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The Enhancement of 5-Fluorouracil Anti-metabolic Activity by Leucovorin, Menadione and α-Tocopherol*

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Abstract—*Studies were designed to develop approaches for increasing the intracellular levels of* 5-10 *methylene tetrahydrofolate and to see if this would augment the anti-metabolic effect of* 5-*fluorouracil* (5-*FU*). *This was studied in the Friend murine erythroleukemia cell line* (*FLC*). *The ability to inhibit tritiated deoxyuridine* ([^{3}H]*UdR*) *incorporation into DNA and a* 72-hr *cell growth curve determination were used as indicators of the* 5-*FU anti-metabolic effect. The cell growth inhibition by continuous* 5-*FU exposure* (3×10^{-7} *to* 2×10^{-6} M) *at* 48 *and* 72 hr *was time and dose-related, and was less in cells depleted of folate. The simultaneous addition of* DL-5-*formyltetrahydrofolate in the form of Leucovorin* (*LV*) (1×10^{-5} M) *enhanced* 5-*FU cell growth inhibition at all times recorded and was greater than* 3-*fold at the highest* 5-*FU concentration. Similarly, a* 1 *to* 6-hr *exposure to this combination, followed by washing, was* 3-*fold greater than that of* 5-*FU cell growth inhibition. The potentiation of* 5-*FU anti-metabolic effect by LV was also demonstrated by further inhibition of* [^{3}H]*UdR incorporation into DNA after* 72 hr *of varying concentrations of drug exposure. The* 5-*FU inhibition of* [^{3}H]*UdR incorporation into DNA was significantly increased and prolonged in the presence of LV after brief exposures of* 1–6 hr. *Under similar conditions, LV did not potentiate the* 5-*FU effect in an LV transport-defective FLC line, demonstrating that LV enhances* 5-*FU by an intracellular mechanism. The addition of menadione* (5×10^{-7} *to* 1×10^{-6} M) *significantly enhanced the anti-metabolic effect of* 5-*FU as measured by* [^{3}H]*UdR incorporation into DNA or by cell growth inhibition curves, but for only the initial* 24 hr. *Levels of menadione greater than* 1×10^{-6} M *were toxic in the FLC system. In contrast,* α-*tocopherol* (1×10^{-5} M), *itself non-toxic, potentiated the anti-metabolic effect of* 5-*FU over a continuous exposure for* 72 hr, *as measured by both inhibition of* [^{3}H]*UdR into DNA and cell growth curves. The* α-*tocopherol potentiation of* 5-*FU was not as great as that of LV, but when added in combination with LV was more effective than* 5-*FU and LV. These data suggest that the intracellular levels of* 5-10 *methylene tetrahydrofolate necessary for the pharmacologic interaction of FdUMP with thymidylate synthetase may be a limiting factor under certain conditions and that it is possible to overcome this metabolic limitation with exogenous reduced folate or by the addition of oxidizing agents which may increase intracellular levels of* 5-10 *methylene tetrahydrofolate.*

INTRODUCTION

THE INHIBITION of thymidylate synthetase (dTMP synthetase) by 5-fluorouracil (5-FU) is thought to be one of the major anti-metabolic sites of action of this drug [1, 2]. The duration of inhibition appears to depend on the 5-FU metabolite fluorodeoxyuridylate (FdUMP) forming a ternary complex with dTMP synthetase and the folate cofactor 5-10 methylene tetrahydrofolate (5-10 methylene H_4PteGlu) [3, 4]. In the presence of this cofactor, complexes contain two tightly bound molecules of FdUMP per dTMP synthetase molecule, with a mol. wt.

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of 70,000 daltons [5–7]. A number of other folate analogues also stimulate binding of FdUMP to dTMP synthetase, albeit to a lesser degree than the physiologic cofactor 5-10 methylene H_4PteGlu [4, 6]. The intracellular pool of 5-10 methylene H_4PteGlu is limited both by its conversion to dihydrofolate during the methylation of deoxyuridylate and by its reduction to 5-methyl H_4PteGlu. Therefore it has been suggested that 5-10 methylene H_4PteGlu may be the limiting factor in the formation of the ternary complex with FdUMP and dTMP synthetase [4, 8, 9].

Studies were designed to develop approaches for increasing the intracellular levels of 5-10 methylene H_4PteGlu or other reduced folates and to see if this would augment the anti-metabolic effect of 5-FU. A stable, reduced form of folate, DL-5-formyl tetrahydrofolate in the form of leucovorin (LV), was added together with the 5-FU in an attempt to increase or to substitute for 5-10 methylene H_4PteGlu so that more ternary complexes might develop and produce a greater anti-metabolic effect. A second approach was to inhibit the reduction of 5-10 methylene H_4PteGlu to 5-methyl H_4PteGlu by the use of oxidizing agents such as menadione or α-tocopherol—compounds which can act as electron acceptors. This approach is based on previous work *in vitro* demonstrating that 5-methyl H_4PteGlu can be enzymatically oxidized to 5-10 methylene H_4PteGlu in the presence of these oxidizing agents [10].

The interaction of 5-FU with these compounds was studied in the Friend murine erythroleukemia cell line (FLC). The ability to inhibit tritiated deoxyuridine ([^{3}H]UdR) incorporation into DNA as well as a 72-hr cell growth determination were used as indicators of the 5-FU anti-metabolic and cytotoxic effect in these cells.

MATERIALS AND METHODS

The cell line used for these experiments was a murine erythroleukemia obtained from Dr. Charlotte Friend (FLC line No. 745A). The cells were maintained as a suspension in tissue culture flasks (Corning Glass, Corning, NY) containing RPMI 1640 medium (Flow Laboratories, Bethesda, MD) supplemented with 10% fetal calf serum (Gibco) and antibiotics (complete medium). The cells were passaged during logarithmic growth at a concentration of 2×10^5 cells/ml in fresh complete medium two times a week. The effect of 5-FU, folate derivatives and various oxidizing agents on cell growth were determined by measuring growth inhibition over a 72-hr observation period. The cells were in logarithmic growth phase and had over 90% cell viability as determined by trypan blue exclusion when each experiment was initiated. Cell growth was monitored with a Coulter Counter, Model ZF (Coulter Electronics, Hialeah, FL). All experiments were carried out in triplicate. Drugs tested in the growth inhibition assays were 5-FU and menadiol sodium diphosphate (Synkayvite) from Roche Laboratories, Nutley, NJ, leucovorin from Lederle Laboratories, Pearl River, NY, and D-α-tocopherol from Sigma Chemical Co., St. Louis, MO.

All drugs, with the exception of α-tocopherol, were serially diluted directly with the RPMI 1640 medium at a concentration for experimentation that did not change the pH of the tissue culture medium. α-Tocopherol was dissolved in ethanol and subsequently diluted in RPMI 1640. The final concentration of ethanol in these experiments was less than 0.1%. Studies showed that the vehicles used for dilutions of ethanol in the final culture medium did not influence cell growth. For all experiments, serial drug solutions were freshly prepared just prior to use.

For the determination of the growth inhibition effects of the various agents, 10 ml cell suspension of logarithmically growing FLC were prepared in individual culture tubes at a density of 2×10^5/ml, to which 0.1 ml of drug solutions at various concentrations were added. The cells were incubated with drugs for periods of time indicated at 37°C and followed by daily cell counts over a period of 72 hr. In some experiments the cells were exposed to the drugs for 1–6 hr, washed with RPMI 1640 and grown in drug-free complete medium for an additional 72 hr. The viable cell count was determined by the trypan blue dye exclusion method and dose–response curves were obtained by calculating the percentage of viable cells in drug-treated tubes as compared to those in untreated control tubes. The additive cytotoxic effect of 5-FU with other agents was determined by dose–response curves with and without additional drug in the presence of 5-FU. All experiments were carried out in triplicate and repeated at least 3 times.

The anti-metabolic effect of 5-FU on *de novo* DNA synthesis was measured by the incorporation of [^{3}H]deoxyuridine ([^{3}H]UdR) into DNA as previously described [11–13]. In this system, abnormal *de novo* DNA synthesis produced by 5-FU is demonstrated by reduced [^{3}H]UdR incorporation into DNA as compared

to control (untreated cells). This was directly correlated with the 5-FU concentration and exposure time.

In these studies, an aliquot of the cells used for the cell growth inhibition curves was taken for the [^{3}H]UdR incorporation assay. The cells were washed three times in Hanks' Balanced Salt Solution, resuspended at a concentration of 1×10^6 cells/ml (in triplicate) and incubated at 37°C for 2 hr in the presence of 0.1 μCi [^{3}H]UdR (Amersham, specific activity 15 C/mM). At the end of the incubation time the cells were washed twice with cold 0.9% NaCl. Two ml of 10% trichloroacetic acid (TCA) was added and the resultant precipitate was washed once with 10% TCA. The acid-precipitable material was dissolved in 1.0 ml NCS reagent (Amersham) and counted in 15 ml of a scintillation mixture (toluene containing 30% ethanol, 0.6% PPO and 0.03% dimethyl POPOP) in a Beckman LS 250 scintillation system with a counting error of 2% or less. Each triplicate experiment had a variation of less than 2%.

RESULTS

Cell growth inhibition studies

The effect of 72 hr of continuous exposure to 5-FU (3×10^{-7} to 2×10^{-6} M) in FLC is shown in Fig. 1. There was a slight inhibition of cell growth with a 5-FU concentration of 3×10^{-7} M. The growth inhibition was dose and time-dependent. LV (1×10^{-5} M) by itself did not significantly effect cell growth. However, LV (1×10^{-7} to 1×10^{-5} M) when added with 5-FU produced an increased inhibition of cell growth at all concentrations of FU. This was more apparent at the higher concentrations of 5-FU (1×10^{-6} and 2×10^{-6} M) and caused greater than 3-fold augmentation of cell growth inhibition.

Similarly, a 1 to 6-hr exposure to 5-FU (2×10^{-5} M), or 5-FU and LV, followed by washing, demonstrated an increase of growth inhibition when LV was in the combination (Fig. 2). The growth inhibition attributed to the presence of LV was greater than 3-fold following the longer exposure time to the two drugs (3 and 6 hr).

Anti-metabolic effects of 5-FU as measured by [^{3}H]UdR into DNA

The potentiation of 5-FU anti-metabolic effect by LV was also demonstrated by additive inhibition of [^{3}H]UdR incorporation into DNA. LV by itself does not effect [^{3}H]UdR incorporation into DNA. The inhibition of [^{3}H]UdR incorporation into DNA by 5-FU is time and dose-dependent. At all times and dosages of 5-FU studied (3×10^{-7} M to 2×10^{-6} M), LV significantly enhanced the inhibition of [^{3}H]UdR incorporated into DNA (Table 1). In the study of 5-FU exposure from 1 to 6 hr, there was a time-related inhibition of [^{3}H]UdR incorporation into DNA. However, that inhibition of [^{3}H]UdR incorporation into DNA by 5-FU present at 24 hr was significantly diminished when studied 48 hr after the 5-FU exposure (Table 2). When LV was added with the 5-FU, the inhibition of [^{3}H]UdR incorporated into DNA was more pronounced and prolonged, so that at 48 hr there continued to be a significant inhibition of [^{3}H]UdR incor-

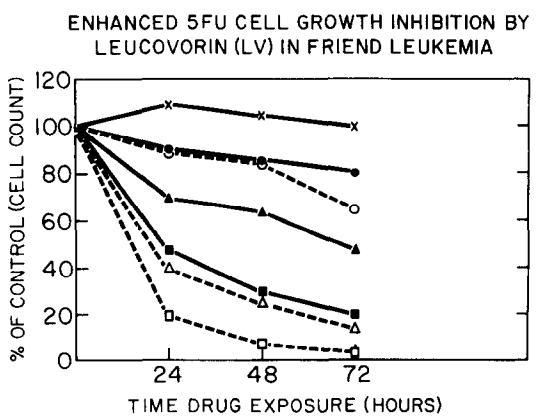

Fig. 1. Cell growth inhibition by continuous exposure to 5-FU and leucovorin for 72 hr. LV 1×10^{-5} M, ×——×; 5-FU 3×10^{-7} M, ●——●; + LV, ○- - -○; 5-FU 1×10^{-6} M, ▲——▲; + LV, △- - -△; 5-FU 2×10^{-6} M, ■——■; + LV, □- - -□. Logarithmic growth FLC cells were continuously exposed to the above agents and compared to control (untreated) FLC cells.

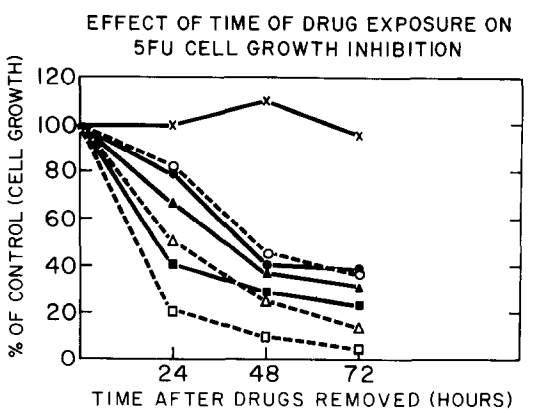

Fig. 2. Effect of time of drug exposure on cell growth inhibition by 5-FU and leucovorin. LV 1×10^{-5} M, ×——×; 5-FU 2×10^{-5} M, ●——●; + LV, ○- - -○; 1-hr exposure. 5-FU 2×10^{-5} M, ▲——▲; + LV, △- - -△; 3-hr exposure. 5-FU 2×10^{-5} M, ■——■; + LV, □- - -□; 6-hr exposure. FLC cells were exposed to the above drugs for the times indicated, washed 3 times and grown in drug-free complete medium for 72 hr, and compared to control (untreated) cells similarly processed.

Table 1. Enhanced 5-FU anti-metabolic effect by leucovorin (LV) in FLC

Drug	Percentage [^{3}H]UdR incorporated into DNA* Time exposed to drugs (hr)		
	24	48	72
Leucovorin (LV) 1×10^{-5} M	105	92	103
5FU (3×10^{-7} M)	68	41	58
+LV	36	21	15
5FU (1×10^{-6} M)	58	39	26
+LV	19	5	3
5FU (2×10^{-6} M)	26	16	19
+LV	3	3	3

*As compared to control (untreated) cells.

Table 2. Enhanced 5FU anti-metabolic effect by leucovorin (LV) in FLC

Drug	Drug exposure time (hr)	Percentage [^{3}H]UdR incorporated into DNA Time after drugs removed (hr)* 24	48
Leucovorin (LV) 1×10^{-5} M		111	96
5FU 2×10^{-5} M		60	70
+LV	1	51	78
5FU 2×10^{-5} M		48	74
+LV	3	48	38
5FU 2×10^{-5} M		21	70
+LV	6	8	23

*The FLC cells were exposed to the drugs for the indicated time, washed 3 times, grown in drug-free complete medium for 48 hr and compared to control (untreated) cells.

poration into DNA, particularly at the higher doses of 5-FU.

Similar studies were done in an FLC mutant cell line which is transport-defective for methotrexate and LV [14]. In this study, where the cells were exposed to drugs for 48 hr, we demonstrated a marked difference between the native FLC No. 745 A and the transport-defective FLC cell lines (Table 3). LV did not augment 5-FU cell growth inhibition or further inhibit [^{3}H]UdR incorporation into DNA in the transport-defective FLC, as compared to the native FLC which can transport LV.

The effect of the addition of menadione and α-tocopherol

Menadione (5×10^{-7} to 1×10^{-6} M) significantly enhanced the anti-metabolic effect of 5-FU as measured by [^{3}H]UdR incorporation into DNA or cell growth inhibition during a 24-hr continuous exposure (Table 4). At these concentrations, menadione had no effect on either parameter by itself. There did not appear to be additional enhancement of 5-FU anti-metabolic effect when menadione was added with the 5-FU and LV combination during the 24-hr drug exposure. The menadione enhancement of the 5-FU effect was only present for 24 hr in the cell culture, but was no longer evident at 48 and 72 hr (data not shown). Levels of menadione greater than 1×10^{-6} M were toxic in the FLC system. In contrast, α-tocopherol (1×10^{-5} M), itself non-toxic, potentiated the anti-metabolic effect of 5-FU (1×10^{-6} M) over a continuous exposure of 72 hr as measured by both enhanced inhibition of [^{3}H]UdR into DNA and cell growth curves. Tocopherol potentiation of 5-FU was not as great as that of LV, but when added in combination with LV, was more effective than the 5-FU and LV combination alone (Fig. 3).

Table 3. 5FU + LV effect in FLC transport defective for reduced folates

Drug	Percentage cell count*	Percentage [^{3}H]UdR incorporated into DNA*
FLC (native)		
LV 1×10^{-5} M	104	105
5-FU 1×10^{-6} M	63	39
5-FU + LV	25	5
FLC (transport defective)		
LV 1×10^{-5} M	101	99
5-FU 1×10^{-6} M	55	25
5-FU + LV	63	26

*As compared to control (untreated) cells.

Table 4. Enhancement of 5-FU anti-metabolic effect by menadione and leucovorin

Drug	Cell count*	[^{3}H]UdR incorporation into DNA*
LV 1×10^{-5} M	96	94
Menadione 1×10^{-6} M	104	105
5-FU 1×10^{-6} M	65	60
5-FU + menadione	38	26
5-FU + LV	41	20
5-FU + menadione + LV	37	21

Results from a 24-hr drug exposure.
*As compared to control (untreated cells).

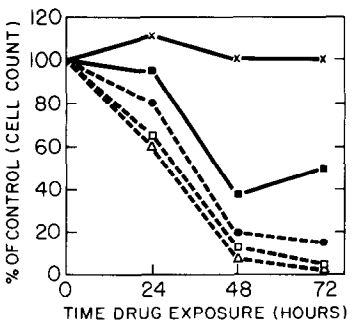

Fig. 3. Enhancement of 5-FU cell growth inhibition by α-tocopherol. α-Tocopherol 1×10^{-5} M, ×——×; *5-FU* 1×10^{-6} M, ■——■; *+ tocopherol,* ●——●; *+ LV* 1×10^{-5} M, □- - - □; *+ tocopherol + LV,* △- - - △. *Logarithmic growing FLC cells were continuously exposed to the above drugs and compared to control (untreated) cells.*

Folate availability and 5FU anti-metabolic effect

FLC cells were adapted to lower folic acid in the tissue culture medium by a period of several passages in decreasing concentrations of folic acid from 1000 ng/ml (the usual concentrations in RPMI 1640) to 10 ng/ml. The cells did not grow as well in the lower concentration of folic acid as in the usual concentration. When 5-FU anti-metabolic and anti-cell-growth effects were studied in cells grown in various concentrations of folate, it was shown that 5-FU was more toxic in cells grown in higher concentrations of folate (1000 ng/ml) as compared to 10 ng/ml of folate. At all concentrations of 5-FU there was a greater cell growth inhibition and decrease in [^{3}H]UdR incorporation into DNA in the folate-replete cells as compared to the folate-depleted cells (Table 5).

DISCUSSION

Studies in tumor-bearing mice have shown that the recovery of dTMP synthetase is a critical factor in determining the activity of 5-FU *in vivo* [15, 16]. dTMP synthetase in various species, including humans, is known to consist of two subunits with different binding affinities for FdUMP [5–7]. There is more ready dissociation of FdUMP from enzyme binding of one site as compared to the second site [6, 17]. *In vitro* studies have demonstrated that stable inhibition of dTMP synthetase requires equimolar concentrations of FdUMP and 5-10 methylene H_4PteGlu to the enzyme in order to form a ternary complex [4]. Kinetic data are available for dTMP synthetase of human

Table 5. Effect of folic acid (FA) availability on 5-FU anti-metabolic effect in FLC

Drug	Percentage control cell growth*			Percentage [^{3}H]UdR incorporated into DNA*		
(in RPMI 1640)	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
1000 ng/ml FA						
+5FU 1×10^{-6} M	70	62	46	58	39	26
2×10^{-6} M	46	31	20	26	16	19
10 ng/ml FA						
+5FU 1×10^{-6} M	91	73	42	72	48	77
2×10^{-6} M	83	58	32	53	56	27
4×10^{-6} M	73	38	26	39	27	24

*Compared to control (untreated cells).

chronic myelocytic leukemia cells [3] and Ehrlich's ascites [18]. The K_m for 5-10 methylene H_4PteGlu in this system is approximately 30–40 μM. The intracellular level of 5-10 methylene H_4PteGlu is unknown but must be less, since in physiologic conditions the total content of folates in L1210 cells is approximately 10 μM [19]. These data support the concept that 5-10 methylene H_4PteGlu may be a limiting factor in the pharmacologic inhibition of dTMP synthetase by 5-FU.

We previously reported that LV enhances the anti-metabolic and cytotoxic effect of 5-FU in murine erythroleukemia (FLC) [20]. In the present study we have demonstrated that FLC growth inhibition by continuous 5-FU exposure is time and dose-related. The simultaneous addition of LV enhanced 5-FU cell growth inhibition at all times recorded, but was most evident (greater than 3-fold) at the higher 5-FU concentrations. Similarly, a 1 to 6-hr exposure to 5-FU (2×10^{-5} M) produced transient cell growth inhibition when followed for 72 hr in tissue culture. This transient inhibition of cell growth by 5-FU alone was markedly prolonged and intensified (greater than 3-fold) by the concomitant administration of LV. The 5-FU anti-metabolic effects as measured by inhibition of [^{3}H]UdR incorporation into DNA were dose and drug-dependent, but showed reversibility as the cells were carried in tissue culture for 72 hr. However, when 5-FU was added concomitantly with LV, the inhibition of [^{3}H]UdR incorporation into DNA was more complete and appeared less reversible. Similar potentiation of 5-FU by LV has also been described in the L1210 murine leukemia system and HEP-2 cells [8, 9]. In the latter study, this enhanced 5-FU effect by LV was found to be associated with a decrease in the recovery of dTMP synthetase activity without having an effect on the initial inhibition of the enzyme. Thus, the FdUMP–enzyme complex appeared to be formed during the treatment with 5-FU and the amount of the folate cofactor within the cells at that time determined the future stability of the complex. In contrast to the Sarcoma 180 cells, the inhibition of dTMP synthetase activity in HEP-2 cells became growth-limiting only when excess LV was added. This suggests that in cells with higher concentrations of deoxyuridylate (dUMP), such as in HEP-2, there is need for additional folate-cofactor to inhibit the enzyme [1, 3].

The importance of intracellular folate pools was demonstrated by a marked reduction in 5-FU activity when studied in FLC cells depleted of folate. We also determined that LV did not potentiate the anti-metabolic and cell growth inhibition effects of 5-FU in a mutant FLC cell line which is transport-defective for reduced folates. This supports the finding of Ullman *et al.* [9] that the levels of folate sufficient for normal growth of L1210 cells could not provide for maximum binding of FdUMP to dTMP synthetase. They also found that folate-depleted L1210 cells, when analysed using sodium dodecyl sulfate polyacrylamide gels, had less than the control levels of FdUMP associated with dTMP synthetase [21].

The intracellular pool of 5-10 methylene H_4PteGlu is subject to marked variations since it is utilized in the methylation of dUMP with oxidation to dihydrofolic acid, and is also reduced to 5 methyl H_4PteGlu. The reduction to 5-10 methylene H_4PteGlu is primarily a unidirectional reaction towards the direction of methyl H_4PteGlu in most mammalian tissues studied [10, 22, 23]. It has been previously demonstrated *in vitro* that 5 methyl H_4PteGlu can be enzymatically oxidized to 5-10 methylene H_4PteGlu in the presence of various oxi-

dizing agents such as menadione. Moreover, α-tocopherol can improve folate-deficient megaloblastic anemia in patients [24]. We have been able to demonstrate that the addition of these oxidizing agents at non-toxic concentrations can enhance the 5-FU anti-metabolic effect and inhibition of cell growth. This potentiation by menadione was reversible after 24 hr in tissue culture conditions and could be due to its conversion to an inactive form by the enzyme menadione reductase, as previously reported *in vivo* [25]. α-Tocopherol enhanced 5-FU and the 5-FU–LV anti-metabolic activity and cell growth inhibition. This enhancement appeared to be persistent during 72 hr of tissue culture incubation. Therefore, the use of non-toxic oxidizing agents for the enhancement of 5-FU activity appears to be a worthwhile consideration in future clinical studies.

Potentiation of 5-FU effect by excess LV has now been reported in four cell lines (L1210, HEP-2, Sarcoma 180 and FLC). The effect appears to be due to the stabilization of the FdUMP–dTMP synthetase complex, which causes a slow-down in the recovery of the enzyme activity resulting in prolonged growth inhibition. Studies in tumor-bearing mice have also shown that the recovery of dTMP synthetase is important in the activity of 5-FU *in vivo*. These data support the concept that LV should alter the biologic effects of 5-FU in the clinical setting. In a preliminary phase I clinical trial, LV was shown to increase the toxicity of 5-FU [26]. A greater degree of stomatitis and bone marrow depression with less 5-FU was observed in the presence of LV as compared to the same patients who received more 5-FU in combination with mitomycin C and hexamethylmelamine. In addition, 5-FU with LV produced two clinically measureable minor responses and two biochemical improvements in patients who failed the combination chemotherapy regimen. Demonstration that LV can increase 5-FU effect on normal and tumor tissue in man further exemplifies a new type of clinical interaction of a specific vitamin which can modulate the pharmacologic effect of an anti-neoplastic drug.

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