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Research Article

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Schedule-dependent Cytotoxicity of Methotrexate and 5-Fluorouracil in Human Colon and Breast Tumor Cell Lines

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ABSTRACT The effect of sequential methotrexate and 5-fluorouracil on the clonal growth of the human colon adenocarcinoma cell, HCT-8, and the hormone-dependent human breast carcinoma cell, 47-DN, was examined. In both cell lines, when 5-fluorouracil was given during the last 6 h of a 24-h methotrexate exposure period, there was marked synergistic inhibition of clonal growth. Shorter intervals or the reverse sequence of drugs were either additive or antagonistic. These results indicate the importance of the drug sequence and time interval between drug administration for optimal cytotoxicity in these human cell lines. This information suggests that the administration of methotrexate 18 h before 5-fluorouracil may have potential application in the design of clinical trials for these malignancies.

INTRODUCTION

Methotrexate (MTX)¹ and 5-fluorouracil (FUra) have been used successfully singly, and in combination, in treating a variety of human neoplasms, including cancers of the breast and colon (1, 2). Biochemical investigations have elucidated the specific molecular mechanisms by which each of these drugs exerts its cytotoxic effect, but there remains some controversy

about the optimum therapeutic effectiveness when these two antimetabolites are combined (3). Previous work from our laboratory has demonstrated that pre-treating cultured murine leukemic cells with MTX results in enhanced intracellular accumulation of FUra and synergistic tumor-cell kill (4). To extend our studies to human tumor cells we have adopted the simple technique of monolayer cloning. Although human tumor-cell lines and suspensions from freshly resected tumor specimens can be cloned in soft agar, this technique is tedious and the cloning efficiency is often very sensitive to specific growth factors (5). The monolayer cloning assay allows more rapid and efficient cloning from any monolayer cell line and enables statistically reliable enumeration by a less expensive automated colony counter. To test our hypothesis that the cytotoxicity of sequenced MTX and FUra is synergistic and schedule dependent, we have used this monolayer cloning assay on two human malignant cell lines, a colorectal adenocarcinoma, HCT-8, and a hormone-dependent breast carcinoma, 47-DN.

METHODS

Cell lines. Both the human colorectal tumor, HCT-8, and the breast tumor, 47-DN, are well characterized, continuously growing monolayer cell lines (6, 7) that under our culture conditions double in 18 and 60 h, respectively, and demonstrate Compertzian growth kinetics. HCT-8 cells were maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) with 10% neonatal calf serum; and the 47-DN cells were grown in RPMI 1640 with 10% fetal bovine serum and 1 nM estradiol (Sigma Chemical Co., St. Louis, Mo.) and 2 IU/ml insulin (Eli Lilly

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¹Abbreviations used in this paper: CH₂FAH₄, methyl-enetetrahydrofolate; FdUMP, fluorodeoxyuridylate; FUra, 5-fluorouracil; MTX, methotrexate.

Co., Indianapolis, Ind.). Stocks of both lines were passaged weekly using a trypsin (0.05%)-EDTA (0.02%) solution and vigorous agitation to obtain a suspension of >90% single cells. Stock cultures and cloning studies were grown in 75 cm² sterile plastic culture flasks (Costar Data Packaging, Cambridge, Mass.) with 25 ml of medium in 5% CO₂ incubators at 37°C. Cell counts were performed on a model ZBI Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Monolayer colony growth. Stock flasks containing 10⁶ cells were rinsed with sterile phosphate-buffered saline (PBS). After cells were suspended by trypsinization and vigorous pipetting, 5 × 10⁴ cells were seeded into the flasks with medium. When untreated cultures exceeded a mean colony density of 500/1,000 mm² and a median clonal diameter of ≥0.25 mm, the medium was decanted, and the colonies were rinsed with PBS, immediately stained with a methanol-crystal violet (2.5%) solution, and counted. Cultures were drug treated between days 0 and 1 when clusters consisted of 4–32 cells; HCT-8 colonies were counted between day 4 and day 5, and the slower growing 47-DN cells were counted between day 10 and day 14. During these posttreatment growth intervals the relationship between the clonal growth rates of treated and control cultures was similar for both cell lines. As seen in Fig. 1, HCT-8 cells were counted between day 4 and day 5, when mean colony count and median colony size were optimal. The monolayer cloning efficiency of both untreated HCT-8 and 47-DN cell lines was 10%, defined as the percent of inoculated cells which reach a clonal diameter of ≥0.20 mm when the mean colony count is highest (5 d in Fig. 1).

Colony counting. Colonies were counted on an automated colony counter (Biotran II; New Brunswick Scientific Co. Inc., Edison, N. J.), which was calibrated by manual counting. Colonies observed through a calibrated lens to be ≥0.2 mm in diameter were counted manually from 12 fields encompassing 50% of the flask surface. Manual counting variability among fields was 30%. The sensitivity of the automated colony counter was adjusted to discriminate colonies ≥0.2 mm in diameter within fields of 1,000 mm². Six non-overlapping fields per flask were counted with a counting variability of 10% among fields of untreated control flasks. Greater variability in colony counts was observed under drug-treated conditions, as is reflected in the standard deviation bars of Figs. 2 and 3. Clonal growth under treated conditions was recorded as mean percent colony count relative to untreated controls. Clonal size determinations were also made with the instrument's adjustable size discriminator and recorded as median diameter of colonies ≥0.2 mm.

Drug treatment. MTX was obtained from Bristol Labs (Syracuse, N. Y.) and Fura was obtained from Sigma Chemical Co. For Figs. 2 and 3, both cell lines received a final MTX concentration of 0.1 μM for 24 h. The Fura concentration and exposure interval for each cell line was chosen to demonstrate sequence-dependent inhibition of clonal growth when combined with MTX treatment; HCT-8 cells received 1 μM Fura and 47-DN cells received 10 μM Fura. Fura was added for 6 h, either alone or in combination with MTX, during one of four 6-h intervals in the 24-h MTX-treatment period. Thus, the dose and exposure time of both MTX and Fura was constant for all treatment conditions and only the sequencing interval was altered.

RESULTS

The results of HCT-8 and 47-DN clonal growth experiments are represented in Figs. 2 and 3, respectively. Fig. 2 shows that 6 h of Fura at 1 μM did not affect

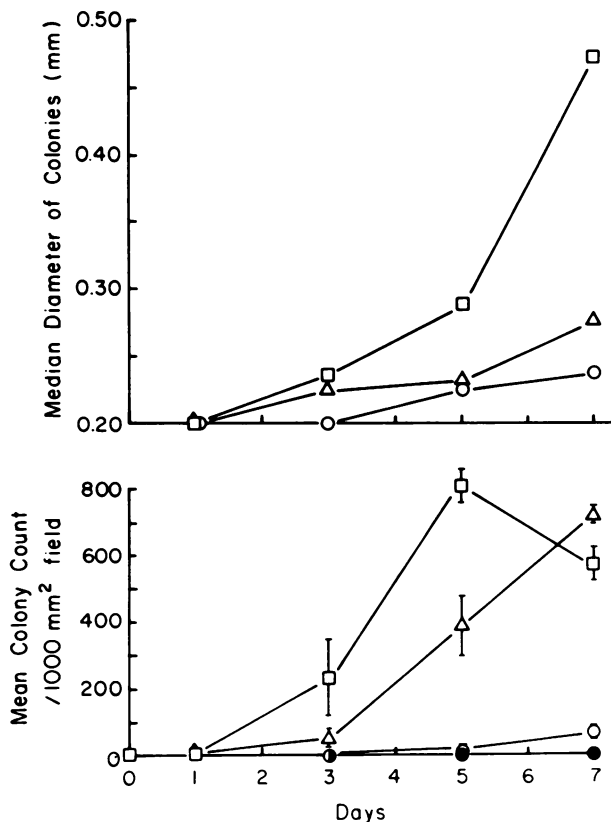


FIGURE 1 Clonal growth of the human colon adenocarcinoma, HCT-8. Cultures were seeded 24 h before day 0 as described in Methods. MTX at various concentrations was added on day 0 and removed by rinsing the cultures on day 1. Colonies were counted and the clonal diameter measured daily. After 5 d the control colonies (□) which were increasing in diameter began to become confluent, which resulted in a decline in the mean colony count. Both mean colony count and median colony diameter were affected by MTX as indicated (0.1 μM, △; 1 μM, ○; 10 μM, ●). The effects of sequential drug treatment on mean colony counts of HCT-8 were measured on days 4 and 5 and represented as percent control in Fig. 2.

clonal growth of HCT-8 cells. 0.1 μM MTX for 24 h resulted in 40% clonal growth. The effect of combining MTX and Fura was found to be schedule dependent. Fura given during the first 6 h of the 24-h MTX exposure did not inhibit clonal growth more than when MTX alone was given. As the 6-h Fura dose was given progressively later into the 24-h MTX-exposure period, synergistic inhibition of clonal growth was observed. 6 h of 10 μM Fura reduced 47-DN clonal growth to 25%, and 24 h of 0.1 μM MTX resulted in 67% clonal growth (Fig. 3). Fura given during the first 6 h of a 24-h MTX-treatment period resulted in no greater growth inhibition than was seen with Fura alone. If the inhibitory effects of MTX and Fura were entirely independent, the com-

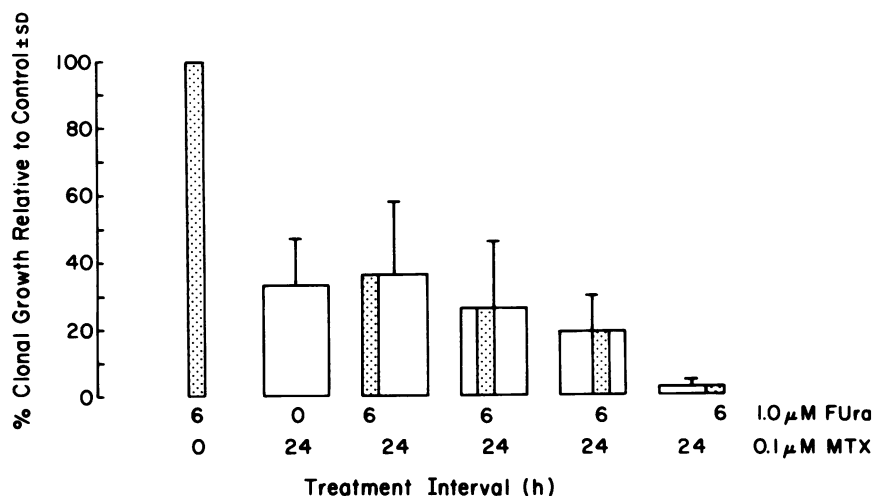


FIGURE 2 Clonal growth of HCT-8. Cells were treated with 0.1 μ M MTX for 24 h (open bars), and 1.0 μ M Fura was added either alone or in combination with MTX during one of four 6-h treatment intervals (stippled bars).

bined addition would have been additive, and closer to 17% of control. Only when the 6-h Fura dose was given later into the 24-h MTX-treatment period did the combined inhibitory effect of these two drugs become synergistic.

DISCUSSION

Our data suggest that a relatively simple monolayer colony-forming assay can be used to assess the cytotoxicity of sequence-dependent combination drug therapy in vitro against human malignant cell lines. In the human colorectal adenocarcinoma, HCT-8, and the hormone-dependent breast carcinoma, 47-DN, combining MTX and Fura resulted in inhibition of clonal growth which ranged from less than additive to synergistic, depending on the sequencing interval. MTX pretreatment for 12–18 h before Fura therapy was needed to produce synergistic growth inhibition in both human cell lines.

Combining MTX and Fura can alter the growth-inhibitory mechanisms of both drugs in a complex fashion. MTX reduces formation of thymidylate and subsequently inhibits nucleic acid and protein synthesis (1). These effects result from the binding of MTX to dihydrofolate reductase (E.C.1.5.1.3.) and the subsequent depletion of intracellular pools of methylenetetrahydrofolate (CH_2FAH_4). Fura is thought to inhibit cell growth after the intracellular conversion to fluorodeoxyuridylate (FdUMP) which binds in a covalent ternary complex with CH_2FAH_4 and the enzyme thymidylate synthetase (E.C.2.7.4.6.) to inhibit DNA synthesis (2). Fura toxicity has also been correlated with the intracellular formation of fluorouridine triphosphate and its incorporation into RNA (8). The relative importance of the two toxic intracellular products,

FdUMP and fluorouridine triphosphate, remains controversial. Since DNA inhibition by FdUMP depends on intracellular pools of CH_2FAH_4 for covalent binding to thymidylate synthetase, MTX pretreatment could antagonize Fura toxicity by depleting these CH_2FAH_4 pools. Alternatively, since FdUMP formation by itself prevents the utilization

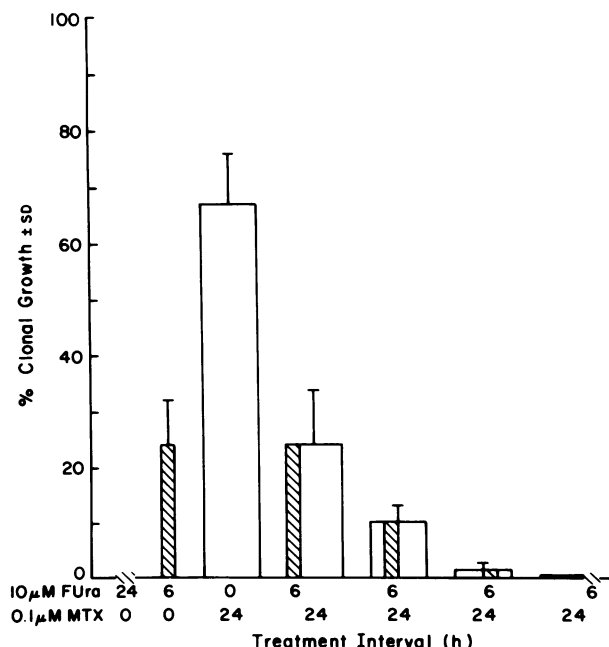


FIGURE 3 Clonal growth of a hormone-dependent human breast carcinoma, 47-DN. Cells were treated with 0.1 μ M MTX for 24 h (open bars) and 10 μ M Fura was added either alone for 6 or 24 h or in combination with MTX during one of four 6-h treatment intervals (hatched bars).

of CH₂FAH₄, pretreating cells with FUra could antagonize the cytotoxicity of MTX. In vitro studies of the interaction between MTX and the ternary complex formed in FUra-treated cells suggest that the drug combination of MTX and FUra is antagonistic regardless of the sequence in which they are given (9). But in vivo studies in rodents have demonstrated synergistic antitumor activity when MTX precedes FUra treatment (10); this agrees with previous work from our laboratory on cultured L1210 cells that correlated synergistic cytotoxicity with enhanced intracellular accumulation of FUra and its nucleotide derivatives incorporated into RNA (4). This enhanced intracellular FUra accumulation was mediated by increased intracellular phosphoribosylpyrophosphate which was the result of the antipurine effect of MTX. The enzyme orotate phosphoribosyltransferase (E.C.2.4.2.10.), which is responsible for the ribosylphosphorylation of FUra (11), therefore, has available increased co-substrate, phosphoribosylpyrophosphate.

Our current finding that synergistic inhibition of clonal growth by combined MTX and FUra therapy is schedule dependent in human malignant cell lines of breast and colon provides a rationale for sequentially combining these two antimetabolites in the treatment of these two cancers. In correlative biochemical studies we have been able to show that FUra cytotoxicity in HCT-8 and 47-DN cells correlates with total intracellular accumulation of FUra and incorporation into cellular RNA (12). Although we found that intracellular pools of FdUMP also accumulate, no enhancement in the inhibition of DNA synthesis was measured in cells treated with combinations of MTX and FUra over those treated with either drug alone. As observed in murine leukemic cells, the appropriate MTX pretreatment interval for maximal FUra accumulation depends on the rate of intracellular accumulation of phosphoribosylpyrophosphate. HCT-8 and 47-DN cells require 12–18 h of MTX pretreatment while cultured L1210 cells require only 4–6 h. This difference in the pretreatment interval required between murine and human cell lines may well be explained by the cell-cycle time and growth fraction of the specific tumor population, as well as the activity of the FUra phosphorylating enzyme, orotate phosphoribosyltransferase.

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