likely that this information will lead to the development of additional inhibitors with similar activity [5].

Although initial studies have shown that flavopiridol is less toxic in noncycling cells, subsequent studies have indicated that this may not be the case in all cell lines [2]. Studies in hematopoietic cancer cell lines have also shown that flavopiridol is a potent inducer of apoptosis in certain cells [1, 8, 13]. Apoptosis induction has been less readily demonstrated in cell lines from solid malig-

nancies, but it has been suggested that apoptosis and cytotoxicity can be enhanced by combined treatment with other agents [3, 18].

Because the effects of flavopiridol apparently differ in cell lines derived from different malignancies, as well as our interest in developing new agents for metastatic bladder cancer, we evaluated the effects of flavopiridol in a series of normal urothelial cells, immortalized urothelial cell lines, and bladder cancer cell lines. We initially hypothesized that flavopiridol's effects would depend on the status of p16, pRb, and/or p53, and thus the lines chosen had all been well characterized in terms of these proteins. We found, in contrast, that flavopiridol leads to G2/M arrest in all cell lines and that cytotoxicity was independent of p16, pRb, or p53 status. We also showed that flavopiridol does lead to modest apoptosis in bladder cancer cell lines, but that this requires 72 h of continuous drug exposure. Unlike other studies, we were unable to demonstrate significant synergy between flavopiridol and radiation or other chemotherapeutic agents at modest concentrations. Importantly, though, we did not find evidence of antagonism. Finally, flavopiridol's cytotoxicity was maintained in a cell line overexpressing the MDR1 multidrug resistance protein. These data, in sum, suggest that flavopiridol may be a useful agent to investigate for the therapy of metastatic bladder cancer, either alone or in combination with other agents.

Methods

Cell culture and materials

Three established bladder cell lines (RT4, UMUC-3, 5637), two immortalized human urothelial cancer cell lines (HUC-E6, and HUC-E7), and primary human urothelial cell (HUC) cultures were used in these experiments. The bladder cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, Va.) and cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, Ga.), 0.1 m*M* nonessential amino acids (Gibco), and 1.0 m*M* sodium pyruvate (Sigma, St Louis, Mo.). HUC-E6 and HUC-E7 were obtained from Dr. C. Reznikoff (University of Wisconsin, Madison) and cultured on a thick layer of rat collagen in a supplemented Ham's F12 medium (F12+; Gibco BRL) containing 1% fetal calf serum, 0.1 m*M* nonessential amino acids, 2.0 m*M* L-glutamine (Sigma), 10 µg/ml insulin (Sigma), 5 µg/ml apotransferin (Sigma), 2.7 mg/ml dextrose (Fisher), and 0.1 µg/ml hydrocortisone (Sigma) [15]. Primary HUC were established on thick collagen gels with kerotinocyte serum-free medium supplemented with 50 µg/ml bovine pituitary extract and 50 ng/ml epidermal growth factor (KSFM+; Gibco) and subcultured in a 50:50 mixture of F12+ and KSFM+ [14]. Flavopiridol was obtained from John Wright, MD (National Cancer Institute, Investigational Drug Branch, CTEP). Methotrexate, cisplatin, doxorubicin, and CPT-11 were obtained from Sigma.

Apoptosis assay

The 5637 cell lines and UMUC-3 were treated with flavopiridol for 72 h. Floating and trypsin-detached cells were fixed with 1% paraformaldehyde and stained with 0.2 µg/ml DAPI (Sigma). Cytospins were visualized and photographed with standard fluorescence microscopy.

Cell cycle analysis

Cells were detached with trypsin, washed with 1 m*M* EDTA/1× phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde/0.1% Triton-X100, and stained for 15 min with 2 µg/ml propidium iodide (Sigma) in 5 m*M* EDTA/PBS. Flow cytometry was performed on a Becton-Dickinson FACScan with CellQuest™ v3.1 acquisition software. Cell cycle distribution was analyzed using the ModFit LT v2.0 program.

Growth assays

Growth inhibition was assayed by colony-forming assays or by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)

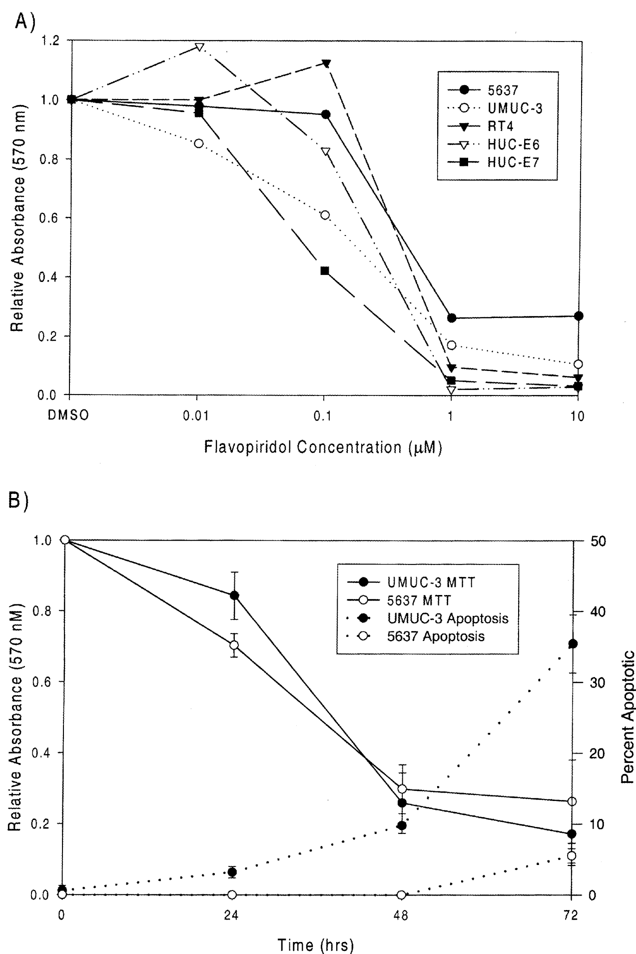

Fig. 1A,B Flavopiridol growth inhibition. **A** Growth inhibition of various bladder cancer cell lines by as a result of exposure to flavopiridol as determined by an MTT assay (see Methods). The assay was performed after 72 h of continuous drug exposure in exponentially growing cells. For clarity of presentation error bars have been omitted, but each point is the mean of six separate observations. Qualitatively similar results were observed for the UMUC-3 and 5637 cell lines when BrdU incorporation as opposed to MTT was used as the experimental endpoint (data not shown). **B** Time-course of flavopiridol growth inhibition and apoptosis in the UMUC-3 and 5637 cell lines. Cells were exposed to 1.0 µ*M* flavopiridol for the indicated times prior to subjecting them to an MTT assay or DAPI staining. For the latter assay the percent apoptotic nuclei is noted. Error bars represent the standard deviations of six (for MTT) or three (for DAPI staining) separate observations

Table 1 Characteristics of bladder cell lines. The status of p53, pRb, and p16 are as reported in references 14, 16, and 20

Cell line	Derived from	p53 status	pRb status	p16 status
RT4	Superficial papillary cancer	Wild type	Wild type	Deleted
UMUC3	Advanced cancer	Mutant	Wild type	Deleted
5637	Advanced cancer	Mutant	Mutant	Wild type
HUC-E6	Immortalized normal urothelium	Inactivated	Wild type	Inactivated
HUC-E7	Immortalized normal urothelium	Wild type	Inactivated	Wild type

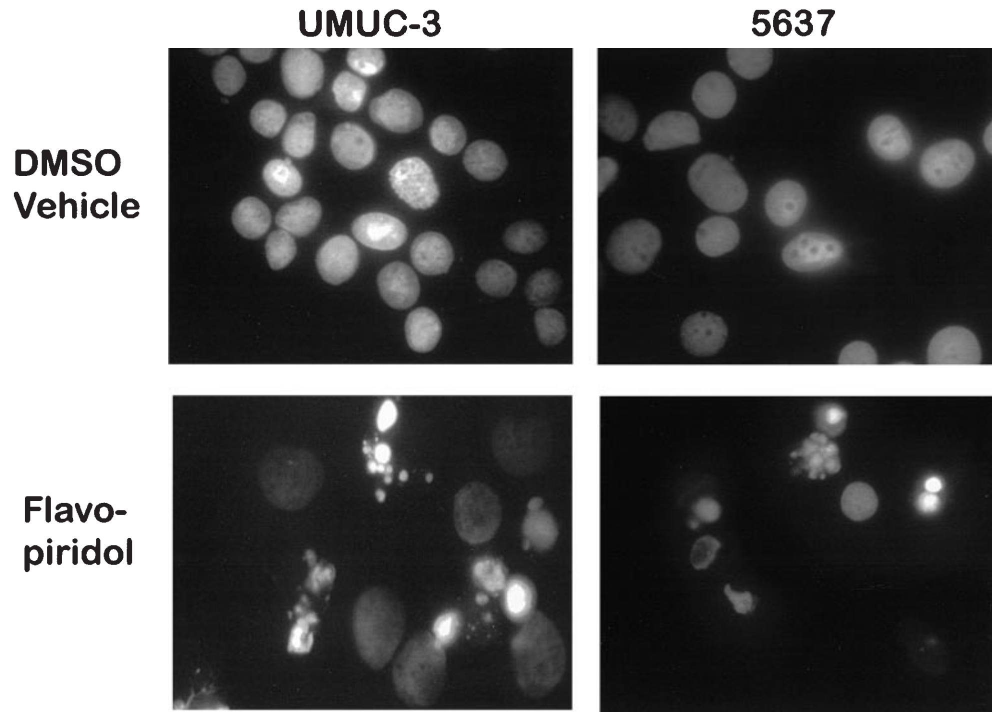

Fig. 2 Flavopiridol exposure leads to apoptosis. DAPI-stained UMUC-3 and 5637 cells following 72 h of treatment with 1.0 μ*M* flavopiridol or DMSO vehicle (see also Fig. 1B)

reduction as previously described [11]. Briefly, cells were plated on 96-well plates at a density of 3000–5000 cells/well, and drug treatment was begun 24 h later. At the experimental endpoint MTT (Sigma) was added to each well at a final concentration of 0.5 mg/ml. After 3 h incubation at 37 °C, the medium was aspirated, the cells were lysed with DMSO, and the absorbance of each well at 570 nm was measured in a BioRad Model 3550 microplate reader. The mean absorbance of six individual wells was used to calculate relative growth.

To assess the combined effects of radiation and flavopiridol, exponentially growing cells were plated at a single cell density on 60 × 15 mm tissue culture dishes. After 24 h, plated cells received 2 Gy of gamma radiation from a cesium-cobalt source, and were then treated with flavopiridol for 48 h immediately, or 24 h, or 48 h postirradiation. After an additional 7–10 days of growth, colonies were stained with 0.5 g crystal violet per 100 ml ethanol. Each experiment was performed twice in triplicate.

Generation of multidrug-resistant cells

UMUC-3 and 5637 bladder cancer cells were exposed to increasing concentrations of doxorubicin. Cells that continued to proliferate in the presence of 0.3 μ*M* doxorubicin were used for further studies. Individual cell clones were not isolated. To determine whether drug resistance was due to overexpression of the MDR1 protein, cells were fixed and stained with the UIC2 antibody (gift from Dr. Igor Roninson, University of Illinois, Chicago) and analyzed by fluorescent activated cell sorting (FACS) as previously described [17].

Results

Three established bladder cancer cell lines, and two immortalized normal urothelial cell lines in which the status of p53, pRb, and p16 are known (Table 1) were used to assess flavopiridol's cytotoxicity in relation to common genetic alterations in human bladder cancer. Figure 1A shows that flavopiridol mediated growth inhibition of these cells as determined in the MTT assay. There were only small differences in the IC_{50} values (concentrations that inhibit growth by 50%) and these did not correlate with the p53, pRb, or p16 status. Figure 1B shows that growth inhibition required at least 48 h of continuous drug exposure, even at highly toxic drug concentrations. To determine whether flavopiridol-induced growth inhibition led to actual cell death, UMUC-3 and 5637 cells were stained with DAPI after exposure to 1.0 μ*M* flavopiridol for various times

(Fig. 2). Figure 1B shows that apoptosis was induced in both cell lines but was evident in only 5.5% of 5637 cells, even after 72 h of exposure.

To determine whether growth inhibition was due to cell cycle arrest, asynchronously growing cells were exposed to 1.0 μ*M* flavopiridol for 24 to 48 h and subjected to cell cycle analysis. As shown in Fig. 3, all lines preferentially arrested in G2/M and this occurred with as little as 24 h of drug exposure. A similar result was obtained with normal cultured urothelial cells suggesting that the G2/M arrest is not a function of G1 checkpoint abnormalities present in the cell lines.

Because γ-radiation can also induce cell cycle arrest, we investigated the combined effects of flavopiridol and radiation. A small dose of γ-radiation (2 Gy) in asynchronous UMUC-3 cells, did not lead to any significant cell cycle arrest within 48 h. However, concomitant flavopiridol and radiation led to slightly greater, but statistically significant, G2/M arrest than flavopiridol alone (Fig. 3B). We thus elected to more carefully examine the combined effects of flavopiridol and radiotherapy on the in vitro colony-forming ability of the UMUC-3 and 5637 cells. Using a dose of radiation that led to approximately a 50% inhibition of colony-forming efficiency, we were unable to demonstrate any significant synergism, although it should be noted that an apparent additive effect (as opposed to antagonistic effect) was evident (Fig. 4A). It should be noted though that only one radiation dose was used and thus formal tests for synergy were not performed. Delaying flavopiridol treatment by 24 or 48 h after radiation did not alter these findings.

Because of bladder cancer's sensitivity to cisplatin, we also examined the combined effect of flavopiridol and cisplatin. Once again we were unable to demonstrate any obvious synergistic effect at a dose of cisplatin that caused 50–75% growth inhibition (Fig. 4B). Preliminary evaluation of doxorubicin, methotrexate, and camptothecin (dosed at their IC_{50}) in combination with flavopiridol also did not reveal any synergistic or antagonistic activity (data not shown). Interestingly, flavopiridol cytotoxicity was maintained in two doxorubicin-resistant cell lines overexpressing the MDR1 protein (Fig. 5A). In fact, overexpression of MDR1 led to a slight increase in overall flavopiridol sensitivity (Fig. 5B). More importantly, we were unable to generate cell lines resistant to flavopiridol despite readily generating doxorubicin-resistant lines from the same parental line.

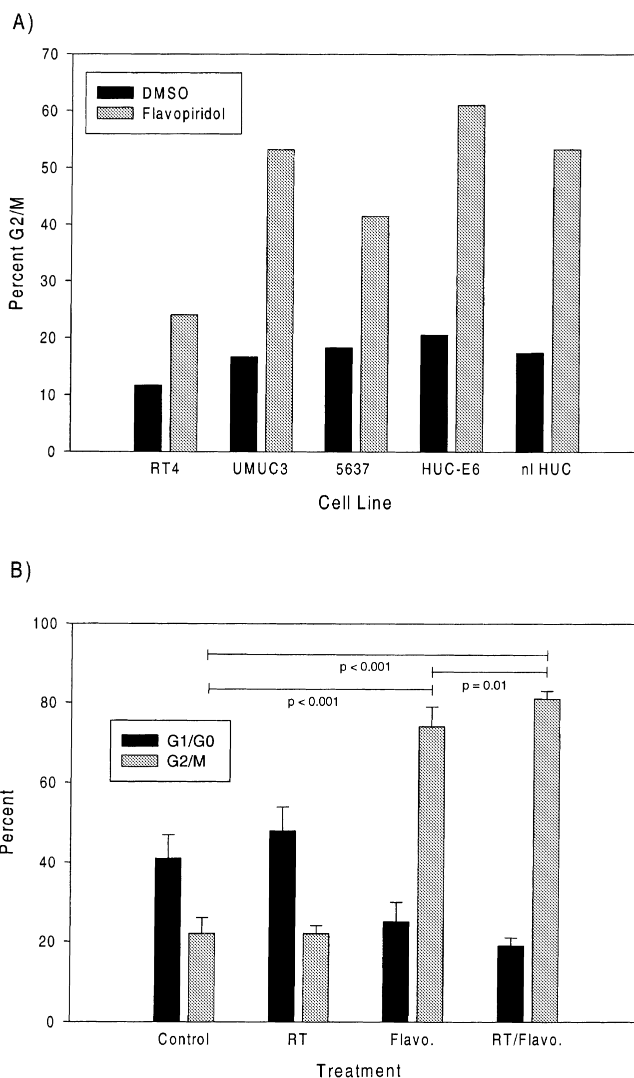

Fig. 3A,B The effect of 1.0 μ*M* flavopiridol on cell cycle distribution. **A** G2/M distribution in the indicated cell lines as well as normal human urothelial cells (*nl HUC*) after 36 to 48 h of drug exposure. Time-course experiments in the UMUC-3 cell line showed that this effect was evident by 24 h of exposure (data not shown). **B** Effect of combined radiation (*RT*) and flavopiridol on UMUC-3 cells. Cells were treated with 1.0 μ*M* flavopiridol or vehicle with or without additional γ-radiation (2 Gy). Differences between control and radiation alone were not significant ($P = 0.10$), but the differences between control and flavopiridol or the combined treatment as well as the difference between flavopiridol and combined treatment were significant with *P*-values as indicated. The standard two-tailed Student's *t*-test was used to assess statistical significance

Discussion

In sum, we showed that flavopiridol is capable of inducing G2/M arrest and growth inhibition in a variety of normal, immortalized, and malignant urothelial cells. This confirms previous observations in cell lines from other solid malignancies [2, 7, 10]. We were also able to demonstrate apoptosis at relevant growth inhibitory concentrations, but unlike the observation in some hematopoietic cell lines, the level of apoptosis was quite modest and required up to 72 h of continuous drug exposure [13]. Growth inhibition did not depend on the status of the commonly altered cell cycle regulatory proteins p53, pRb, or p16. Given the increasing evidence that p53 alterations confer resistance to a number of "standard" chemotherapeutic agents, flavopiridol may thus be an important candidate for therapy of p53 mutant tumors [12].

We were unable to demonstrate obvious synergy between flavopiridol and radiation, cisplatin, metho-

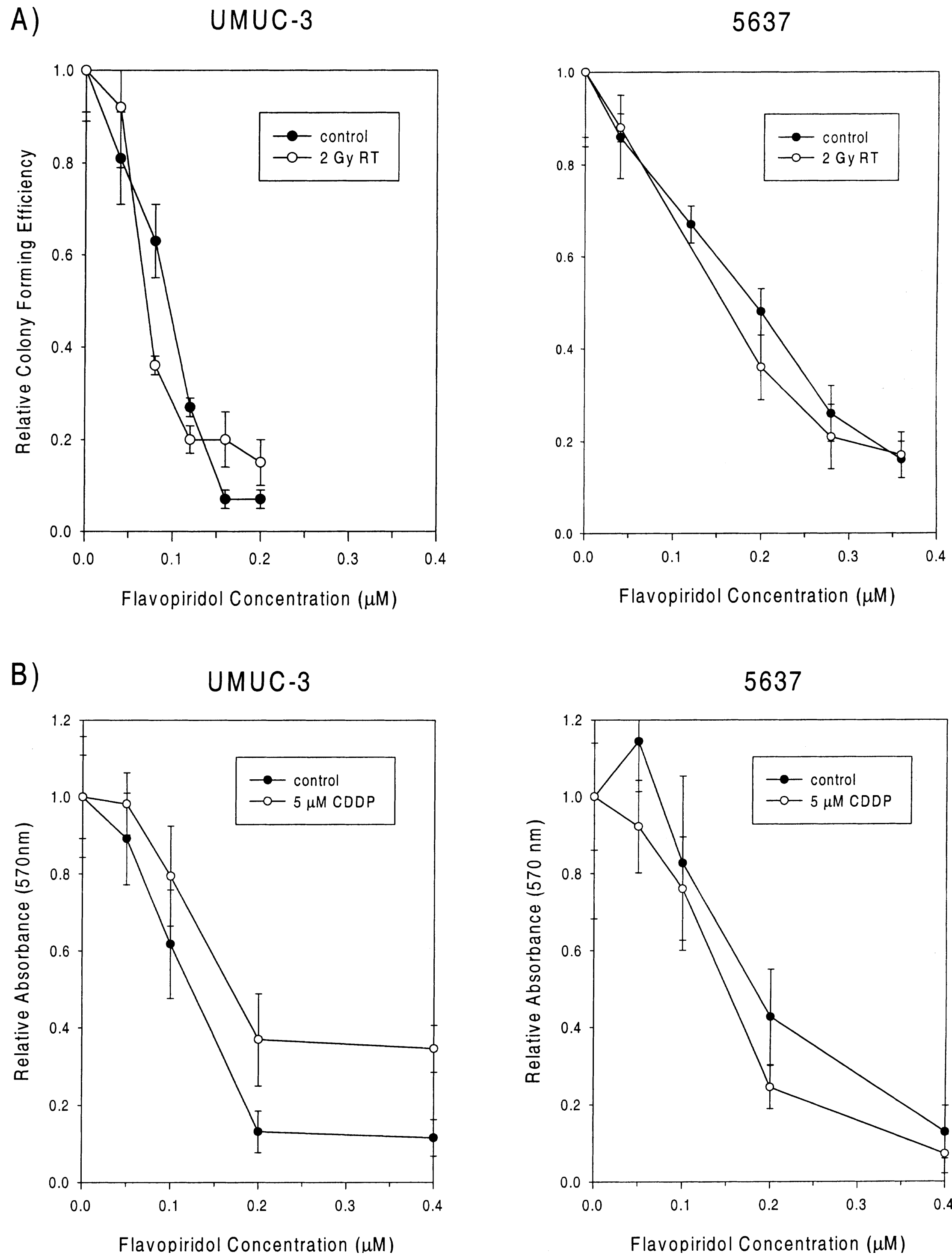

Fig. 4A,B Combination therapy with flavopiridol and radiation or cisplatin does not lead to synergistic toxicity. **A** Colony-forming efficiency of UMUC-3 and 5637 cells continuously exposed to flavopiridol for 48 h immediately following 2 Gy γ-radiation or mock treatment. In both cases the absolute colony-forming efficiency of the radiation-treated cells is approximately 50% of the control cells. **B** MTT assay for UMUC-3 and 5637 cells exposed to both flavopiridol and cisplatin for 72 h. The cisplatin dose led to a 50–70% growth inhibition by itself

trexate, doxorubicin, and camptothecin in the induction of cytotoxicity. We should note, however, that our studies were all performed at a single dose of the concomitant agent that led to approximately a 50% growth inhibition. Thus formal tests of synergism were not performed. Previous studies using more formal synergism analysis have suggested that doses of several concomitant agents that lead to higher levels of growth inhibition do demonstrate synergistic toxicity with flavopiridol [3]. Our data also suggest that exposure to

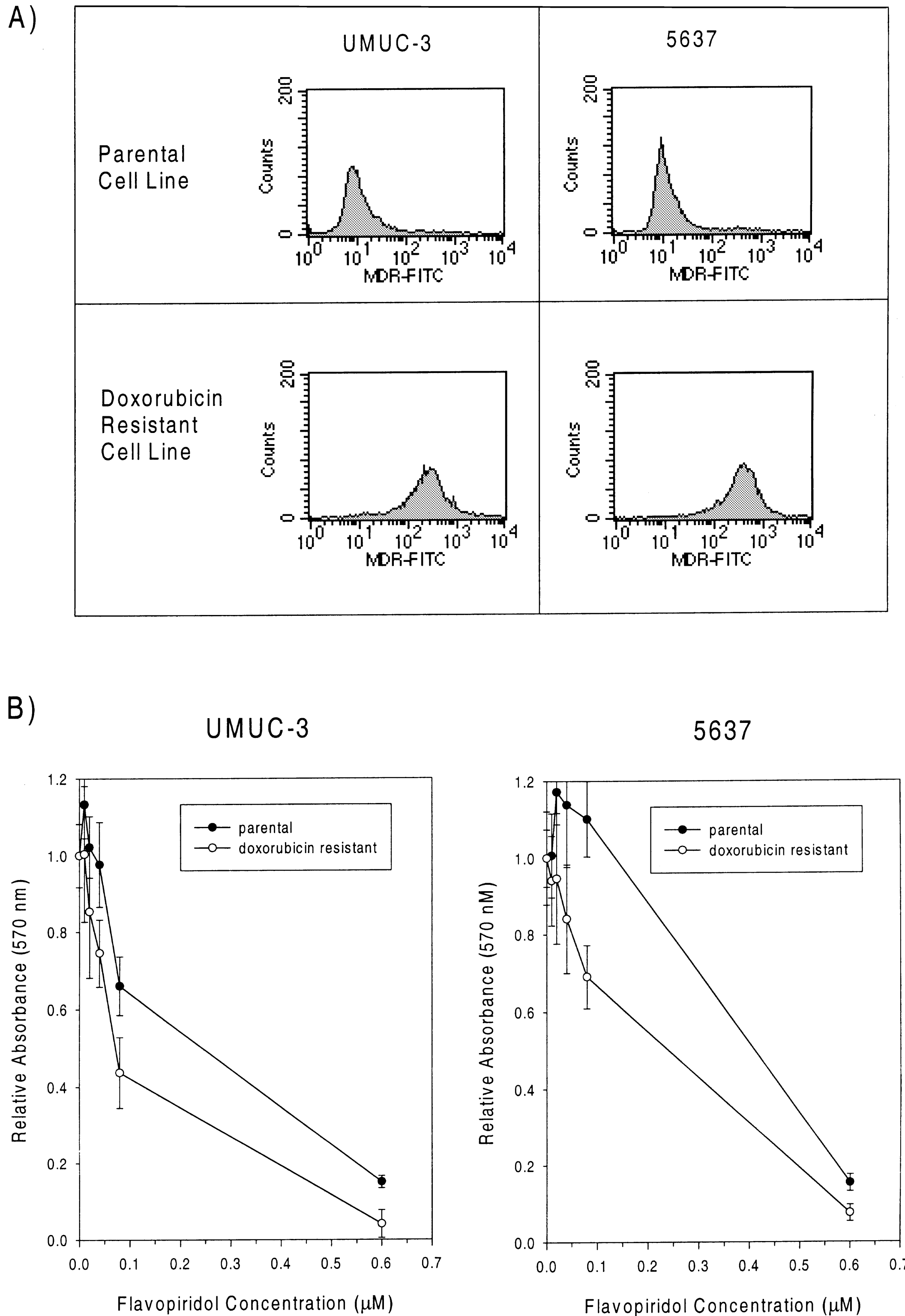

Fig. 5A,B Overexpression of the multidrug-resistance protein MDR1 does not inhibit flavopiridol growth inhibition **A** Parental and doxorubicin-resistant 5637 and UMUC-3 cells were stained with an MDR1 antibody and detected with a fluoroscein-labeled secondary antibody using FACS. **B** Flavopiridol mediated growth inhibition in the parental and doxorubicin-resistant 5637 and UMUC-3 cell lines

radiation and conventional chemotherapeutic agents is not antagonistic to flavopiridol toxicity. This is a critical observation given that initial studies of flavopiridol have shown decreased cytotoxicity in growth-arrested cells [7]. Furthermore, we demonstrated that resistance to flavopiridol is difficult to generate in vitro, despite the fact that the cells we used were p53 mutant, a phenotype

for which drug-resistant mutants are readily generated [9, 22]. In addition, cells overexpressing the multidrug-resistance protein, one of the most common mechanisms of chemotherapeutic drug resistance, were not resistant to flavopiridol, and in fact may even have been modestly more sensitive. This is somewhat surprising given the previous observation that flavopiridol stimulates the ATPase activity of the human MDR1 protein [6].

In sum, our observations suggest that flavopiridol may be an interesting agent to explore in clinical trials with conventional chemotherapeutic agents or radiation therapy in bladder cancer patients.

Acknowledgement The authors would like to thank Dr. Eileen Dolan for critical reading of the manuscript.

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