Cancer Chemother. Pharmacol. 4, 111–116 (1980)

Cancer Chemotherapy and Pharmacology

The Interaction Between Fluoropyrimidines and Methotrexate, and [^{14}C]-Formate Incorporation into Nucleic Acids and Protein

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Summary. *Changes are reported in [^{14}C]-formate incorporation into nucleic acids and protein of Ehrlich ascites tumor cells during exposure to methotrexate (MTX) and fluoropyrimidines. The rate of [^{14}C]-formate incorporation into RNA, DNA, and protein in the presence of only MTX was inhibited by 82%, 91%, and 75% respectively, when compared with control rates. However, in the presence of 5-fluorodeoxyuridine (FdUrd) plus MTX, formate incorporation into RNA, DNA, and protein was inhibited by 67%, 85%, and 66%. Incubation of cells in vitro with [^{3}H]-dihydrofolate (DHF) results in its rapid conversion to [^{3}H]-tetrahydrofolate (THF). The THF/DHF ratio from the soluble fraction of cells that were incubated with [^{3}H]-DHF was 43% greater in the presence of FdUrd and MTX than in the presence of MTX alone. As the rate of [^{3}H]-dUrd incorporation into DNA was reduced by 88% and 99% by pretreating cells with 0.1 μM and 1 μM FdUrd, respectively, the inhibitory effect of MTX on [^{14}C]-formate incorporation into (a) RNA was decreased by 63% and 46%; (b) DNA was decreased by 74% and 61%; and (c) protein was decreased by 63% and 32%. These data suggest that fluoropyrimidines can antagonize the effects of MTX on purines or nucleic acid synthesis and protein synthesis by preventing the consumption of THF for dTMP synthesis.*

Introduction

The combination of fluoropyrimidines and MTX[1] has been used to treat a variety of neoplasms. Recently, the combination of MTX and FUra along with cyclophosphamide has been used extensively as adjuvant therapy for breast cancer [6]. In experimental systems, however, the report of conflicting evidence as to whether the combination of MTX and fluoropyrimidines is additive [1, 2, 4, 9, 13, 15], less than additive, or antagonistic [4, 22, 23] stimulated a series of investigations into the biochemical effects of the MTX-fluoropyrimidine combination.

Previous studies by Bowen et al. [8] demonstrated a biochemical basis for fluoropyrimidine-induced antagonism to MTX based upon the effects of FdUrd in reducing the basal rate of dTMP synthesis from deoxyuridylate. When the inhibitory effect of FdUrd on dTMP synthesis was less than the inhibitory effect of MTX, true antagonism to MTX suppression of dTMP synthesis by FdUrd could be shown when these agents were combined. While a basis for antagonism to MTX induced by fluoropyrimidines on dTMP synthesis has been attributed to the rate at which folate coenzymes are utilized for nucleic acid synthesis, a biochemical basis for antagonism to MTX induced by fluoropyrimidines on RNA and protein synthesis in contrast to DNA synthesis has not been clarified.

The role of MTX beyond that necessary for saturation of high-affinity DHFR sites will be evaluated, since it has been demonstrated that free intracellular MTX is critical in the suppression of THF synthesis and THF-dependent processes within the intact cell [10, 11, 20, 24, 25]. In studies employing L-cell mouse fibroblasts in which MTX levels in excess of the apparent binding capacity for DHFR were eliminated from the intracellular and extracellular compartments, DNA synthesis was minimally perturbed [11]. To achieve complete inhibition of DNA synthesis, a concentration of exchangeable MTX orders of magnitude above the Ki for DHFR was needed. In this report, the consequence(s) of FdUrd inhibition of dTMP synthesis on MTX suppression of nucleic acid and protein synthesis will be assessed in Ehrlich ascites tumor cells.

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1 The abbreviations used are: MTX, methotrexate; FdUrd, 5-fluorodeoxyuridine; FUra, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridine monophosphate; dUrd, deoxyuridine; dUMP, deoxyuridine monophosphate; dTMP, thymidylate; DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-CH_2THF, 5,10-methylene tetrahydrofolate; DHFR, dihydrofolate reductase

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Materials and Methods

Cells, Media, and Incubation

Ehrlich ascites tumor cells were obtained from CFl mice 6–12 days after IP injection of 0.2 ml undiluted ascitic fluid and prepared for experimentation as previously described [7, 12]. Cells were suspended into a modified Eagle's medium [11] free of folates, serum or methionine, with the following electrolyte composition: 135 m*M* NaCl, 4.4 m*M* KCl, 16 m*M* $NaHCO_3$, 1.1 m*M* Na_2HPO_4, 1.9 m*M* $CaCl_2$, and 1.0 m*M* $MgCl_2$. The cell suspension was stirred continuously with a motor-driven Teflon paddle as reported previously [12]. A pH of 7.2–7.4 was maintained during incubation by passing warmed and humidified 95% O_2/5% CO_2 over the cell suspension at 37° C. All incubations employing DHF were performed in the dark.

[^{14}C]-Formate incorporation into RNA, DNA, and protein, and [^{3}H]-dUrd incorporation into DNA was determined by measuring ^{14}C or ^{3}H in a dilute acid extract of a perchlorate precipitate as reported previously [25]. Data are expressed as the mean ± SE.

Chemicals

[^{14}C]-Formate, sodium salt (52 mCi/mmol), and [^{3}H]-dUrd (21.9 mCi/mmol) were obtained from New England Nuclear Corporation, Boston, Mass. MTX, obtained from Lederle Laboratories, was purified by fractionation on a DEAE-cellulose ion exchange column as previously described [12]. FdUrd was obtained from Sigma Chemical Company, St. Louis, Mo. [^{3}H]-DHF was prepared from (3′,5′,9-^{3}H) folic acid (500 Ci/mol) by dithionite reduction according to Blakley [5]. The radiochemical purity of [^{3}H]-DHF was 92%.

Chromatographic Techniques

After incubation with [^{3}H]-DHF, cells were washed twice with 0.85% NaCl at 0° C (to remove any extracellular adsorbed DHF) and then resuspended into 5 m*M* Tris-HCl buffer (pH 7.2) at 0° C, containing 0.2 *M* mercaptoethanol. Then, the cells were subjected to sonic oscillation four times for 15 s on a Heat Systems ultrasonic oscillator. After centrifugation for 1 h at 12,000 *g*, the supernatant fraction and the nonlabeled reference compounds were chromatographed [24].

Results

Effects of FdUrd and MTX on DHF Metabolism

Studies were undertaken to establish whether FdUrd-induced antagonism to MTX suppression of nucleic acid and protein synthesis is a result of decreased THF consumption. The synthesis and consumption of THF were evaluated by incubating cells with 0.05 μ*M* FdUrd for 15 min and then resuspending them into a medium containing 50 μ*M* MTX for 30 min to generate intracellular MTX in excess of the high-affinity binding sites. The cells were then incubated with [^{3}H]-DHF for 15 min (to minimize nonenzymatic degradation of DHF), and the composition of intracellular ^{3}H was then analyzed. Control cells converted all [^{3}H]-DHF to [^{3}H]-THF-[^{3}H]-DHF could not be detected (Fig. 1A). Cells exposed to FdUrd alone also converted all [^{3}H]-DHF to [^{3}H]-THF (Fig. 1B). When cells were continuously exposed to MTX (Fig. 1C), [^{3}H]-THF was decreased and the ^{3}H-labeled peak coincident with the nonlabeled DHF marker was increased to a very high level within the cell. When FdUrd was combined with MTX (Fig. 1D), a small increase in the [^{3}H]-THF level occurred compared with cells exposed to MTX alone. The ratio of THF (340.1 ± 87.2 cpm) to

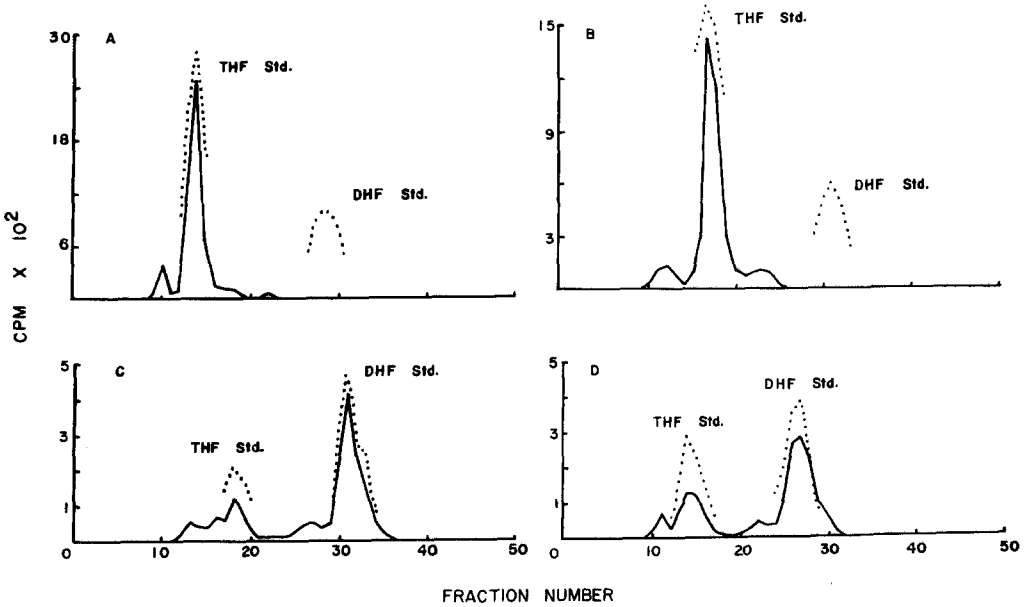

Fig. 1A–D. Effect of FdUrd and MTX on the metabolism of [^{3}H]-DHF. Cells were treated with (**B** and **D**) or without (**A** and **C**) 0.05 μ*M* FdUrd for 15 min. After separation from the FdUrd medium and two-fold washing at 37° C, cells were then exposed to 50 μ*M* MTX (**C** and **D**) for 30 min. The cells were then treated with 50 μ*M* [^{3}H]DHF for 15 min and subjected to sonication. Extracts and nonlabeled reference compounds were then chromatographed as described in 'Materials and Methods'

DHF (492.7 ± 265.8 cpm) in cells exposed to FdUrd plus MTX was 43 ± 3% ($P < 0.1$; $n = 3$), greater than cells exposed only to MTX [THF (256.4 ± 55.6 cpm)/DHF (597.1 ± 252.7 cpm)]. The total counts in cells exposed to FdUrd plus MTX or only to MTX were 1,100.5 ± 369.6 and 1,205.9 ± 356.9, respectively.

Effects of MTX on [^{14}C]-Formate Incorporation into RNA, DNA, and Protein after FdUrd Exposure

Fig. 2 illustrates the effects of FdUrd or 50 μ*M* MTX on [^{14}C]-formate incorporation into RNA, DNA, and protein, respectively. Ehrlich ascites tumor cells were exposed to FdUrd for 15 min to inhibit dTMP synthesis and to prevent a depletion of intracellular reduced folates. The cells were then resuspended into medium without FdUrd but in the presence or absence of MTX. Thirty minutes later, when MTX exceeded the capacity of the high-affinity intracellular binding sites, [^{14}C]-formate incorporation was monitored. In four or more separate experiments, the rate of [^{14}C]-formate incorporation into RNA, DNA, and protein, in the presence of 50 μ*M* MTX alone, was inhibited 82.2 ± 3.4%, 91.1 ± 1.7%, and 75.0 ± 5.8%, respectively; whereas, in the presence of FdUrd plus MTX, [^{14}C]-formate incorporation into RNA, DNA, and protein was inhibited 67.1 ± 5.6%, 85.5 ± 1.9%, and 65.8 ± 1.2%. A similar effect was observed after exposure of cells to FUra.

Dose-Response Effect of FdUrd on MTX Suppression of [^{14}C]-Formate Incorporation into RNA, DNA and Protein

To assess the relationship between suppression of the basal rates of dTMP synthesis and the effects of MTX on THF-dependent RNA, DNA, and protein synthesis, Ehrlich tumor cells were exposed to varying concentrations of FdUrd (0.1 μ*M* and 1 μ*M*) for 15 min to achieve increasing suppression of dTMP synthesis. The cells were then separated by centrifugation and resuspended into a FdUrd-free medium but in the presence of 50 μ*M* MTX. A 15-min exposure of cells to 0.1 μ*M* and 1 μ*M* FdUrd decreased the rate of [^{3}H]-dUrd incorporation into DNA by 88% and 99%, respectively. Fig. 3 illustrates a representative experiment showing the effects of 0.1 μ*M* and 1 μ*M* FdUrd on MTX suppression of [^{14}C]-formate incorporation into RNA, DNA, and protein. In five experiments, the mean depression of ^{14}C incorporation into RNA by 0.1 μ*M* and 1 μ*M* FdUrd in combination with MTX was 63.0 ± 8.5% and 46.3 ± 12.5%, respectively; whereas the inhibitory effect of MTX alone was 81.9 ± 3.3%. The high standard error from cells exposed to 0.1 μ*M* FdUrd or 1 μ*M* FdUrd and MTX reflects the variability from day to day. However, the difference between the rate of ^{14}C incorporated into RNA in cells exposed to 0.1 μ*M* FdUrd plus MTX is significant to $P < 0.05$. In the presence of MTX and 0.1 μ*M* FdUrd or 1 μ*M* FdUrd, the rate of ^{14}C incorporation into DNA was inhibited by 74.2 ± 6.7% and 61.3 ± 7.1%, respectively (three experi-

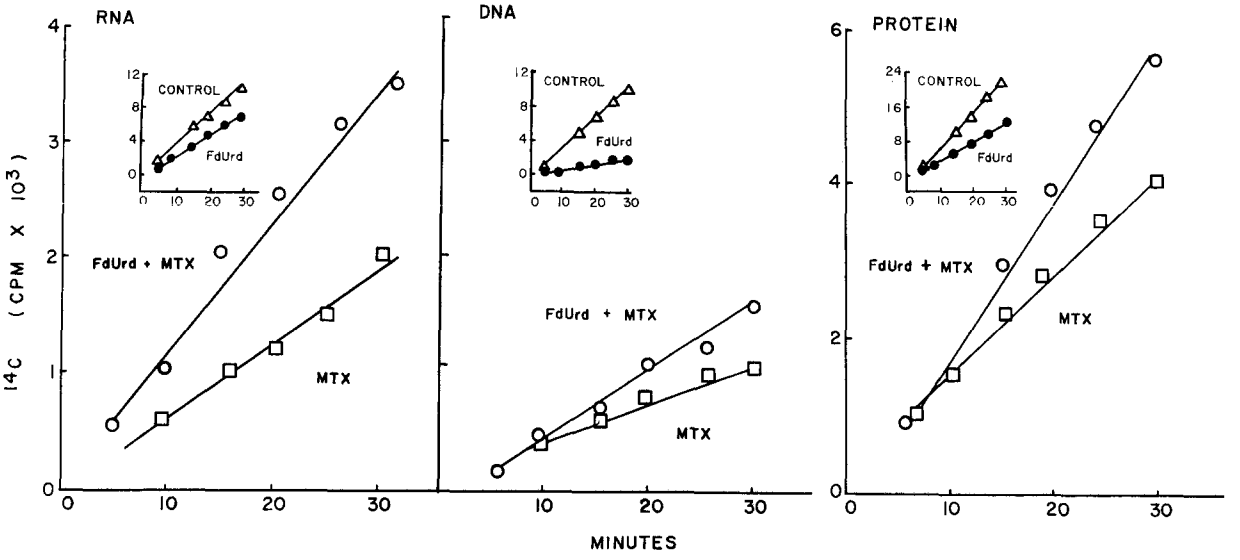

Fig. 2. Effect of MTX on [^{14}C]-formate incorporation into RNA, DNA, and protein after exposure of cells to FdUrd. Ehrlich tumor cells were exposed to 0.05 μ*M* FdUrd for 15 min. The cells were washed twice in a fresh medium at 37° C, divided into two fractions, and then resuspended into fresh medium in the presence (○) and absence (●) of 50 μ*M* MTX for 30 min. Cells treated similarly but not exposed to FdUrd were incubated only in the presence (□) or absence (△) of MTX. [^{14}C]-formate was added to a final concentration of 100 μ*M*, and ^{14}C incorporation into nucleic acids and protein was monitored

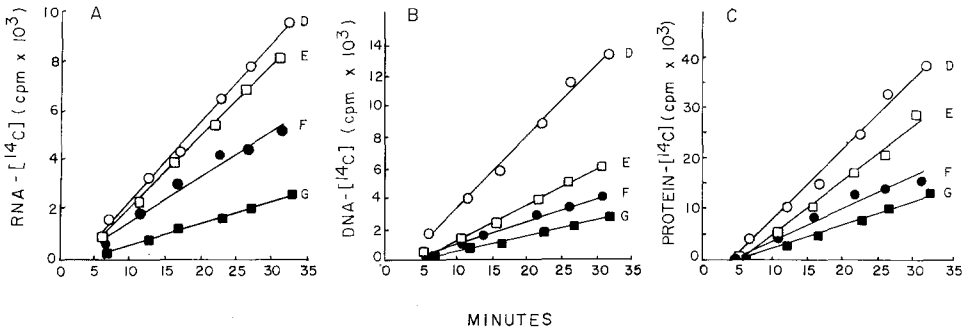

Fig. 3. Effect of FdUrd dose on MTX suppression of [^{14}C]-formate incorporation into RNA, DNA, and protein. The experimental protocol is as described in the legend to Fig. 2, except that cells were incubated with 0.1 μM and 1 μM FdUrd. D, control cells; E, cells exposed to 1 μM FdUrd and MTX; F, cells exposed to 0.1 μM FdUrd and MTX; G, cells exposed to MTX alone

ments); the rate of ^{14}C incorporation in the presence of MTX alone was inhibited by 87.1 ± 3.6%. Hence, the rate of [^{14}C]-formate incorporation into DNA was augmented ($P < 0.1$) by 1 μM FdUrd and MTX, as compared with cells exposed to 0.1 μM FdUrd and MTX. Similarly, inhibition of ^{14}C incorporation into protein was increased from 32.1 ± 6.9% in the presence of 1 μM FdUrd plus MTX to 63.2 ± 5.2% (three experiments, $P < 0.01$) in cells exposed to 0.1 μM FdUrd plus MTX; MTX alone inhibited ^{14}C incorporation into protein by 79.7 ± 6.1%. Hence, as the concentration of FdUrd is increased and the basal rate of dUrd incorporation is reduced, the inhibitory effect of MTX on the rate of [^{14}C]-formate incorporation into RNA, DNA, and protein is progressively reduced.

Discussion

The incorporation of [^{14}C]-formate into RNA, DNA, and protein occurs as a result of the insertion of the formate carbon via tetrahydrofolates into purine nucleotides at C-2 and C-8 as well as into thymidine, methionine, and serine [21]. Data from this study support the concept that fluoropyrimidine-induced antagonism to MTX suppression of [^{14}C]-formate incorporation into nucleic acids and protein is related, in part, to the rate of dTMP synthesis from deoxyuridylate. Hence, as the basal rate of dTMP synthesis from dUMP is decreased by FdUMP, the oxidation of 5,10-CH_2THF should be decreased, and the rate of DHF accumulation caused by MTX will be suppressed. Consequently, fluoropyrimidines should reduce the inhibitory effect of MTX by diminishing depletion of intracellular THF or reduced folates for RNA, DNA, and protein biosynthesis.

To determine whether decreased oxidation of THF cofactors after FdUrd exposure was responsible for minimizing the effect of MTX on nucleic acid and protein synthesis, the rate of [^{14}C]-formate incorporation into RNA, DNA, and protein was studied under conditions in which tumor cells were first exposed to FdUrd and then to MTX. The rate of [^{14}C]-formate incorporation into RNA, DNA and protein (Fig. 2) is consistent with the formulation that fluoropyrimidines diminish the depletion of reduced folates and decrease the effect of MTX on nucleic acid and protein biosynthesis. This is further supported by the demonstration of a decreased MTX effect on RNA, DNA, and protein synthesis as the dose of FdUrd was increased (Fig. 3). Since three of the major substrates (dATP, dGTP, and dCTP) for DNA polymerase are obtained by direct reduction of the corresponding ribonucleotides, the rate at which ^{14}C appears in purines (adenine and guanine) for RNA, therefore, should exceed those for DNA because of fluoropyrimidines limiting the availability of dTMP for polymerization into DNA. Although dTMP can also arise from thymidine, this reaction is probably used as a salvage pathway for dTMP formed from thymidylate synthetase.

The greater difference in antagonism to MTX observed from [^{14}C]-formate incorporation into RNA and protein than DNA suggests that the rate of THF utilization for RNA and protein exceeds that for DNA when cells are pretreated with FdUrd. DNA synthesis is the least sensitive to fluoropyrimidine-induced antagonism to MTX, mainly because of the block of dTMP synthesis by fluoropyrimidines. The low sensitivity of [^{14}C]-formate incorporation into protein to fluoropyrimidine-induced antagonism to MTX may be a result of interconversion enzymes sustaining 5-methyl THF levels [19] (the major reduced folate within the mammalian cell [14]). Since the pathways of dTMP synthesis and methionine synthesis have a common origin in 5,10-CH_2THF, the relative flux of folate coenzymes through these pathways can determine the rate at which folate coenzymes are utilized for dTMP and methionine synthesis [16, 18]. When dTMP synthesis is reduced by fluoropyrimidines, the portion of 5,10-CH_2THF that is not part of the FdUMP-5,10-CH_2THF-thymidylate synthetase ternary complex [17] should be converted to 5-methyl THF [16], which in the

presence of methyl THF: homocysteine methyl-transferase synthesizes a molecule of methionine and THF. Thus, for every molecule of methionine that is synthesized and enters, in part or completely, into protein, a molecule of THF is available and could be utilized for purine synthesis. This is consistent with data from five experiments obtained in a similar way to data in Fig. 2, which indicated that the rate of [^{14}C]-formate incorporation into protein and RNA in the presence of FdUrd plus MTX was inhibited by 66% and 67%, respectively. Hence, the incorporation of ^{14}C into RNA and protein in these cells should be (a) least sensitive to depletion of THF by MTX [25] (Fig. 2), and (b) most sensitive to fluoropyrimidine-induced antagonism to MTX, because the specific coenzymes necessary to sustain these reactions depend only in part upon the sustained regeneration of THF from DHF.

The critical role of MTX in suppression of THF synthesis was examined in contrast to conditions in which cells were exposed to FdUrd and to FdUrd plus MTX. In the absence of FdUrd and MTX, Ehrlich tumor cells converted DHF to THF – demonstrating that DHF is at a concentration below its Km and that DHFR is at a low state of saturation. In the presence of MTX alone, DHF reached its maximum level in the cell; this was accompanied with the suppression of THF production. A similar response to DHF metabolism occurred in the presence of FdUrd plus MTX; however, the ratio of THF to DHF was 43% greater in the presence of FdUrd and MTX than with MTX alone. The presence of THF in cells treated only with MTX may be a result of competition between a high concentration of DHF extracellularly and increasing intracellular levels of DHF and MTX for DHFR. When the cells are exposed to MTX and the drug enters the intracellular compartment, the following sequence of events would be expected to occur: association of MTX with DHFR, which results in a small transient reduction in the rate of THF synthesis with a subsequent increase in the DHF level; eventually, as MTX binds to the major portion of DHFR, the rate of THF synthesis will decrease. Since the interaction between DHFR and MTX is not stoichiometric at physiological pH [3], and because cellular DHF has risen to high levels from MTX binding to DHFR and a high extracellular DHF concentration, DHF can compete with MTX for the few remaining DHFR-binding sites and maintain residual THF synthesis. Similar conditions, therefore, should exist when cells are exposed to FdUrd and MTX. However, the THF level should be less in cells treated only with MTX, because of continual production of DHF from the oxidation of 5,10-CH_2THF. This was reflected in the lower THF/DHF ratio in cells treated with MTX than FdUrd and MTX.

This and other studies continue to indicate that the biochemical mechanism by which fluoropyrimidines and MTX derive their antitumor effects is a complex process and warrants further study.

Acknowledgements: This research was supported by Grant CA-24192 from the National Cancer Institute.

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Received August 1, 1979/Accepted November 5, 1979