

POTENTIATION OF CYTOTOXICITY AND RADIOSENSITIZATION OF (E)-2-DEOXY-2'-(FLUOROMETHYLENE) CYTIDINE BY PENTOXIFYLLINE *IN VITRO*

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(E)-2'-deoxy-2'-(fluoromethylene) cytidine (FMdC), a novel inhibitor of ribonucleotide-diphosphate reductase, has been shown to have anti-tumor activity against solid tumors and sensitize tumor cells to ionizing radiation. Pentoxifylline (PTX) can potentiate the cell killing induced by DNA-damaging agents through abrogation of DNA-damage-dependent G₂ checkpoint. We investigated the cytotoxic, radiosensitizing and cell-cycle effects of FMdC and PTX in a human colon-cancer cell line WiDr. PTX at 0.25–1.0 mM enhanced the cytotoxicity of FMdC and lowered the IC₅₀ of FMdC from 79 ± 0.1 to 31.2 ± 2.1 nM, as determined by MTT assay. Using clonogenic assay, pre-irradiation exposure of exponentially growing WiDr cells to 30 nM FMdC for 48 hr or post-irradiation to 0.5 to 1.0 mM PTX alone resulted in an increase in radiation-induced cytotoxicity. Moreover, there was a significant change of the radiosensitization if both drugs were combined as compared with the effect of either drug alone. Cell-cycle analysis showed that treatment with nanomolar FMdC resulted in S-phase accumulation and that such an S-phase arrest can be abrogated by PTX. Treatment with FMdC prior to radiation increased post-irradiation-induced G₂ arrest, and such G₂ accumulation was also abrogated by PTX. These results suggest that pharmacological abrogation of S and G₂ checkpoints by PTX may provide an effective strategy for enhancing the cytotoxic and radiosensitizing effects of FMdC. *Int. J. Cancer* 80:155–160, 1999.

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(E)-2'-deoxy-2'-(fluoromethylene) cytidine (FMdC) has been synthesized as a mechanism-based inhibitor of ribonucleotide-diphosphate reductase (Bitonti *et al.*, 1995), and is presently in Phase-I clinical trial. Ribonucleotide reductase is a key enzyme in DNA synthesis. This enzyme has increased activity in rapidly growing tumors, and represents a potential target for chemotherapy. In contrast to other inhibitors of RR, such as hydroxyurea and ara-C, which are widely used in the treatment of leukemias and show limited or no anti-tumor activity against solid tumor, FMdC is a promising novel chemotherapeutic agent with striking activity in a variety of solid-tumor cells (Bitonti *et al.*, 1995; Piepmeier *et al.*, 1996). Several experimental studies have demonstrated that FMdC sensitized human colon- and cervical-cancer cells and mouse mammary-tumor cells to radiation *in vitro* (Snyder, 1994; Li *et al.*, 1996), and we have reported that the anti-tumor and radiosensitizing effects are also evident *in vivo* (Sun *et al.*, 1997).

Methylxanthines such as caffeine enhance cytotoxicity induced by DNA-damaging agents (Lau and Pardee, 1982). Caffeine and pentoxifylline (PTX) potentiate the cytotoxicity of several chemotherapeutic drugs such as cisplatin, camptothecin and alkylating compounds (Fan *et al.*, 1995; Teicher *et al.*, 1991). In contrast, those compounds antagonize the cytotoxic effect of doxorubicin. Preferential sensitization has been reported using a combination of caffeine and ionizing radiation or caffeine and cisplatin (Russell *et al.*, 1995). In all these studies, the sensitization is explained by the preferential abrogation of the G₂ checkpoint in cells lacking *p53* function. FMdC acts as a novel anti-tumor drug and the modulation of its cytotoxicity and radiosensitization by PTX has never been evaluated.

In the present study, we used a human colon-cancer cell line (WiDr), known to be *p53*-mutated, to investigate whether PTX enhances FMdC-mediated cytotoxicity and radiation sensitization.

In addition, we tried to assess whether PTX can affect cell-cycle distribution induced by FMdC and irradiation. From our findings, we may conclude that PTX abrogates FMdC-induced S-phase arrest and radiation-induced G₂ arrest. These effects on cell-cycle blocks may explain, at least in part, the enhancement of both the cytotoxicity and the radiosensitizing potential of FMdC on WiDr cells *in vitro*.

MATERIALS AND METHODS

Chemicals and cell culture

FMdC (MDL 101,731), kindly provided by Hoechst Marion Roussel (Cincinnati, OH), was dissolved in distilled water. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Buchs, Switzerland). MTT was dissolved at a concentration of 5 mg/ml in RPMI-1640 medium without phenol red, filtered, then frozen at –80°C for storage. PTX was a gift from Hoechst (Frankfurt, Germany). PTX was dissolved in PBS to 100 mM stock solution and stored at –20°C.

The human colon-cancer cell line WiDr was obtained from the ATCC (Rockville, USA). Cells were maintained in minimum essential medium containing 10% fetal bovine serum (FBS), 1% non-essential aminoacids and 2 mM L-glutamine. The cells were incubated in a humidified 5% CO₂ atmosphere at 37°C. In these conditions, the mean doubling time of the exponentially growing cells was 23 hr.

MTT assay

The cytotoxic interaction of FMdC and PTX was analyzed using a MTT assay as described (Li *et al.*, 1997). Briefly, the WiDr cells (4000 cells/well) were seeded in duplicate in 96-well plates; at the same time, drug-containing medium was added. FMdC was used in concentrations ranging from 10 nM to 320 nM, and PTX was used at a concentration from 0.25 mM to 4.0 mM. FMdC and PTX were added simultaneously. After 3-day incubation, 100 µl MTT were added to each well and incubated for 4 hr at 37°C. The supernatants were removed and formazan crystals were re-suspended in 200 µl of DMSO. The plates were shaken for 5 min, then the optical densities (OD) of each well were read at 570 nm, with the reference wavelength set at 690 nm using a SLT SPECTRA II (Tecan, Hombrechtikon, Switzerland) plate recorder. Cell survival was calculated as the percentage absorbance of untreated cells (Li *et al.*, 1997). The IC₅₀ (50% inhibitory concentration) values were

Abbreviations: ara-C, arabinosylcytosine; DMSO, dimethyl sulfoxide; dNTP, deoxyribonucleoside 5'-triphosphate; ER, enhancement ratios; FMdC, (E)-2'-deoxy-2'-(fluoromethylene) cytidine; IC₅₀, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PE, plating efficiency; PTX, pentoxifylline; UCN-01, 7-hydroxystaurosporine.

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defined as the drug concentrations inducing a 50% reduction in the OD as compared with untreated cells.

Clonogenic assay

Assessment of the radiosensitization observed with FMdC alone, with PTX alone and with the combination of both drugs was made by clonogenic assay. The WiDr cells, in conditions of exponential growth, were treated with FMdC prior to irradiation, trypsinized, and re-suspended in fresh medium. In preliminary experiments, we observed, as expected for a nucleoside analogue, the marked effect of prolonged exposure prior to irradiation on the radiosensitizing capacity of FMdC. After trypsinization, an appropriate number of cells was plated into 60-mm² tissue-culture dishes. After 3-hr incubation, introduced to obtain maximal cell attachment, the dishes were irradiated at a dose rate of 80.2 cGy/min with an Oris IBL cesium-37 source at room temperature. PTX was added immediately following irradiation, since we expected PTX to be active on the post-irradiation G₂/M block. After 2 weeks, colonies were visualized by staining with crystal violet, and colonies of 50 cells or more were counted. All colony counts were adjusted for plating efficiency (PE) to yield corrected survival of 100% for untreated controls. Similarly, colony counts for PTX-treated cells were adjusted for drug toxicity to yield corrected survival of 100% for unirradiated PTX-treated controls. Radiosensitization is expressed as an enhancement ratio, defined as the mean survival fraction (SF) for control/mean SF for FMdC and/or PTX.

Flow cytometry

Cells were processed in the same way as for cell survival. WiDr cells in the exponential-growth phase were treated by FMdC alone, by irradiation alone, or by FMdC + irradiation in the presence or the absence of PTX. The cells were harvested at the indicated time, fixed in 70% alcohol, and stored in the dark at 4°C for at least 24 hr until analysis.

After fixation, cells were washed twice in ice-cold PBS containing 200 U/ml RNase A. Cellular DNA was stained with 3 ml 0.5 mg/ml propidium-iodide solution. Cell-cycle determination was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), using the cellfit software. Results are expressed as a percentage of total cells at a specific cell phase.

Statistical analysis

Each experiment was repeated 2 or 3 times. Data are presented as the mean \pm the standard error of 2 or independent experiments. Means were compared by the Student's 2-tailed *t*-test. A difference was considered significant if a value of 0.05 was reached. ANOVA was used to test the main effects of FMdC and PTX alone, and their interaction of radiosensitization. Isobologram analysis was used to evaluate the cytotoxic interaction of FMdC and PTX (Chou and Talalay, 1984).

RESULTS

Potentiation of FMdC cytotoxicity by PTX

The cytotoxic effect of FMdC and PTX on WiDr was measured with an MTT assay after 3-day exposure. The IC₅₀ was 79.0 \pm 1.0 nM for FMdC and 2.7 \pm 0.1 mM for PTX. PTX alone, up to concentrations of 1 mM, had a minimal effect on the viability of WiDr-cells, with approximately 80% cell survival. A 3-day exposure to 0.25–1.0 mM PTX resulted in a significant increase of FMdC cytotoxicity in a dose-dependent manner (Fig. 1a). Addition of 0.25, 0.5 and 1.0 mM PTX to various concentrations of FMdC lowered the IC₅₀ of FMdC to 54.3 \pm 2.2 nM, 37.0 \pm 1.2 nM, and 31.2 \pm 2.1 nM respectively. Isobologram analysis showed that the cytotoxicity of FMdC and PTX was supra-additive (Fig. 1b).

Enhancement of FMdC radiosensitization by PTX

We investigated first the importance of the duration of exposure to FMdC on cytotoxicity and on radiosensitization. As found for

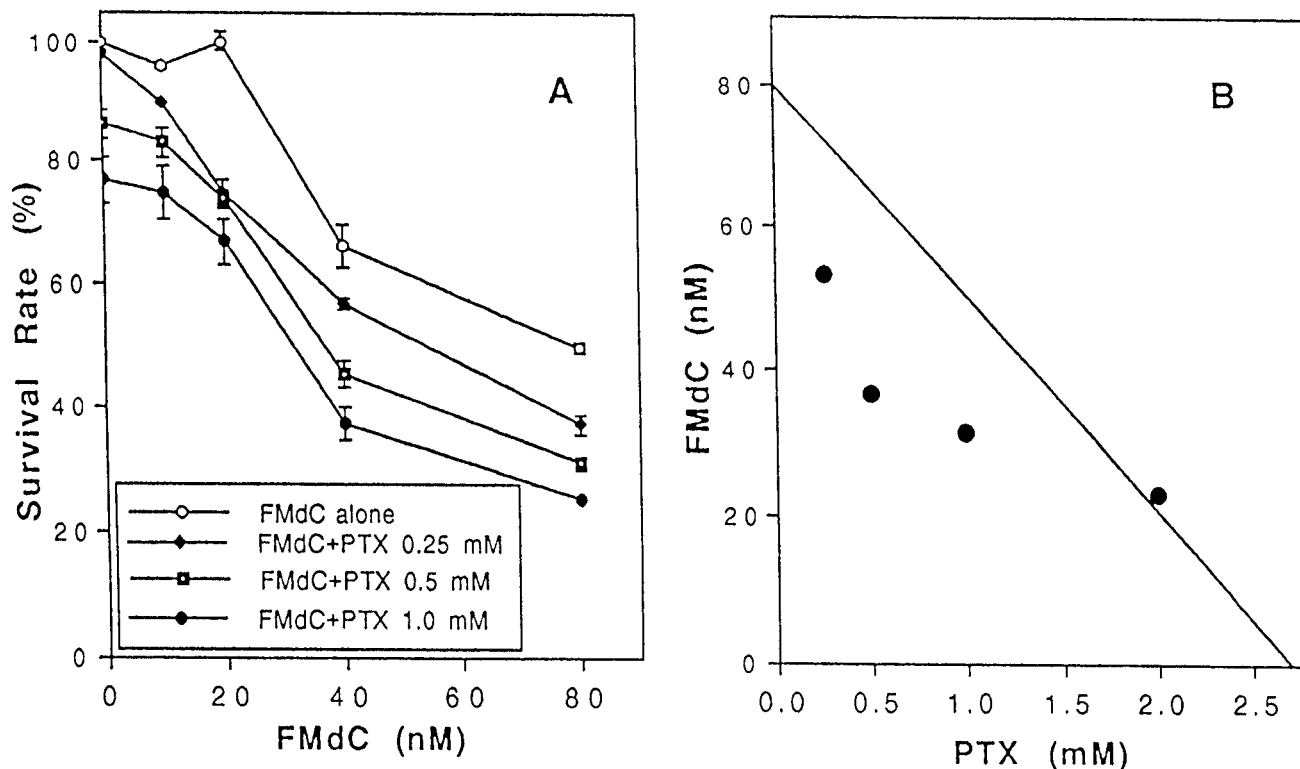


FIGURE 1 – Effect of PTX on FMdC cytotoxicity using a 3-day MTT assay. (a) Survival rate of FMdC in the presence of 0.25 to 1.0 mM PTX. Cells were treated with different concentrations of FMdC with or without PTX. (b) Isobologram analysis showing a supra-additive cytotoxic effect of FMdC and PTX.

plating efficiency (PE), the decrease in the surviving fraction after irradiation, compared with corresponding controls (Fig. 2a), did not occur if cells were exposed for 6 to 24 hr to 30 nM FMdC (Fig. 2b). In contrast, after 48 hr exposure to 30 nM FMdC there was a significant decrease in the surviving fraction, compared with irradiated controls, preceding in time the significant reduction in PE observed in unirradiated but FMdC-treated cells (Fig. 2a,b).

To assess the effect of PTX on FMdC radiosensitization, cells were incubated for 48 hr prior to radiation with 30 nM FMdC and PTX was added immediately after irradiation. As noted above, no significant cytotoxicity but measurable radiosensitization was observed with FMdC at this time point by clonogenic assay. PTX alone at concentrations of 0.25 to 1.0 mM had minimal cytotoxicity, with a PE of 92%. In Figure 3a, the radiosensitizing effect of 30 nM FMdC in the presence or the absence of 0.5 mM PTX on WiDr cells has been plotted. PTX alone at concentrations of 0.5 mM and 1.0 mM enhanced the cell killing induced by irradiation. The enhancement ratios (ER) at the clinical relevant fraction dose of 2 Gy were 1.22 ± 0.03 for 30 nM FMdC alone, 1.23 ± 0.03 for 0.5 mM PTX and 1.4 ± 0.15 for 1.0 mM PTX alone. In contrast, 0.25 mM PTX alone had no significant effect on radiosensitization (data not shown). However, addition of 0.5 to 1.0 mM PTX resulted in an increase of FMdC-mediated radiosensitization. When cells were treated with 30 nM FMdC in the presence of 0.5 and 1.0 mM PTX, the corresponding ER ranged from 1.69 ± 0.03 to 2.04 ± 0.32 . In Figure 3b, we reported the radiosensitization of WiDr cells as a function of FMdC concentration in the presence of 0.5 mM PTX. WiDr cells were exposed to varying concentrations of FMdC (10–50 nM) for 48 hr prior to a single dose of 2 Gy irradiation. The survival of irradiated cells decreased gradually as the drug concentration increased from 20 to 50 nM. Whatever the concentration of FMdC used, 0.5 mM PTX yielded an additive effect on FMdC radiosensitization.

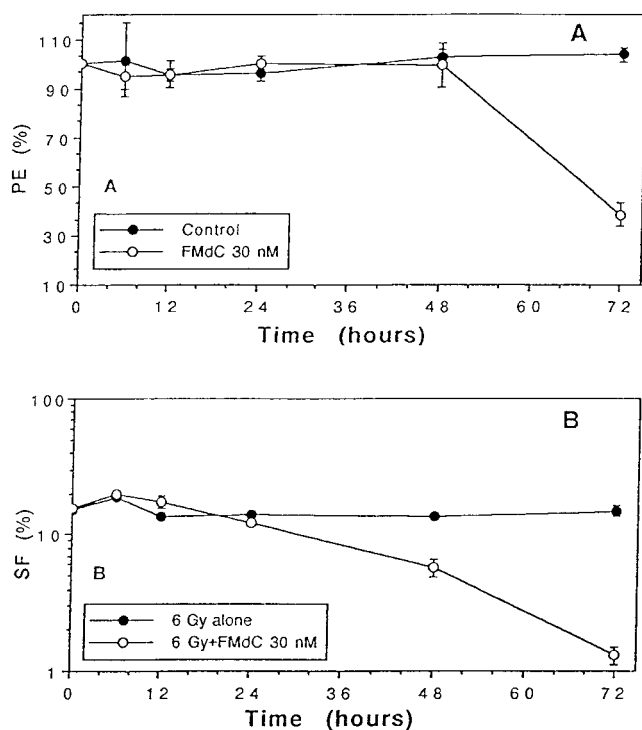


FIGURE 2—Cytotoxicity and radiosensitization of FMdC as a function of time. Cells were exposed to 30 nM FMdC for the indicated time periods, and clonogenicity was assessed with or without 6 Gy. (a) Effect of FMdC alone on cell survival or plating efficiency (PE). (b) Survival of cells treated with 6 Gy with or without 30 nM FMdC at the indicated time point.

Effect of FMdC alone on cell-cycle distribution

To investigate the effect of FMdC on cell-cycle progression, asynchronous exponentially growing cells were treated with FMdC for 6 to 48 hr, then subjected to DNA analysis by flow cytometry. Exposure of WiDr cells to 20 to 40 nM FMdC alone resulted in an arrest of cells in S phase in a concentration-dependent manner, as compared with the control (Fig. 4). In contrast, FMdC at the lower concentration of 10 nM had no detectable effect on cell-cycle distribution. At 20 nM FMdC, significant S-phase accumulation is observed only at 48 hr after treatment. S-phase arrest induced by 30 nM and 40 nM FMdC appeared at 12 hr, peaks at 24 hr, and reached a plateau at 48 hr after treatment. Such S-phase arrest was paralleled by depletion of G₁ and G₂M phase (data not shown).

PTX abrogation of FMdC-induced S-phase arrest

To examine the potential of PTX to influence FMdC-induced cell-cycle perturbation, cells were treated for 24 hr with 30 nM FMdC in the presence of PTX. At concentrations up to 1 mM, PTX alone had no detectable effect on cell-cycle progression of untreated control cells (data not shown). At 24 hr after treatment with 30 nM FMdC, addition of 0.25 to 1.0 mM PTX abrogated FMdC-induced S-phase arrest in a dose-dependent manner (Fig. 5). PTX at concentrations of 0.5 mM and 1.0 mM reduced S-phase arrest from $66.5 \pm 0.9\%$ to $54.4 \pm 1.2\%$ and to $39.3 \pm 1.4\%$ respectively. The reduction of S-phase arrest by PTX was paralleled by an increase in G₁ and G₂M phase (Fig. 5b).

Effect of PTX on G₂ arrest induced by radiation with or without FMdC

We have demonstrated that irradiation resulted in dose-dependent G₂ arrest in WiDr cells and that this G₂ arrest can be over-ridden by PTX (Li *et al.*, 1998). In the present study, we investigated further whether pre-irradiation exposure of WiDr cells to FMdC affects irradiation-induced G₂ arrest, and whether PTX abrogates this G₂ arrest after FMdC treatment. Cells were treated with 30 nM FMdC for 48 hr prior to radiation, then irradiated incubated with 0.5 mM PTX for 24 hr. Flow-cytometry analysis confirms our observation that PTX inhibits the G₂ arrest induced by radiation (Fig. 6). Moreover, the pre-irradiation treatment with 30 nM FMdC for 48 hr results in an increase in G₂ arrest in irradiated cells. Such G₂/M accumulation observed after FMdC treatment and irradiation is significantly abrogated by 0.5 mM PTX (Fig. 6).

DISCUSSION

The data reported in this study demonstrate that PTX as a biological response modifier is able to modify the intrinsic toxicity of FMdC and sensitize WiDr cells *in vitro* to ionizing irradiation. The human colon-cancer WiDr cells exposed to nanomolar concentrations of FMdC are characterized by S-phase arrest. This latter can be abrogated by exposure to PTX, and this correlates with enhancement of the cytotoxic effect of FMdC, while the radiosensitizing effect of FMdC is also enhanced by PTX. This observation is compatible with the observed abrogation of G₂ arrest by PTX, determined by flow cytometry on cells exposed to irradiation and FMdC. Our experiments therefore suggest that PTX is a potent inhibitor of cell-cycle arrest, both in S and in G₂, and that this abrogation of cell-cycle blocks increases the cytotoxic and radiosensitizing effects of FMdC.

The mechanism by which FMdC produces cytotoxicity and radiosensitization is much less clear. While FMdC shares some metabolic similarities with other ribonucleotide-reductase inhibitors, such as ara-C, hydroxyurea and gemcitabine, these drugs have markedly different anti-tumor effects (Bitonti *et al.*, 1995; Shewach *et al.*, 1994; Iwasaki *et al.*, 1997). Presumably, FMdC is a mechanism-based, irreversible inhibitor of ribonucleotide reductase. It is possible that this drug acts in a manner similar to other ribonucleotide-reductase inhibitors, such as 2',2'-difluorocytidine (gemcitabine). The latter has been shown to cause inhibition of DNA synthesis without significantly inhibiting either protein or RNA synthesis (Huang *et al.*, 1991). Inhibition of DNA synthesis with

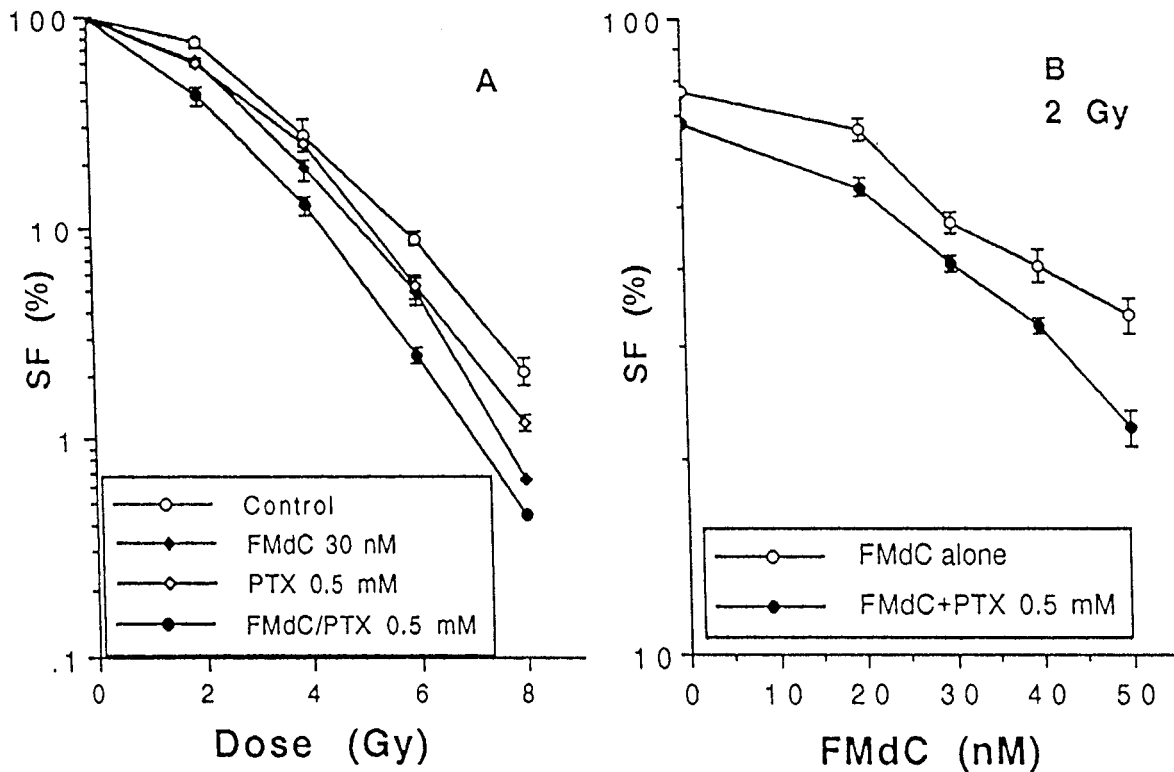


FIGURE 3 – Potentiation of FMdC radiosensitization by PTX. Cells were treated with FMdC for 48 hr and irradiated, after which PTX was added immediately. (a) Survival fraction of cells exposed to 30 nM FMdC in the presence of 0.5 to 1.0 mM PTX. (b) Surviving fraction of cells as a function of dose of FMdC (0–50 nM) with or without 0.5 mM PTX. Cells were exposed to FMdC for 48 hr before irradiation at 2 Gy.

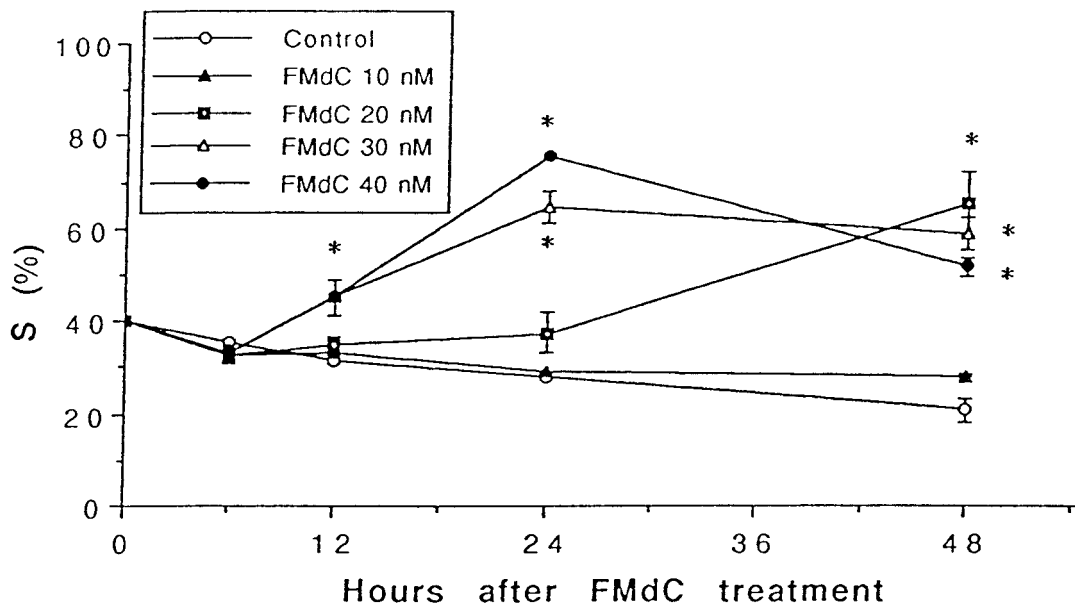


FIGURE 4 – Effect of FMdC alone on cell-cycle distribution as a function of concentration. Cells were treated with varying concentrations of FMdC for the different time periods. *Values significantly different from control ($p < 0.05$).

gemcitabine might be due to decreased deoxynucleotide pools (Fox, 1993), or to chain termination after being converted to the triphosphate (Huang *et al.*, 1991). Shewach *et al.* (1994) reported that the depletion of deoxyribonucleoside 5'-triphosphate (dNTP) pools by gemcitabine may also contribute to its radiosensitizing effect.

The S-phase arrest induced by nanomolar FMdC in the present study is in accordance with other data: whatever the exact

mechanism of DNA-synthesis inhibition, the ultimate effect is an S-phase block (Plunkett *et al.*, 1989; Paulovich and Hartwell, 1995). S-phase arrest can also be induced by other DNA-damaging agents, such as camptothecin, cisplatin and bleomycin (Perras *et al.*, 1993; Shao *et al.*, 1997; Paulovich and Hartwell, 1995; Goldwasser *et al.*, 1996). Block in the DNA-synthesis phase of the cell cycle by FMdC is expected, since the ribonucleotide-reductase

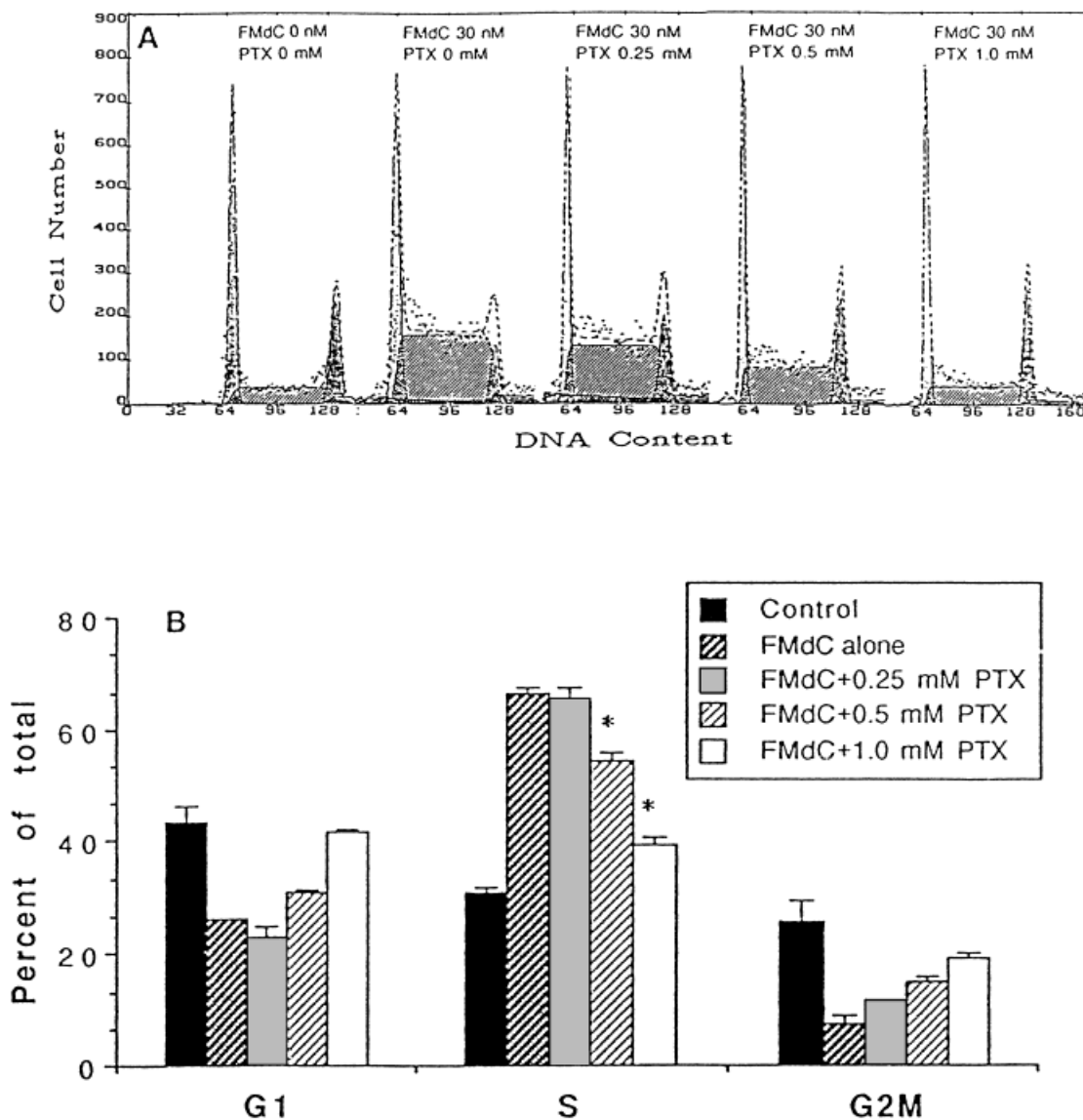


FIGURE 5 – Alleviation of FMdC-induced S-phase arrest by PTX. Cells were treated with 30 nM FMdC in the presence of 0.25 to 1.0 mM PTX for 24 hr, and then harvested. (a) Cells analyzed by flow cytometry. (b) Quantitation of data in (a), * $p < 0.05$ compared with S-phase in cells treated with FMdC alone.

activity is cell-cycle dependent, being induced essentially when cells are passing from G₁ to S (Eriksson *et al.*, 1984). However, more basic research will be required before we can reach a conclusion on the metabolism and mechanisms of cytotoxicity and radiosensitization of FMdC.

DNA-damaging agents are known to result in perturbation of cell-cycle progression. Cells with damaged DNA arrest at checkpoints in the G₁ or the S phase, to prevent replication of damaged DNA, or in G₂ to prevent aberrant mitosis (Hartwell and Kastan, 1994). The current analysis of cell-cycle distribution in WiDr cells shows that FMdC alone causes predominantly S-phase arrest, and that the addition of PTX abrogates this arrest in a dose-dependent manner, resulting in enhancement of FMdC cytotoxicity. Similarly, other authors have observed enhancement of the cytotoxicity of DNA-damaging agents by 7-hydroxystaurosporine (UCN-01), a selective protein-kinase-C inhibitor. In their experiments, UCN-01 was used to abrogate S-phase arrest in order to enhance the cytotoxicity of camptothecin or cisplatin in human colon-cancer cells and in Chinese-hamster ovary cells (Bunch and Eastman, 1997; Shao *et al.*, 1997). The preferential potentiation of camptothe-

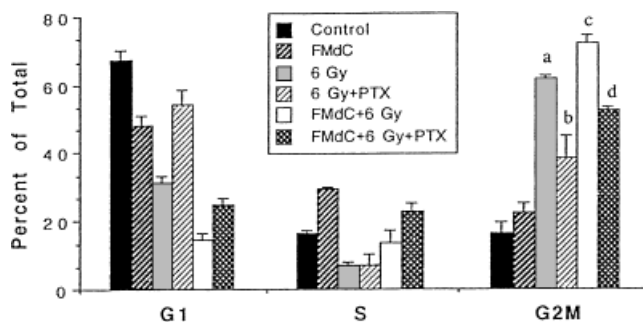


FIGURE 6 – Effect of PTX on cell-cycle distribution of WiDr cells treated with FMdC and irradiation. Cells were treated with 30 nM FMdC for 48 hr, then irradiated, and 0.5 mM PTX was added immediately. Cells were harvested 24 hr after irradiation before flow cytometry. a, $p < 0.01$ relative to control; b, $p < 0.01$ relative to 6 Gy; c, $p < 0.05$ relative to 6 Gy; d, $p < 0.05$ relative to FMdC-treated and irradiated (6 Gy) cells.

cin cytotoxicity by UCN-01 is observed in *p53*-defective tumor cells (Shao *et al.*, 1997). Perras *et al.* (1993) reported that PTX enhances cisplatin cytotoxicity in a human ovarian cell line (BG-1), and postulate that this toxicity enhancement is probably due to the suppression of DNA synthesis or DNA repair as cells enter the S phase. Our results thus represent independent observations that enhancement of the cytotoxic effect of different DNA-damaging agents occurs by abrogating S-phase arrest. The hypothesis that S-phase-specific drugs act partially by blocking tumor cells in S phase suggests that studies combining those drugs with methylxanthine derivatives such as PTX should be seriously considered. This concept is in accordance with findings of our group showing a synergistic effect between PTX and 5-fluorouracil (data not shown).

The human colon-cancer cell line WiDr has a mutated *p53* gene, and fails to arrest in G₁ after ionizing radiation (Li *et al.*, 1995). If cells lack a post-irradiation G₁ block, they are still able to arrest the cell cycle at the G₂/M transition. We have shown the ability of PTX to modify the radiation-induced G₂ delay and hence to act as a radiosensitizer (Li *et al.*, 1998). Loss of the *p53*-dependent G₁ arrest makes the ability of the cells to arrest in G₂ crucial for survival after irradiation. We analyzed radiation-induced G₂ arrest in human colon-cancer (WiDr) and cervical-cancer (C33-A, C4-I)

cell lines with defective *p53* (Li *et al.*, 1998). PTX is effective at abrogating this arrest and enhances radiation-induced cytotoxicity. The importance of the G₂ delay following FMdC and irradiation is suggested in the present study by the correlation between PTX abrogation of G₂ arrest and radiosensitization. Alleviation of the G₂/M block by PTX might explain, at least in part, the combined radiosensitizing effect of FMdC and PTX. As reported by Smeets *et al.* (1994), radiation-induced G₂ arrest is associated with down-regulation of cyclin B/cdc2 kinase activity, and methylxanthines such as caffeine can reverse this effect and enhance cytotoxicity (Hain *et al.*, 1993).

Taken together, our findings suggest, first, the existence of both S-phase and G₂-phase arrest induced by FMdC and irradiation respectively. Second, the S-phase and G₂-phase blocks can be abrogated by PTX. A checkpoint-based strategy may therefore offer a way to selectively enhance the cytotoxicity of DNA-damaging agents, provided less toxic derivatives of PTX can be developed.

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