[CANCER RESEARCH 43, 4011–4013, September 1983]

# Sequence-dependent Synergism between Dichloromethotrexate and 5-Fluorouracil in a Human Colon Carcinoma Cell Line[1]

**Alberto F. Sobrero[2] and Joseph R. Bertino[3]**

*Yale University School of Medicine, Department of Pharmacology, New Haven, Connecticut 06510*

## ABSTRACT

Prior to their use in patients with hepatic metastasis of colon cancer, we studied the cell-killing effects of 5-fluorouracil, 5-fluoro-2′-deoxyuridine, 3′,5′-dichloromethotrexate (DCM), and combinations of these fluoropyrimidines with DCM on a human colon carcinoma cell line (HCT-8). Sequential exposure of these cells to DCM followed by 5-fluorouracil resulted in significant synergistic cell killing at every dose and time studied, and the synergy was more evident with increasing doses of both drugs. In contrast, simple additive effects were observed when DCM was given together with or following 5-fluorouracil. When the antifolate was given before 5-fluoro-2′-deoxyuridine, simple additive effects, rather than synergy, were observed. Mild antagonism and even strong antagonism were found when 5-fluoro-2′-deoxyuridine preceded DCM administration or when the cells were exposed to the drugs simultaneously, respectively.

## INTRODUCTION

The fluoropyrimidines FUra[4] and FdUrd, as well as DCM, are potentially useful drugs for treatment of patients with hepatic metastasis due to gastrointestinal cancer (12). These drugs are less toxic to nondividing hepatic parenchymal cells than to dividing tumor cells; in addition, they are extensively metabolized by normal liver (3, 6) and thus cause less systemic toxicity when administered via the hepatic artery as compared to i.v. administration.

Since the sequential use of MTX and FUra has been shown to give synergistic tumor cell kill in *in vitro* (1, 5) as well as *in vivo* (2, 11, 15) experimental systems and is in clinical use (8, 16, 18), we studied the cell-killing effects of the more rapidly metabolized MTX analogue DCM alone and in combination with FUra and FdUrd on the human colon tumor cell line HCT-8, prior to use in patients with hepatic metastasis of colon carcinoma.

## MATERIALS AND METHODS

FUra and FdUrd were obtained from commercial sources and were the materials available for clinical use. DCM was a gift of Lederle Laboratories and was greater than 95% pure, as measured by high-pressure liquid chromatography.

Tissue culture cells utilized in this investigation were from the continuously growing human colon adenocarcinoma line HCT-8 (19). Cells were maintained in 25-sq cm sterile plastic flasks (Costar, Cambridge, Mass.) as monolayer cultures in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% horse serum and subcultured weekly. Under these conditions, the doubling time was 18 hr.

**Clonogenic Assay.** A monolayer clonal growth technique was used (17). Cell suspensions were obtained by trypsinization of stock cultures for 5 min. A high degree of monocellular dispersion was obtained by pipeting trypsinized cells followed by passages through needles of decreasing size, from 19 to 25 gauge. Portions of 500 cells in 5 ml of medium containing 10% horse serum were pipeted into sterile 60-mm Petri dishes (Costar) and incubated at 37° and 100% humidity, with 7.5% $CO_2$. Eighteen hr later, when the cells were firmly attached to the bottom of the Petri dish but had not yet divided, 0.1 ml of an appropriate dilution of drug in PBS was added to each dish. Control dishes received the same volume of PBS. After the designed incubation period, the medium was decanted, the dishes were washed twice with 5 ml of PBS, and 5 ml of fresh medium were replaced. In sequential treatment experiments, this procedure was repeated after the exposure to the second drug. Ten days after the initial plating, colonies were stained with orcein, and those colonies with more than 30 cells were counted at ×10 using a dissecting microscope. Each experimental point was determined in triplicate with 5 replicate controls, and experiments were repeated at least 3 times.

**Experimental Design.** Times and sequences of exposure to DCM, FUra, and FdUrd are outlined in Tables 1 and 2. The tables also show some of the doses selected for drug combination experiments; in general, they were chosen to give a cell kill between 30 and 70%, so that maximum synergy could be observed. The quantitation of drug interaction was done by calculating the ratio between the product of the survival fraction of each individual drug and the survival fraction of the drug combination (14). Values greater than one were considered to show synergism, those less than one, antagonism, and those of about one, an additive effect.

## RESULTS

**Cell Kill as a Function of Concentration and Time of Exposure to DCM, FUra, and FdUrd.** HCT-8 cell kill produced by DCM, FUra, and FdUrd as a function of concentration and time of exposure is summarized in Charts 1 to 3. The $ED_{50}$ for DCM and FUra decreased by one log when HCT-8 cells were exposed to either of these drugs for 24 hr as compared to 4 hr. Cell kill by FdUrd was even more time dependent; the $ED_{50}$ was 300 times lower when cells were exposed to this drug for 24 hr, as compared to a 4-hr exposure. The $ED_{50}$ values for a 24-hr exposure to FdUrd, DCM, and FUra were 0.006, 0.4, and 3.5 $\mu$M, respectively. While exposure of these cells for longer than 24 hr produced a substantial increase in cell kill in the case of DCM, relatively modest increments of cell kill were obtained with FUra and FdUrd.

**DCM-FUra Combinations.** A summary of the results obtained with combinations of DCM and FUra administered in different

[1] Supported by Grant CA 24887-05.

[2] Fellow of the Associazione Italiana per la Ricerca sul Cancro.

[3] American Cancer Society Professor. To whom requests for reprints should be addressed, at Yale University School of Medicine, Department of Pharmacology, P.O. Box 3333, 333 Cedar Street, New Haven, Conn. 06510.

[4] The abbreviations used are: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; DCM, 3′,5′-dichloromethotrexate; MTX, methotrexate; PBS, phosphate-buffered saline (0.8% NaCl-0.02% KCl-0.115% $Na_2HPO_4$-0.02% $KH_2PO_4$); $ED_{50}$, concentration of drug killing 50% of the cells compared to untreated controls; FdUMP, 5-fluoro-2′-deoxyuridylate.

Received February 17, 1983; accepted May 25, 1983.

Table 1

*Percentage of survival of HCT-8 cells exposed to DCM, FUra, and their combinations*

Survival was measured by a monolayer clonal growth technique and expressed as a percentage of untreated controls (see "Materials and Methods").

A. 4-hr exposure: sequence-dependent effects

| Treatment | % of survival |
|---|---|
| DCM | |
| 0.3 μM | 93.7 ± 5.7[a] |
| 1.0 μM | 69.8 ± 5.6 |
| 3.0μM | 50.9 ; pm 5.9 |
| FUra (30μM) | 57.2 ± 4.6 |
| DCM (1.0 μM) + FUra (30 μM) | 35.1 ± 3.4 (1.0)[b] |
| DCM (3.0 μM) + FUra (30 μM) | 23.7 ± 2.5 (0.9) |
| FUra (30 μM) → DCM (0.3 μM) | 53.6 ± 7.8 (1.1) |
| FUra (30 μM) → DCM (1.0 μM) | 41.9 ± 3.5 (0.9) |
| FUra (30 μM) → DCM (3.0 μM) | 26.3[c] (1.0) |
| DCM (0.3 μM) → FUra (30 μM) | 27.9 ± 9.0 (1.8) |
| DCM (1.0 μM) → FUra (30 μM) | 20.2 ± 9.8 (2.3) |
| DMC (3.0 μM) → FUra (30 μM) | 5.8 ± 5.0 (2.2) |

B. Sequential DCM → FUra for various time periods

| | | % of survival | | |
|---|---|---|---|---|
| DCM (μM) | FUra (μM) | DCM (24 hr) → FUra (4 hr) | DCM (48 hr) → FUra I24 hr) | DCM (48 hr) → FUra (168 hr) |
| 0.03 | 0 | | 90.7 ± 3.9 | 90.7 ± 3.9 |
| 0.1 | 0 | 78.0 ± 11.0 | 30.2 ± 7.9 | |
| 0.3 | 0 | 57.6 ± 5.7 | | |
| 0 | 3 | | 62.0 ± 22.0 | 35.4 ± 6.1 |
| 0 | 60 | 29.5 ± 11.0 | | |
| 0.03 | 3 | | 26.9 ± 15.3 (1.8) | 16.2 ± 11.3 (2.1) |
| 0.1 | 3 | | 5.7 ± 2.0 (2.0) | 4.1[c] (4.1) |
| 0.1 | 60 | 3.2 ± 2.2 (5.6) | | |
| 0.3 | 60 | 2.2 ± 1.9 (6.5) | | |

[a] Mean ± S.E. of at least 3 experiments.
[b] Numbers in parentheses, coefficient of drug interaction.
[c] Only one value available.

doses and sequences and for different time periods is presented in Table 1. Sequential exposure of HCT-8 cells to DCM followed by FUra resulted in significant synergism at every dose and time studied and was more evident with increasing doses of both drugs. (Data with increasing doses of FUra are not shown.) Marked synergy was observed with 24-hr exposure to DCM (0.1 or 0.3 μM) with a 4-hr exposure to FUra during Hr 20 to 24. A simple additive effect was observed when DCM was given with or after FUra.

**DCM-FdUrd Combinations.** Similar concentrations of DCM and 6 concentrations of FdUrd, ranging between 1 nM and 1 μM, were tested. Only data obtained with 0.003 and 0.3 μM FdUrd are shown (Table 2). A simple additive effect on cell kill was observed when DCM preceded FdUrd. Conversely, a mild antagonism occurred with the sequence FdUrd-DCM; the exposure to these drugs given simultaneously produced a very strong antagonism (Table 2A).

## DISCUSSION

These data clearly demonstrate synergistic cell kill on HCT-8 cells when DCM is given before FUra. These results are not surprising, in view of the data of Benz and Cadman (1), who also noted synergistic cell killing with sequential use of MTX and FUra on the same cell line. The exact mechanism of this synergy observed in several experimental systems (2, 5, 11) is not entirely clear, but increased FUra nucleotide formation has been shown to correlate with increased levels of intracellular phosphoribosyl-1-pyrophosphate that result when certain cells are treated with

Table 2

*Percentage of survival of HCT-8 cells exposed to DCM, FdUrd, and their combinations*

Survival was measured by a monolayer clonal growth technique and expressed as a percentage of untreated controls (see "Materials and Methods").

A. 4-hr exposure: sequence-dependent effects

| Treatment | % of survival |
|---|---|
| DCM | |
| 0.3 μM | 93.7 ± 5.7[a] |
| 1.0 μM | 69.8 ± 5.6 |
| 3.0 μM | 50.9 ± 5.9 |
| FdUrd (0.3 μM) | 68.7 ± 3.4 |
| DCM (1.0 μM) + FdUrd (0.3 μM) | 66.5 ± 4.4 (0.5)[b] |
| DCM (3.0 μM) + FdUrd (0.3 μM) | 51.5 ± 10.3 (0.4) |
| FdUrd (0.3 μM) → DCM (0.3 μM) | 48.6 ± 11.8 (0.8) |
| FdUrd (0.3 μM) → DCM (1.0 μM) | 44.6 ± 11.4 (0.8) |
| DCM (0.3 μM) → FdUrd (0.3 μM) | 42.2 ± 1.6 (1.2) |
| DCM (1.0 μM) → FdUrd (0.3 μM) | 39.2 ± 3.6 (0.9) |
| DCM (3.0 μM) → FdUrd (0.3 μM) | 31.5[c] (1.2) |

B. Sequential DCM → FUrd for various time periods

| | | % of survival | | |
|---|---|---|---|---|
| DCM (μM) | FdUrd (μM) | DCM (24 hr)→ FdUrd (4 hr) | DCM (48 hr)→ FdUrd (24 hr) | DCM (48 hr)→ FdUrd (168 hr) |
| 0.03 | 0 | | 90.7 ± 3.9 | 90.7 ± 3.9 |
| 0.1 | 0 | 78.0 ± 5.7 | 30.2 ± 7.9 | 30.2 ± 7.9 |
| 0.3 | 0 | 57.6 ± 5.7 | | |
| 0 | 0.3 | 68.7 ± 3.4 | | |
| 0 | 0.003 | | 65.3 ± 7.8 | 54.6 ± 7.7 |
| 0.03 | 0.003 | | 54.8 ± 6.7 (0.9) | 53.1 ± 7.6 (1.1) |
| 0.1 | 0.003 | | 13.2 ± 1.7 (0.8) | 18.2 ± 2.5 (1.2) |
| 0.1 | 0.3 | 67.4 ± 7.5 (1.0) | | |
| 0.3 | 0.3 | 64.3 ± 4.4 (0.7) | | |

[a] Mean ± S.E. of at least 3 experiments.
[b] Numbers in parentheses, coefficient of drug interaction.
[c] Only one value available.

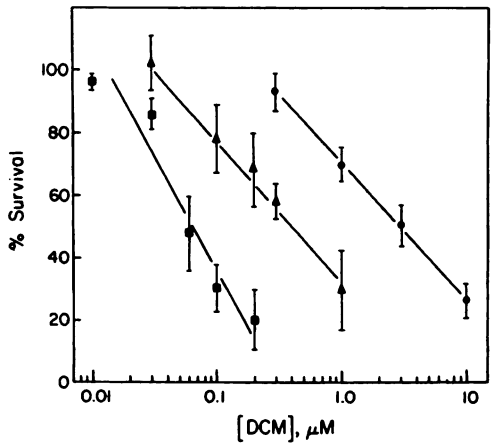


Chart 1. Survival of HCT-8 cells exposed to DCM. ●, 4-hr exposure; ▲, 24-hr exposure; and ■, 48-hr exposure. Survival was evaluated by a monolayer clonal growth technique. *Bars*, S.E.

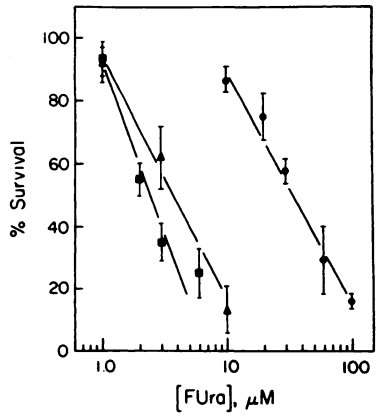


Chart 2. Survival of HCT-8 cells exposed to FUra. ●, 4-hr exposure; ▲, 24-hr exposure; and ■, 7-day exposure. Survival was evaluated by a monolayer clonal growth technique. *Bars*, S.E.

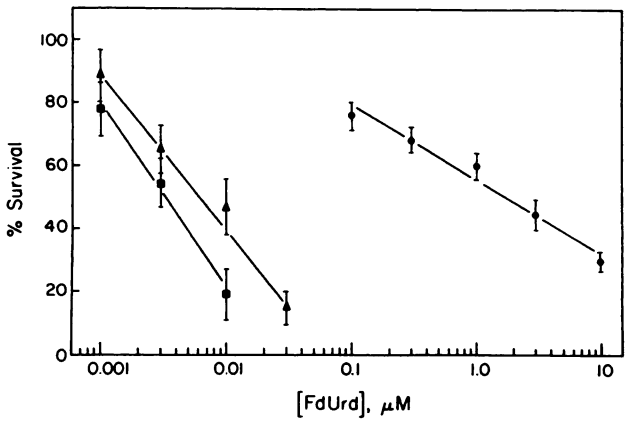


Chart 3. Survival of HCT-8 cells exposed to FdUrd. ●, 4-hr exposure; ▲, 24-hr exposure; and ■, 7-day exposure. Survival was evaluated by a monolayer clonal growth technique. *Bars*, S.E.

MTX and then given FUra (5). As a consequence of this increase in FUra nucleotide formation, more FdUMP, an inhibitor of thymidylate synthase (9), is formed, as well as more 5-fluorouridine triphosphate, resulting in an increased incorporation of this compound into RNA. Fernandes and Bertino (7) have also shown that dihydrofolate pentaglutamate that would be expected to rapidly accumulate when dividing cells are given MTX as a consequence of inhibition of dihydrofolate reductase can enhance tight binding of FdUMP to thymidylate synthase, possibly replacing the depleted folate cofactor $N^5$,$N^{10}$-methylentetrahydrofolate in this ternary complex.

Since FdUrd is not primarily activated by a phosphoribosyl-1-pyrophosphate-dependent enzymatic mechanism but rather by the enzyme thymidine kinase (10), antifolate pretreatment followed by FdUrd may not result in increased FdUMP formation.

The sequence FdUrd-DCM might be expected to be antagonistic. In fact, inhibition of thymidylate synthase by FdUMP prevents the oxidation of $N^5$,$N^{10}$-methylentetrahydrofolate to dihydrofolate (4); the subsequent administration of DCM should have little effect in the presence of decreased DNA synthesis. The antagonistic effects on cell kill produced by simultaneous exposure of HCT-8 cells to DCM and FdUrd or to the sequence FdUrd-DCM are of clinical interest, since an attractive drug combination for hepatic infusion treatment would be the concomitant use of DCM and FdUrd, since they may easily be administered by the implantable pump. Conversely, sequential DCM and FUra may be valuable for intraarterial therapy of hepatic metastasis of gastrointestinal carcinoma. When given by this route, these drugs are both metabolized moderately rapidly, and appropriate dose rates would not produce enhanced cell kill on normal renewal tissues, a problem encountered when MTX and FUra have been administered i.v. in some studies using these drugs in sequence (13, 20).

Since these data were obtained with a human colon carcinoma line propagated in culture, caution must be exercised in extrapolating these results to the clinical situation. Nevertheless, these data should be kept in mind when designing clinical protocols and should encourage further *in vitro* and *in vivo* testing of these regimens.

## REFERENCES


1. Benz, C., and Cadman, E. Modulation of 5-fluorouracil metabolism and cytotoxicity by antimetabolite pretreatment in human colorectal adenocarcinoma HCT-8. Cancer Res., *41:* 994–999, 1981.
2. Bertino, J. R., Sawicki, W. L., Lindquist, C. A., and Gupta, V. S. Schedule-dependent antitumor effects of methotrexate and 5-fluorouracil. Cancer Res., *37:* 327–328, 1977.
3. Blakley, R. L. Biochemistry and pharmacology of folate analogues. *In:* R. L. Blakley (ed.), The Biochemistry of Folic Acid and Related Pteridines, pp. 454–517. Amsterdam: Elsevier/North-Holland Biomedical Press, 1969.
4. Bowen, D., White, C., and Goldman, I. D. A basis for fluoropyrimidine-induced antagonism to methotrexate in Ehrlich ascites tumor cells *in vitro*. Cancer Res., *38:* 219–222, 1978.
5. Cadman, E., Heimer, R., and Davis, L. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation for drug synergism. Science (Wash. D.C.), *205:* 1135–1137, 1979.
6. Ensminger, W. D., Rosowsky, A., Raso, V., Levin, D. C., Glode, M., Come, S., Steele, G., and Frei, E., III. A clinical-pharmacological evaluation of hepatic arterial infusion of 5-fluoro-2′-deoxyuridine and 5-fluorouracil. Cancer Res., *38:* 3784–3792, 1978.
7. Fernandes, D. J., and Bertino, J. R. 5-Fluorouracil-methotrexate synergy: enhancement of 5-fluorodeoxyuridylate binding to thymidylate synthetase by dihydropteroylpolyglutamates. Proc. Natl. Acad. Sci. U. S. A., *77:* 5663–5667, 1980.
8. Gewirtz, A. M., and Cadman, E. Preliminary report on the efficacy of sequential methotrexate and 5-fluorouracil in advanced breast cancer. Cancer (Phila.), *47:* 2552–2555, 1981.
9. Hartman, K. V., and Heidelberger, C. Studies on fluorinated pyrimidines. VIII, Inhibition of thymidilate synthetase. J. Biol. Chem., *235:* 3005–3013, 1961.
10. Heidelberger, C. Fluorinated pyrimidines and their nucleosides. *In:* A. C. Sartorelli (ed.), Antineoplastic and Immunosuppressive Agents. Handbook of Experimental Pharmacology, Vol. 38, pp. 193–231. New York: Springer-Verlag, 1975.
11. Heppner, G. H., and Calabresi, P. Effect of sequence of administration of methotrexate, leucovorin, and 5-fluorouracil on mammary tumor growth and survival in syngeneic C3H mice. Cancer Res., *37:* 4580–4583, 1977.
12. Lee, Y. T. N. Nonsystemic treatment of metastatic tumors of the liver. A review. Med. Pediatr. Oncol., *4:* 185–203, 1978.
13. Mehrotra, S., Rosenthal, C. J., and Gardner, B. Biochemical modulation of antineoplastic response in colorectal carcinoma: 5-fluorouracil, high-dose methotrexate with calcium leucovorin rescue in two sequences of administration. Proc. Am. Soc. Clin. Oncol., *1:* 100, 1982.
14. Momparler, R. L. *In vitro* systems for evaluation of combination chemotherapy. Pharmacol. Ther., *8:* 21–35, 1980.
15. Mulder, J. H., Smink, T., and Van Putten, L. M. 5-Fluorouracil and methotrexate combination chemotherapy: the effect of drug scheduling. Eur. J. Cancer Clin. Oncol., *17:* 831–837, 1981.
16. Pitman, S. W., Kowal, C., Papac, R. J., and Bertino, J. R. Sequential MTX-5-fluorouracil: a highly active drug combination in advanced squamous cell carcinoma of the head and neck. Proc. Am. Assoc. Cancer Res., *21:* 473, 1980.
17. Reid, L. C. M. Cloning. Methods Enzymol. *58:* 152–162, 1979.
18. Tisman, G., and Wu, S. G. Effectiveness of intermediate-dose methotrexate and high-dose 5-fluorouracil as sequential combination chemotherapy in refractory breast cancer and as primary therapy in metastatic adenocarcinoma of the colon. Cancer Treat. Rep., *64:* 829–835, 1980.
19. Tompkins, W. A. F., Watrach, A. M., Shmale, J. D., Shultz, R. M., and Harris, J. A. Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. J. Natl. Cancer Inst., *52:* 1101–1106, 1974.
20. Weinerman, B., Shacter, B., Schipper, H., Bowman, D., and Levitt, M. Sequential methotrexate and 5FU in the treatment of colorectal cancer. Cancer Treat. Rep., *55:* 1553–1555, 1982.