limetric lines of the HCO or formyl radical.

It has been pointed out that radio, television, radar, microwave link, and other terrestrial transmissions are expanding into space at 1 light-year per year (*2*). Another technological society near a neighboring star could receive the strongest of these directly with substantial effort and could learn a great deal about the earth and the technology of its inhabitants. The concepts presented here suggest that on an imaginary screen sufficiently far behind that star, the short-wavelength end of this terrestrial activity is now being played out at substantial amplifications. Properly placed receivers with antennas of modest size could in principle scan the earth and discriminate between different sources, mapping such activity over the earth and learning not only about the technology of its inhabitants, but also about their thoughts. It is possible that several or many such focused stories about other worlds are now running their course on such a gigantic screen surrounding our sun, but no one in this theater is observing them (*8*).

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2. For example, see G. Cocconi and P. Morrison, *Nature (London)* **184**, 844 (1959); I. S. Shklovskii and C. Sagan, *Intelligent Life in the Universe* (Holden-Day, San Francisco, 1965); A. G. W. Cameron, Ed., *Interstellar Communication* (Benjamin, New York, 1963); C. Sagan and F. Drake, *Sci. Am.* **237**, 80 (May 1975); B. M. Oliver and J. Billingham, "Project Cyclops: A design study of a system for detecting extraterrestrial intelligent life," *NASA Contract Rep. CR114445* (1973); W. T. Sullivan III, S. Brown, C. Wetherill, *Science* **199**, 377 (1978); B. Murray, S. Gulkis, R. E. Edelson, *ibid.*, p. 485; R. N. Bracewell, *The Galactic Club* (Freeman, San Francisco, 1974). The messenger probe concept introduced by Bracewell would require communications to a home base over interstellar distances. Thus a gravitational lens could have application here, and also in astronomical observations of the more usual type.
3. V. R. Eshleman, G. L. Tyler, W. T. Freeman, *Icarus* **37**, 612 (1979).
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5. There are some interesting questions about how a two-way exchange might be initiated and maintained with a single lens used for both directions, since the paths are so directive that separated transmitting and receiving locations would be required on at least one end of the path. Perhaps a symmetrical situation would be preferable, where a lens at each end would be used but only for reception, with transmissions originating from the respective home planets. Of course, in all cases the local links would involve two-way communications.
6. By traveling this far along a radius from the sun, it would be possible to use most of the nearer stars as lenses to observe regions along a very narrow angular strip within a few tenths of a degree from the directions of these stars as viewed from the earth. However, it would not be possible to dwell on a particular direction or come back to a previous one without a large change in the spacecraft velocity. If the lens of a nearby star that is similar to the sun were used, the large value of $z$ would mean that coronal effects would be reduced considerably.
7. Using the example of $z = 4z_{min}$, a wavelength of less than 1 or 2 cm is needed for energy to even reach the focal zone. Coronal irregularities would break up the principal Fresnel zone annulus of the idealized coherent case into a large number of incoherent smaller zones for wavelengths from these values to several orders of magnitude smaller, with the number of pieces decreasing as the wavelength is reduced. The maximum value of the average signal intensification would still be appreciable, varying inversely with the number of zones. Thus, while our idealized example of $I_{max} \cong 10^8$ at $\lambda = 10^{-3}$ m and $z = 4z_{min}$ would not be realized, this value of $I_{max}$ might be obtained at a somewhat shorter wavelength.
8. As an extension of the conclusions of Einstein (*1*) and Liebes (*4*) concerning the improbability of observing the flash of two suitably aligned stars, it is interesting that if there were a technological society orbiting each visible star in our galaxy, and if every one of them were communicating with every other one except ours by use of stellar gravitational lenses (quintillions of paths), it is highly unlikely that the earth, by chance, would have intercepted any of the focal lines since the time when we first developed radio receivers.
9. I thank S. Liebes, Jr., and G. L. Tyler for helpful discussions. The Center for Radar Astronomy is supported, in part, by the NASA Planetary Atmospheres Program, grant NGL 05-020-014.

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## Enhanced 5-Fluorouracil Nucleotide Formation After Methotrexate Administration: Explanation for Drug Synergism

Abstract. *Exposure of L1210 leukemia cells first to 0.1 to 100 micromolar methotrexate and then to 10 micromolar 5-fluorouracil produces a synergistic effect on the number of cells killed in culture. Methotrexate dose-related increases occur in the concentrations of intracellular 5-fluorouracil ribonucleotides and 5-fluoro-2'-deoxyuridylate and in the incorporation of 5-fluorouracil into RNA. These increases are correlated with increased concentrations of intracellular phosphoribosylpyrophosphate. It is proposed that the enhanced formation of ribonucleotides of 5-fluorouracil and the subsequent incorporation of these compounds into RNA in methotrexate-treated cells may account for synergism between these agents.*

Methotrexate (MTX) and 5-fluorouracil (5-FU) are often used in combination for the treatment of breast cancer (*1*). However, recent biochemical studies demonstrating antagonism of MTX and 5-FU with thymidylate synthetase question the rationale for concurrent use of these chemotherapeutic drugs (*2, 3*). Inactivation of thymidylate synthetase by 5-fluoro-2'-deoxyuridylate (FdUMP) requires the formation of a ternary complex among FdUMP, 5,10-methylenetetrahydrofolate ($CH_2FAH_4$), and thymidylate synthetase, leading to the covalent binding of FdUMP to this enzyme (*4-6*). By inhibiting dihydrofolate reductase, MTX prevents regeneration of $CH_2FAH_4$ from the oxidized folate ($FAH_2$) formed during thymidylate synthesis. It is this depletion of $CH_2FAH_4$ that has been proposed (*3*) as a basis for the biochemical antagonism observed in vitro when MTX is administered before 5-FU. Since MTX is only 21 percent as efficient as $CH_2FAH_4$ in promoting the binding of FdUMP to thymidylate synthetase (*7*), and possibly does not permit the covalent association of FdUMP with this enzyme, it is unlikely that a direct interaction between MTX and thymidylate synthetase can compensate for the reduced concentrations of $CH_2FAH_4$. Methotrexate also increases intracellular deoxyuridylate (dUMP) (*8, 9*), which

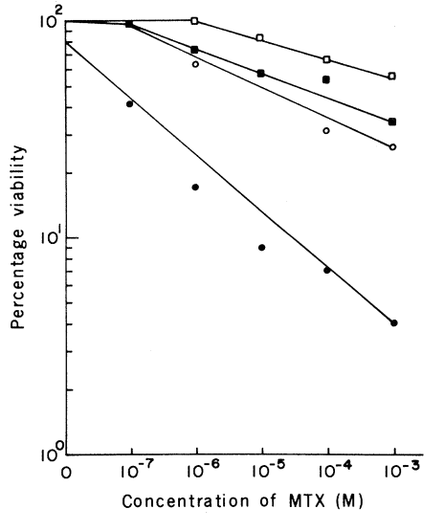


Fig. 1. Soft agar cloning of L1210 cells. The L1210 cells in Fischer's medium were exposed to MTX at the indicated concentrations for 2 or 4 hours. The drug-containing medium was then removed and the cells were washed twice with drug-free medium before cloning. Symbols: □, cell viability after 2 hours, and ■, after 4 hours of exposure to MTX. The MTX at the designated concentrations was present in the medium for 3 hours before 1 $\mu M$ (○) or 10 $\mu M$ (●) 5-FU was added for 1 hour. The cells were then cloned in drug-free medium. This sequence increased the number of cells that were killed, with 10 $\mu M$ of 5-FU having the greatest effect. The viability of cells exposed to either dose of 5-FU alone for 1 hour was 100 percent of the no-drug control. Cloning efficiency was 90 percent. Cell viability was determined from the number of clones formed from drug-treated cells divided by the number of clones formed from control cells multiplied by 100.


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may compete with FdUMP for binding to thymidylate synthetase. The reverse drug sequence, with 5-FU administered before MTX, is also biochemically antagonistic (*2*). Inhibition of thymidylate synthesis by FdUMP also results in an accumulation of dUMP and diminishes utilization of $CH_2FAH_4$ (*10*), decreasing the rate of $FAH_2$ production. This enables cells to sustain their concentrations of tetrahydrofolate cofactor with a lower level of dihydrofolate reductase activity which thus reduces the pharmacologic effect of subsequently added MTX (*2*). This may account for the observation that when 5-FU precedes MTX administration, there is no synergistic killing of tumor cells (*11*). Hence, irrespective of the order in which the drugs are administered, there is no currently recognized biochemical interaction among these drugs and thymidylate synthetase that accounts for the synergism observed in tumor-bearing rodents when MTX is given before 5-FU (*11–13*) and supports the use of these drugs in humans.

We examined the effect of treating logarithmically growing L1210 murine leukemia cells (*14*) first with MTX and then with 5-FU. We used the soft agar cloning system (*15*), and also observed in these cells the accumulation of 5-FU nucleotide derivatives. When MTX at varying doses preceded either 1 $\mu M$ or 10 $\mu M$ 5-FU by 3 hours, cell viability was reduced over that produced by MTX alone (Fig. 1). Exposure to 5-FU alone for 1 hour under these conditions did not reduce cell viability, and 5-FU preceding MTX was no better than MTX alone. This synergistic antitumor activity could be correlated with enhanced accumulation of 5-FU nucleotides in cells previously treated with MTX.

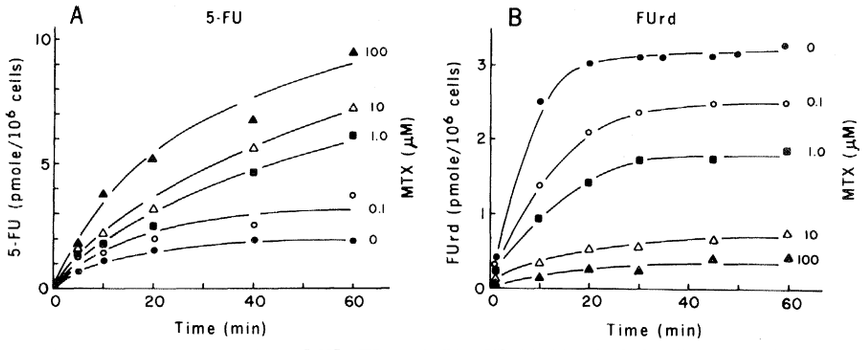


Fig. 2. The accumulation of (A) [$^3$H]5-FU and (B) [$^3$H]FUrd by L1210 cells exposed to MTX. The curves show 5-FU and FUrd (1 $\mu M$) uptake by (●) cells not previously exposed to MTX; and cells exposed to (○) 0.1 $\mu M$, (■) 1.0 $\mu M$, (△) 10 $\mu M$, and (▲) 100 $\mu M$ MTX. All MTX exposure was for 3 hours.

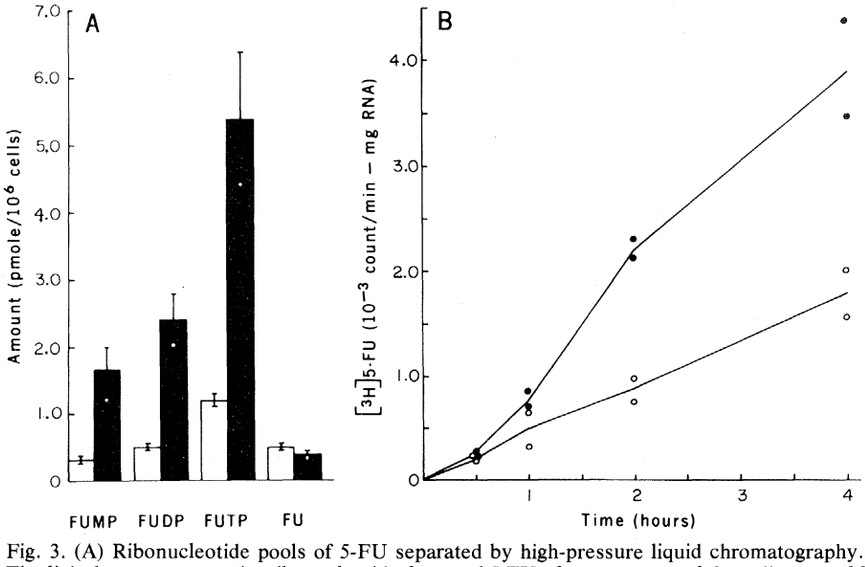


Fig. 3. (A) Ribonucleotide pools of 5-FU separated by high-pressure liquid chromatography. The light bars represent the ribonucleotide forms of 5-FU after exposure of the cells to 1 $\mu M$ [$^3$H]5-FU for 1 hour. The dark bars represent 5-FU ribonucleotides after exposure to 1 $\mu M$ MTX for 3 hours. (B) The 5-FU content of RNA from MTX-treated L1210 cells. The RNA fraction of the duplicate experiment in (A) was separated from the DNA and protein fractions. Symbols: ●, incorporation of [$^3$H]5-FU (2 Ci/mmole, 1 $\mu M$) into RNA of cells previously exposed to MTX; ○, cells not previously exposed to MTX. The RNA was quantitated by the Orcinol reaction.

Cells were exposed to MTX for 3 hours at concentrations of 0.1 to 100 $\mu M$. We then determined the intracellular accumulation of [$^3$H]5-FU nucleotides after exposing the cells to 1 $\mu M$ concentrations of $^3$H-labeled 5-FU (2 Ci/mmole) (*16*). The rate of accumulation and net amount of cellular [$^3$H]5-FU derivatives increased with successively higher concentrations of MTX (Fig. 2A). It is of interest that augmentation of [$^3$H]5-FU derivative accumulation continued at MTX concentrations above those that should deplete all $CH_2FAH_4$ (*17*). Mono-, di-, and triphosphorylated ribonucleotide derivatives of 5-FU were quantitated by high-pressure liquid chromatography (HPLC) (*18*) after exposing the cells to 1 $\mu M$ [$^3$H]5-FU (2 Ci/mmole) for 1 to 4 hours with and without exposing them first to 1 $\mu M$ MTX for 3 hours. The concentrations of 5-FU ribonucleotides were fivefold higher in MTX-treated cells than control cells after the first hour (Fig. 3A), and accumulation of these ribonucleotides continued at a constant rate for 4 hours. Exposure of cells to 1 $\mu M$ MTX for 3 hours resulted in a 30 percent reduction in the rate of RNA synthesis as determined by the incorporation of [$^{14}$C]glycine into RNA (*19*), but this treatment enhanced incorporation of [$^3$H]5-FU into RNA (Fig. 3B). No $^3$H was detected in the DNA or protein fraction.

The nonenzyme-bound FdUMP pool was monitored by a new rapid technique utilizing periodate ribonucleotide oxidation and HPLC separation (*18*). Cells were exposed to [$^3$H]5-FU (1 $\mu M$, 25 Ci/mmole) for 1 hour with and without prior exposure to 1 $\mu M$ MTX for 3 hours. The amount of FdUMP in cells not exposed to MTX was 27 ± 3 fmole per $10^6$ cells; in cells exposed to MTX the amount was 134 ± 15 fmole per $10^6$ cells. This fivefold increase in FdUMP is similar in magnitude to the increase in fluoropyrimidine ribonucleotides induced by MTX. This suggests that the increase in FdUMP is secondary to the increase in ribonucleotides and occurs in proportion to the conversion of fluorouracil diphosphate (FUDP) to FdUDP by ribonucleotide reductase with subsequent conversion to FdUMP.

These data suggest that exposure of L1210 cells in logarithmic growth to MTX enhances phosphoribosyl transfer to 5-FU and that the increase in the number of cells killed when MTX is administered before 5-FU may result from increased incorporation of fluoropyrimidine into RNA. We propose that this

increased incorporation of fluoropyrimidine is due to an increase in cell phosphoribosylpyrophosphate (PRPP). The PRPP provides the phosphoribosyl moiety for the conversion of 5-FU to 5-fluorouridylate (5-FUMP) in some cells. Our observation that enhanced phosphorylation of 5-FU occurs at doses of MTX which inhibit thymidylate and purine synthesis (*17*) suggested that the PRPP which would have been utilized for purine synthesis was now available for the transfer of the phosphoribosyl moiety to acceptable bases, in this instance, 5-FU. To evaluate this further, we measured the PRPP utilized in the conversion of [$^3$H]adenine to [$^3$H]adenosine monophosphate in the 40 to 70 percent ammonium sulfate fraction of an L1210 leukemia cell homogenate, using a modification of a technique previously reported (*20*). As shown in Fig. 4, PRPP increased in response to 3 hours of exposure to increasing concentrations of MTX. Supporting the role of PRPP in the enhanced formation of 5-FU nucleotides in L1210 cells treated with MTX is the observation that hypoxanthine (10 $\mu M$), which readily accepts the phosphoribosyl moiety from PRPP, decreased PRPP concentrations and diminished the increase in 5-FU (5 $\mu M$) nucleotide formation seen in cells exposed to 1 $\mu M$ MTX. Cells were exposed to 1 $\mu M$ 6-methylmercaptopurine riboside for 3 hours. This agent inhibits amidophosphoribosyltransferase (*21*), the first reaction unique to purine nucleotide synthesis, and prevents utilization of PRPP in de novo purine synthesis. The cells showed an increase in PRPP and ribonucleotide derivatives of 5-FU that was identical to the increase that followed exposure of the cells to 1 $\mu M$ MTX for 3 hours. These observations confirm the importance of 5-FU ribonucleotides and support the concept that MTX enhances accumulation of ribonucleotide derivatives of 5-FU in cells that utilize PRPP to form 5-FUMP by increasing the concentration of this substrate. The subsequent enhanced incorporation of 5-fluorouracil triphosphate (5-FUTP) into RNA may explain the synergistic effect on cell death that is observed when cells are exposed to MTX prior to 5-FU. It is unlikely that the higher concentrations of FdUMP in MTX-treated cells contribute to cytotoxicity since MTX decreases $CH_2FAH_4$ and the reduction in this cofactor may limit the covalent association of FdUMP with thymidylate synthetase. This suggests that effects of FdUMP on thymidylate synthetase are not as critical to cytotoxicity under these conditions as is the MTX-induced increase in the incorporation of 5-FUTP into RNA.

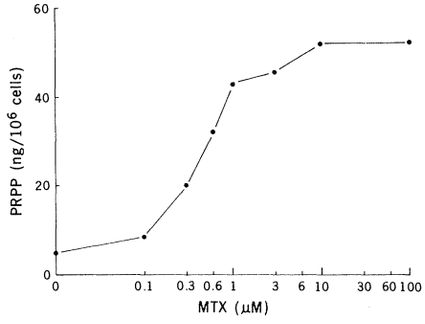


Fig. 4. The concentrations of PRPP in L1210 cells exposed to varying concentrations of MTX for 3 hours.

Increased formation of 5-FU nucleotides and synergistic effects on cell death by MTX would not be expected in cells that form 5-FUMP by mechanisms independent of PRPP. Because the ribonucleotide pools of uridine triphosphate and cytidine triphosphate, the natural feedback inhibitors of the enzyme uridine-cytidine kinase, are unchanged in the presence of high concentrations of MTX given for the limited duration of our experiments (*22*), cells that use this enzyme to phosphorylate the riboside derivative of 5-fluorouridine (FUrd) would not be expected to have an augmented accumulation of FUrd in response to MTX. In fact, the intracellular accumulation of ribonucleotide derivatives of [$^3$H]FUrd (1 $\mu M$, 20 Ci/mmole) is inhibited in L1210 leukemia cells by MTX (Fig. 2B). The magnitude of this inhibition is also related to the concentration of MTX, with the highest concentration of MTX (100 $\mu M$) resulting in the greatest inhibition; this effect is the opposite of the MTX effect on 5-FU accumulation.

These observations confirm that MTX administered before 5-FU can act synergistically in killing cells that use PRPP as a ribose and phosphate donor, but not in killing cells that do not require this pathway for the formation of 5-FUMP. These results can explain the discrepancy between the biochemical antagonism of MTX and 5-FU with thymidylate synthetase and the synergistic effects of these drugs on cancers in rodents reported by others (*11–13*). The data also emphasize the possible importance of 5-FU incorporation into RNA as a determinant of the cytotoxicity of this agent.

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18. Fifty milliliters of L1210 cells in the logarithmic phase of growth ($3 \times 10^5$ cell/ml) were exposed first to MTX (1 $\mu M$) for 3 hours and then to 5-FU (1 $\mu M$, 2 Ci/mmole; Amersham/Searle) for 1 hour. The cells were then centrifuged at 1000*g* for 5 minutes, and the fraction soluble in 0.5*M* $HClO_4$ was obtained. The ribonucleotides were separated by HPLC (Altex model 100 pump) with a linear gradient (0.01*M* to 1.0*M*) of sodium phosphate buffer, *p*H 3.31, and a flow rate of 0.9 ml per minute on a 25 cm by 4.6 mm Partisil SAX (10 $\mu M$ particle size) column. Absorbance was recorded at 254 and 280 nm and fractions collected of the entire column flow. We used FU, FUMP, UDP, UDPG, UTP, and FUDR as unlabeled markers; radioactivity was only present in the FU, FUMP, UDP, UDPG, and UTP regions and accounted for 99 percent of the radioactivity. Acid-soluble fractions were also prepared for FdUMP analysis from cells under identical conditions and treatment, except 5-FU of higher specific activity was used (1 $\mu M$, 25 Ci/mmole; Moravek Biochemicals). Periodate oxidation of this acid-soluble fraction (12 m*M* $NaIO_4$ at 37°C for 30 minutes followed by 0.4*M* $CH_3NH_2$ plus 0.01*N* NaOH for 15 minutes) cleaved the base and phosphates from the ribonucleotides but not the deoxyribonucleotides. Unlabeled FdUMP was used to mark the region containing the [$^3$H]FdUMP. Separation by HPLC of the oxidation products from the remaining FdUMP was achieved with a polystyrene column (BA-X4; James Benson) eluted at 50°C with ammonium acetate (*p*H 7.0, 0.5*M*) at a rate of 1 ml/min. The radioactivity remaining after periodate treatment was in the FdUMP region and the void volume. No radioactivity was present in the UTP, UDP, or UDPG region when the samples were examined on the Partisil SAX column, indicating the completeness of the periodate oxidation of the ribonucleotide forms of 5-FU. The 5-FUMP at 40,000 count/min was reduced to background counts (40 count/min) by the periodate oxidation, demonstrating that this reaction was greater than 99.8 percent complete.
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23. The MTX was provided by the Drug Development Branch of the National Cancer Institute, Bethesda, Md. The 5-FU was purchased from Hoffmann-La Roche, Nutley, N.J. The work was supported by a Swebelius award from the Yale Comprehensive Cancer Center, and NIH grants CA 09200-03 and CA 24187-01.

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