Eur J Cancer Clin Oncol, Vol. 25, No. 6, pp. 977–982, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Sequence-dependent Enhancement of HCT-8 Cell Kill by Trimetrexate and Fluoropyrimidines: Implications for the Mechanism of this Interaction

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Abstract—*The new folate antagonist trimetrexate is an inhibitor of dihydrofolate reductase, but unlike methotrexate (MTX) it is not polyglutamylated. We have compared the cell killing effects of MTX and trimetrexate/5-fluorouracil (FUra) and 5-fluoro-2'-deoxyuridine (FdUrd) combinations on HCT-8 cells* in vitro, *in an attempt to explore indirectly the role of polyglutamylation of the antifol in determining the known sequence-dependent synergism between MTX and FUra. The comparisons were made in a number of equitoxic concentrations and times of exposure. Trimetrexate given for* 4, 24 *or* 48 h *followed by FUra, for* 4, 24 *or* 196 h, *produced synergistic HTC-8 cell kill, whereas antagonism was observed when FUra preceded or was given simultaneously with trimetrexate. The degree of interaction was essentially identical to those obtained when MTX was combined with FUra. The interactions between MTX/FdUrd and trimetrexate/FdUrd were also similar: synergistic cell kill resulted from the sequences trimetrexate or MTX followed by FdUrd, while additive effects were produced by trimetrexate or MTX + FdUrd combinations or FdUrd followed by MTX or trimetrexate.*

Because the same interactions observed with MTX/FUra or FdUrd combinations were also obtained when trimetrexate was combined with the fluoropyrimidines, it is unlikely that polyglutamylation of the antifols plays a significant role in determining the different sequence-dependent effects of these antimetabolites. However, these studies do not rule out the possibility that increased levels of dihydrofolate polyglutamates increase fluoropyrimidine cytotoxicity.

INTRODUCTION

TRIMETREXATE is a new folate antagonist that unlike methotrexate (MTX) is not polyglutamylated and enters cells via a transport system different from that utilized by MTX and reduced folates [1]. Because of these properties trimetrexate is effective against MTX-resistant cells by virtue of impaired transport and low level increase in DHFR [2]. In the clinic this compound has produced encouraging results in phase II trials conducted on patients with head and neck, breast and non-small cell lung carcinomas [3–5] and it is now entering clinical trials in combination with other drugs. FUra could be a good candidate drug for combination studies with trimetrexate: the fluoropyrimidine is in fact known to synergize with MTX in *in vitro* [6, 7] as well as *in vivo* [8] experimental tumor systems and some clinical studies [9–12] have encouraged further testing of this combination in breast, gastric colon and head and neck carcinomas.

Given the analogous mechanism of action of MTX and trimetrexate and their different pharmacological properties, we have compared the cell killing effects of MTX and trimetrexate/FUra combinations on a colon carcinoma cell line *in vitro* with two objectives:

(a) To investigate whether or not synergy occurs with the sequence trimetrexate → FUra and compare this to the sequence MTX → FUra. This comparison may provide information on the role of MTX polyglutamates in determining, or contributing to the synergy.
(b) To provide experimental support to the design of phase II and III studies employing trimetrexate/FUra combinations.

Accepted 20 January 1989.
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Supported by the Associazione Italiana per la Ricerca sul Cancro and by grant 87.00230.04, Consiglio Nazionale delle Ricerche, Roma, Italy.

Furthermore, additional insight on the mechanism of interaction between antifolates and fluoropyrimidines could be provided by the use of FdUrd instead of FUra. This nucleoside should theoretically kill the cells via inhibition of thymidylate synthase with no substantial incorporation of FUra nucleotides in RNA; if no conversion of FdUrd to FUra occurs, any interaction of the nucleoside with MTX or trimetrexate should be interpreted as the result of enhanced or reduced inhibition of thymidylate synthesis.

MATERIALS AND METHODS

Chemicals

FUra and MTX were obtained from commercial sources and were the material available for clinical use. FdUrd was purchased from Sigma Chemical Co., St. Louis, MO; and trimetrexate was kindly supplied by Warner-Lambert/Parke Davis, Ann Arbor, MI. [^{14}C]FdUrd (56 mCi/mmol) was purchased from Moravek Biochemicals, City of Industry, CA.

Methods

The colon adenocarcinoma line HCT-8 [13] was grown as a monolayer culture in 25 cm^2 sterile plastic flasks (Costar, Cambridge, MA) in RPMI medium 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% horse serum and subcultured weekly. Under these conditions, the doubling time was approximately 18 h, and the cloning efficiency in monolayer about 30%.

Clonogenic assay

A monolayer clonal growth technique was employed [14]. Monocellular dispersion was obtained by passing the trypsinized cells (3 min incubation with 5% trypsin in 0.9% NaCl) through a 23 gauge needle. Five hundred to 1000 cells in 5 ml of medium containing 10% horse serum were dispensed into sterile 60 mm Petri dishes (Costar) and incubated at 37°C and 100% humidity with 5% CO_2. Eighteen hours later, when the cells were attached to the bottom of the Petri dish but had not yet divided, 0.1 ml of an appropriate dilution of drug in H_2O or 0.9% NaCl solution was added to each dish. Control dishes received the same volume of saline. After the designated incubation period, the medium was decanted, the dishes were washed twice with 5 ml of 0.9% NaCl solution, and 5 ml of fresh medium was replaced. In sequential treatment experiments, this procedure was repeated for all cultures after the exposure to the second drug. Ten days after the initial plating, colonies were stained with orcein and those colonies with >100 cells were counted at 10× magnification using a dissecting microscope. Each experimental point was determined in triplicate with four replicate controls: experiments were repeated at least twice. In most instances the different experiments were repeated 2–4 times. Thereafter, when the optimal concentrations of each agent were defined, a comprehensive cloning experiment (almost 200 dishes) was done, producing results similar to the preliminary series. This comprehensive experiment was selected for presentation.

Thymidine phosphorylase

Cytosol extract of HCT-8 cells was prepared from exponentially growing cells in 600 cm^2 tissue culture dishes (Nunc, Rockilde, Denmark). Cells were washed twice with 50 ml of ice cold 0.9% NaCl solution and collected by scraping the attached cells off the plate with a rubber blade into 2 ml of an ice cold solution of 50 mM Tris–HCl, pH 7.4, with 4 nM NaF and 3 mM dithiothreitol. Cells were homogenized for 5 s (Tissumizer, Tekmar Co., Cincinnati, OH), and the extracts were frozen and thawed twice in dry ice and methanol; no intact cells could be seen by light microscopy after this procedure. The extract was then centrifuged at 100,000 ***g*** for 60 min at 4°C, and the supernatant was used for enzyme assay.

Thymidine phosphorylase was assayed in 100-μl reaction mixtures containing 50 mM Tris–HCl, pH 7.4. The reaction mixtures contained 2 mM inorganic phosphate, 50 μM [^{14}C]FdUrd and 25 or 50 μl of cell extract. Reactions were initated by the addition of the cell extract and incubated at 37°C; 5 μl were removed at each time point, applied to thin-layer chromatography plates (Merck; silica gel with 60 F254 fluorescent indicator) with unlabeled standards of FUra and FdUrd and immediately dried at 80°C to stop the reaction. The plates were developed in a chloroform:methanol:acetic acid (17:3:1) solvent. The standards were visualized by u.v. light, and radioactivity in each spot was measured in a Beckman liquid scintillation spectrometer. The assays were performed at two enzyme concentrations so that rates were derived from less than 25% substrate conversion. The rate was linear for at least two time points (15 and 30 min). Activity is expressed as nmol of product formed per mg of protein per h. Protein concentration was estimated by the method of Bradford [15].

Experimental design

Times and sequences of exposure to trimetrexate MTX, FUra and FdUrd are shown in the figures. In general, the doses selected for drug combination experiments were chosen to give a cell kill between 20 and 80%, so that synergy could be observed. The expected survival rates for the drug combinations were calculated by multiplying the observed survival value of each drug alone. When the

observed survival determined was lower than the expected survival, the two drugs were considered to synergize. When the observed survival was greater than expected, this was considered to be antagonism, and when the two values were similar, this was considered to be additive [16]. This simple method of comparing expected and observed interactions was used because our goal was not to reiterate the occurrence of a well known synergism between MTX and FUra in HCT-8 cells, but rather to see whether or not two substantially different antifols behave similarly in their pharmacological interactions with fluoropyrimidines.

RESULTS

Thymidine phosphorylase activity in HCT-8 cells

Since FdUrd may be rapidly degraded to FUra by thymidine phosphorylase, we measured the activity of this enzyme in exponentially growing HCT-8 cells. The data indicate a very low conversion of the nucleoside to FUra as a result of low activity of this enzyme *in vitro* (3.4 ± 0.8 nmol/mg protein) as measured by thin-layer chromatography.

Effects of trimetrexate, FUra and FdUrd on colony formation of HCT-8 cells

Cell kill as a function of concentration and time of exposure to FUra and FdUrd on HCT-8 cells has been published [17] and the current experiments confirm the strong time-dependent cytotoxicity of the fluoropyrimidines on this cell line (data not shown). Table 1 shows the comparison between the ED_{50} values observed with trimetrexate and those obtained with MTX after different periods of incubation. Trimetrexate is approximately two to six times more potent than MTX after short (4 h) and longer incubation times (24, 48 h, continuous exposure). In another paper [18] we describe the pharmacodynamics of this phenomenon: the influx of trimetrexate into HCT-8 cells is much faster than that of MTX, and the quinazoline achieves intracellular conentrations approximately 10 times higher than those of MTX. In addition, efflux experiments have indicated longer intracellular retention of trimetrexate at concentrations well above the level of dihydrofolate reductase.

Comparison between the interactions of MTX and trimetrexate with fluoropyrimidines

The major goal of this work was to focus on the differences that might exist between trimetrexate and MTX in their interactions with the fluoropyrimidines FUra and FdUrd. Figures 1, 2 and 3 show comparable results obtained with the combinations trimetrexate/FUra-FdUrd and MTX/FUra–FdUrd. Since the degree of interaction between two drugs may be different at different levels of cell kill, we compared essentially equitoxic doses. It is remarkable how similar these interactions are, except for the sequence FdUrd → trimetrexate vs. FdUrd → MTX (Fig. 2), where minor differences were observed. Trimetrexate given 4 h before, after or simultaneously with FUra or FdUrd produced additive, synergistic and antagonistic effects identical to those observed when MTX was combined with the fluoropyrimidines (Figs. 1 and 2). Figure 3 also indicates that this analogy between MTX and trimetrexate/fluoropyrimidines combinations is noted even in a longer incubation time (24 h).

Synergistic drug combinations: trimetrexate → FUra, trimetrexate → FdUrd

Trimetrexate causes synergistic cell kill with FUra and FdUrd when given before the fluoropyrimidines. The degree of synergistic HCT-8 cell killing is similar for both sequences trimetrexate → FUra and trimetrexate → FdUrd, and the synergism occurred at every concentration and treatment schedule tested (Figs. 1–3). Maximal synergy was observed at high level of cell kill in the sequence trimetrexate for 48 h followed by FUra or FdUrd for 5 days (Fig. 3). Under these conditions the observed survival of the drug combination was approximately five times lower than the calculated expected survival.

Antagonistic drug combinations: trimetrexate + FUra and FUra → trimetrexate

When FUra is given for 4 h either simultaneously or before tremetrexate, antagonism is observed at

Table 1. *Inhibitory effects of MTX and trimetrexate on the colony formation of HCT-8 cells, median* ED_{50} *values,* μM ± *S.E.* (n = 3)*

	Incubation time			
	4 h	24 h	48 h	Continuous
MTX	2.4 ± 1.3	0.25 ± 0.17	0.031 ± 0.01	0.015 ± 0.005
Trimetrexate	0.3 ± 0.18	0.05 ± 0.02	0.02 ± 0.007	0.005 ± 0.003

*Concentration of drug required to inhibit colony formation by 50% compared to untreated control cells.

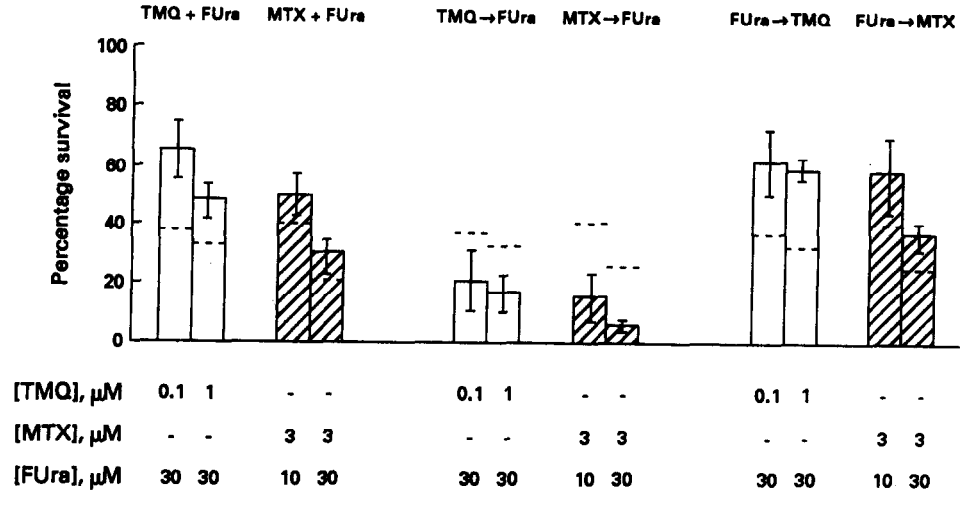

Fig. 1 Sequence-dependent effects of trimetrexate–FUra and MTX–FUra combinations on the survival of HCT-8 cells: 4 h exposure. Dashed lines indicate expected survival; bars, S.D. in a representative experiment.

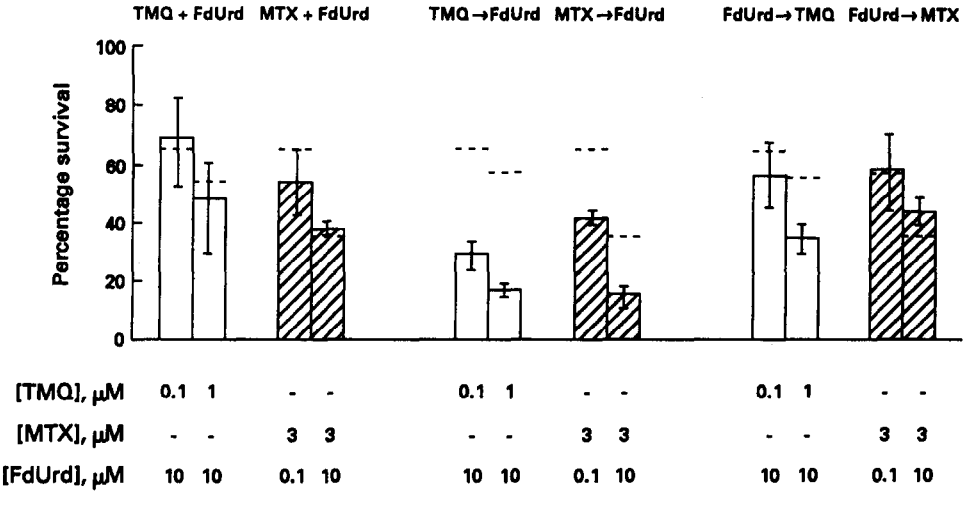

Fig. 2. Sequence-dependent effects of trimetrexate-FdUrd and MTX-FdUrd combinations on the survival of HCT-8 cells: 4 h exposure. Dashed lines indicate expected survival; bars, S.D. in a representative experiment.

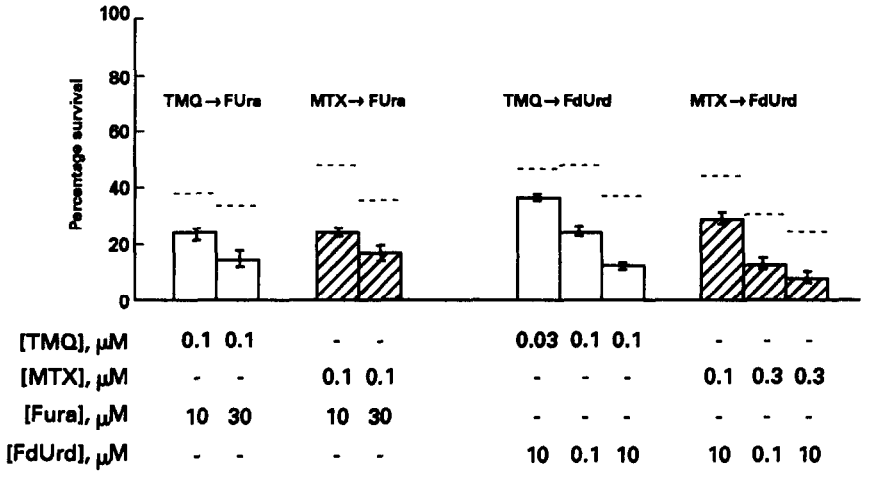

Fig. 3. Effects of trimetrexate and MTX/FUra and FdUrd combinations on the survival of HCT-8 cells: the antifols were given for 24 h and the fluoropyrimidines during 20–24 h. Dashed lines indicate expected survival; bars, S.D. in a representative experiment.

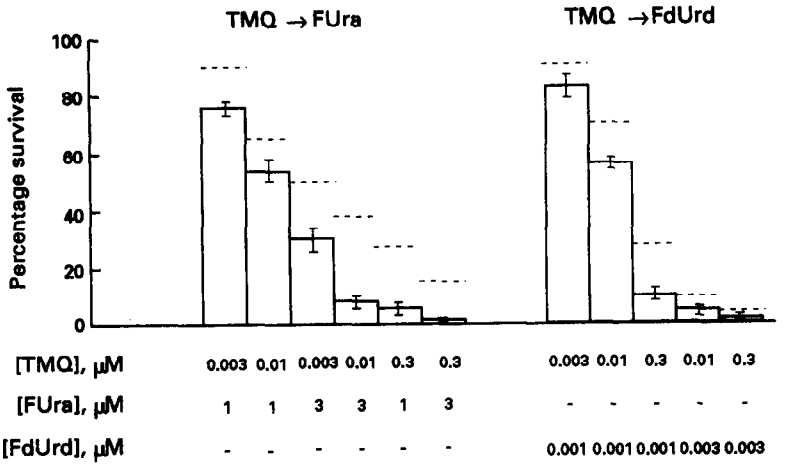

Fig. 4. Effects of trimetrexate–FUra, and trimetrexate–FdUrd combinations on the survival of HCT-8 cells: the quinazoline was given for 48 h *followed by the fluoropyrimidines for* 5 *days. Dashed lines indicate expected survival; bars, S.D. in a representative experiment.*

every concentration of both drugs (Fig. 1). There is no substantial difference in the degree of antagonism produced by the two schedules of drug administration.

The simultaneous exposure of HCT-8 cells to trimetrexate + FUra for 24 h or to the sequence FUra → trimetrexate for 24 h also produced equivalent antagonistic effects on cell kill (data not shown).

Additive drug combinations: trimetrexate + FdUrd and FdUrd → trimetrexate

Unlike FUra, when FdUrd preceded the antifol or was given simultaneously with it for 4 h, no antagonism was obtained, but only an additive effect on survival of HCT-8 cells. This is confirmed by the results obtained with several concentrations of FdUrd (data not shown) where the slight but consistent synergy observed at 1 μM trimetrexate and 10 μM FdUrd does not occur.

DISCUSSION

Several mechanisms by which MTX synergizes with FUra in the known sequence-dependent manner have been proposed:

(a) The depletion of tetrahydrofolates, resulting from the inhibition of dihydrofolate reductase by MTX, may lead to a block in the *de novo* synthesis of purines with accumulation of 5-phosphoribosyl-1-pyrophosphate. This co-substrate might then be utilized for a more efficient conversion of FUra to 5-fluorouridylate by the enzyme orotate phosphoribosyltransferase, resulting in increased 5-fluoronucleotide formation and enhanced 5-fluorouridylate incorporation into RNA [19].

(b) MTX polyglutamates formed by pretreating the cells with MTX may enhance the binding of 5-fluorodeoxyuridylate to the enzyme thymidylate synthase, producing synergistic cell kill via enhanced inhibition of thymidylate synthesis [20].

(c) In a similar way, the accumulation of dihydrofolate and dihydrofolate polyglutamate derivatives produced by MTX inhibition of dihydrofolate reductase activity may also enhance ternary complex formation with 5-fluorodeoxyuridylate and thymidylate synthase [21].

Since the same interactions observed with MTX–FUra combinations also occurred when an antifol lacking the glutamyl residue was combined with FUra, it is therefore unlikely that polyglutamylation of the antifol, plays a significant role in the synergism itself.

The fact that trimetrexate and MTX also synergize when followed by FdUrd, in a cell line with low level of thymidine phosphorylase, is consistent with enhanced DNA inhibition as the major contributor to the synergy. However, the present study cannot distinguish between the relative contribution of elevated levels of dihydrofolate polyglutamylates versus enhanced levels of 5-fluorodeoxyuridylate due to accumulation of 5-phosphoribosyl-1-pyrophosphate, or increased fluorouridine triphosphate incorporation into nucleic acids in determining the observed drug interactions.

These *in vitro* data are strengthened by the observation that sequential trimetrexate → FUra syner-

gizes *in vivo* in CDF1 mice bearing P388 leukemic cells [22]. These positive results should thus encourage the design of chemotherapeutic regimens including trimetrexate and FUra, administered in sequential combination: the enhancement of trimetrexate activity by FUra may in fact be particularly valuable for the treatment of MTX-resistant tumors.

Acknowledgements—The authors wish to thank Miss Maurizia Pratolongo for skillful assistance in the preparation of this manuscript.

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