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Thymidine enhancement of methotrexate and 5-fluorouracil toxicity in cultured human colon carcinoma

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Summary. *Cultures of human colon carcinoma, HCT-8, were treated with millimolar concentrations of thymidine by different schedules designed to cytokinetically and biochemically modulate methotrexate (MTX) and 5-fluorouracil (FUra) toxicity. Thymidine (dThd)-synchronized HCT-8 cells monitored by flow cytofluorometry showed increased sensitivity to MTX and synergistic cytotoxicity to the combination MTX-FUra. FUra toxicity in synchronized cells showed no significant phase specificity overall, but a pattern of relative G_2/M resistance was correlated with decreased intracellular FUra accumulation, incorporation into RNA, and formation of FdUMP. In asynchronous cultures dThd reduced MTX toxicity when given within the first 12 h of a 24-h MTX exposure, and also appeared to reduce the MTX-induced synergistic enhancement of FUra toxicity. When dThd was administered with FUra alone in asynchronous cultures, progressive, and synergistic enhancement of FUra toxicity was observed only after 6 h dThd pretreatment. Unlike MTX-FUra synergy, this schedule-dependent synergism between dThd and FUra did not correlate with intracellular FUra accumulation or specific incorporation into total cellular RNA. These results suggest that less well studied mechanisms of dThd modulation, other than enhanced deoxynucleotide formation or total RNA incorporation, may biochemically enhance FUra toxicity in HCT-8 cells.*

Introduction

The cytokinetic and biochemical modulating effects of thymidine (dThd) continue to interest investigators probing into the mechanisms underlying methotrexate (MTX) and 5-fluorouracil (FUra) cytotoxicity. In a recent review, Martin et al. showed that in tumor-bearing mice, combining dThd with sequentially administered MTX-FUra produced greater anticancer activity than either MTX-FUra or dThd-FUra combinations [40]. Studies with human tumor cell lines have shown that 12–24 h of MTX pretreatment synergistically enhances FUra cytotoxicity by increasing intracellular accumulation and incorporation of FUra into RNA [4, 7, 30]. Addition of dThd to this synergistic sequence may modify the effects of MTX and FUra by a variety of possible mechanisms, summarized in Table 1. If dThd precedes MTX, cytokinetic modulation may affect the S-phase-specific toxicity of MTX. On the other hand, if dThd is administered concurrently with MTX, biochemical modulation might result in either increased [48] or decreased cytotoxicity [9, 16, 29, 42, 51].

Since FUra metabolism in tumor cells depends on the activity of intracellular enzymes used for the catabolism as well as synthesis of pyrimidines and nucleic acids, dThd administration may potentially enhance [13, 40, 46, 49, 50, 57] or inhibit FUra toxicity [17, 28, 42, 44]. In vivo studies all suggest that high-dose dThd infusions increase the plasma half-life of FUra by competing for the primarily hepatic enzymes which catabolize FUra [3, 40, 55, 58]. This increase in FUra C × T has also been correlated with both increased incorporation of FUra into tumor RNA, and increased antitumor activity [49, 50]. Recent attempts to demonstrate dThd enhanced FUra toxicity in vitro have not been successful [20, 44]. We have been investigating the mechanisms underlying two schedules of in vitro dThd administration with MTX and FUra. Each of these dThd schedules increases the in vitro antitumor effects of FUra or MTX-FUra against the human colorectal adenocarcinoma, HCT-8. The first schedule employs cytokinetic

Table 1. Mechanisms of growth modulation by thymidine

Cytokinetic	Biochemical
1. G_1-S-phase block [8, 22, 59]	1. ↑ dTTP inhibits:
2. Delayed arrest in early G_1 phase [52]	Ribonucleotide reductase [19, 41]
3. Unbalanced growth [32]	Deoxycytidylate deaminase [39, 47]
	Thymidine kinase [27]
	Aspartate transcarbamylase [10]
	Dihydroorotase [11]
	CTP synthetase [37]
	2. Competition for pyrimidine catabolism:
	Thy dehydrogenase [43]
	dThd phosphorylase [21, 54, 60]
	dTMP 5′nucleotidase [38]
	dTTP phosphatase [38]
	3. Inhibition of RNA synthesis:
	Early nucleolar [18, 31]
	Late extranucleolar [18]

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Abbreviations used in this paper: MTX, methotrexate; FUra, 5-fluorouracil; FUTP, 5-fluorouridine triphosphate; FdUMP, 5-fluorodeoxyuridylate; dThd, thymidine; dTTP, thymidine triphosphate; PBS, phosphate-buffered saline ($CaCl_2$ 100 mg, KCl 200 mg, KH_2PO_4 200 mg, $MgSO_4$ 59 mg, NaCl 8,000 mg, and Na_2HPO_4 1,150 mg in 1 l sterile aqueous solution)

modulation by dThd; the second may involve less well studied mechanisms of biochemical modulation by dThd.

Materials and methods

The human colorectal adenocarcinoma HCT-8 is a well characterized continuously growing monolayer cell line which doubles every 18 h [53]. Cultures were maintained in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, NY) with 10% neonatal calf serum, and subcultured weekly. Cells for stocks and studies were grown in 75-cm^2 sterile plastic culture flasks (Costar; Cambridge, MA) with 25 ml medium in 5% CO_2 incubators at 37° C. Cell counts were performed on a Coulter Model ZBI counter (Hialeah, FL).

Clonal growth of HCT-8. Cells from stock cultures were suspended by trypsinization and vigorous pipetting before 5×10^4 cells were seeded into sterile flasks with medium. Cultures were drug treated on day 2 or 3 when clusters were between four and 32 cells in size, as previously described [4, 6, 7]. After the indicated time of drug treatment, cultures were rinsed with PBS and replaced with fresh media with or without other drugs. At 7 days, when untreated cultures reached the optimal mean colony density of 500 (per 1,000−mm^2 counting field) and median clonal diameter of approximately 0.25 mm, the medium was decanted and the colonies rinsed with PBS, then stained with methanol-crystal violet (2.5%). An automated colony counter (Biotran II; New Brunswick Scientific, Edison, NJ) was used to enumerate colonies. Clonal growth in treated conditions was recorded as mean percentage of colony count relative to untreated controls with SD's $< 5\%$.

Drug pretreatment and intracellular FUra accumulation. FUra, MTX, and dThd were purchased from Sigma Chemical Co. (St Louis, MO). [^{3}H] FUra (18 Ci/mmol) was purchased from Moravek Biochemicals (City of Industry, CA). The intracellular accumulation of FUra was measured by the microfuge method which we have reported previously [4, 7, 14]. Following drug pretreatment, the radiolabeled FUra was addd to a concentration of 100 μ*M*, a clinically achievable dose. The cells were harvested by rapid trypsinization (0.05% solution) and counted at the indicated time. The intracellular accumulation of FUra was linear to 6 h and reported as nmol FUra/10^6 cells/h [4].

FUra metabolism and incorporation into RNA. Monolayer cells given radiolabeled FUra were rinsed with cold PBS, trypsinized, counted, and then precipitated in 0.5 *M* perchloric acid at 4° C at variable times. The soluble extract containing free drug, nucleoside, and nucleotide derivatives was neutralized with KOH, and the salt removed by centrifugation. The acid-insoluble precipitate was washed in 2 ml perchloric acid until the radioactivity remaining in 0.5 ml of the wash did not exceed background counts. The precipitate was then hydrolyzed in 1 *N* KOH at 37° C for 5 h. After reacidification, the amount of radiolabeled FUra incorporated into RNA was counted in the soluble portion of the hydrolyzed precipitate and recorded as pmol/10^6 cells/h. RNA was also quantitated by the orcinol reaction, and specific incorporation of FUra into RNA was recorded as pmol/μg D-ribose/h.

The acid-soluble cell extract was analyzed by high-pressure liquid chromatography (Altex Instruments, Berkeley, CA) using a Partisil SAX column (10 μ*M* particle size; 250 × 4.6 mm) and a 2%/min linear phosphate buffer gradient (0.01−0.7 *M* NaH_2PO_4, pH 3.3) to quantitate mono-, di-, and triphosphate ribonucleotides or deoxyribonucleotides of FUra. Absorbance was measured at 254 and 280 nm, and 1-min fractions were collected for radiochromatograms. All retention times were calibrated using either cold or radiolabeled standards. Intracellular FdUMP was measured nonenzymatically by the above HPLC technique after periodate oxidation of the acid-soluble fraction of cells treated with [^{3}H]FUra (100 μ*M*, 3 h) with or without dThd treatment or synchronization. This technique has previously been described in detail [4, 14] and measures soluble-free FdUMP, as well as lesser amounts of FdUDP and FdUTP which may form under certain treatment conditions [26, 35], but this method may not detect enzyme-bound deoxynucleotide and may underestimate total intracellular FdUMP.

Flow cytofluorometry (FCM). Monolayer cultures of HCT-8 either untreated or synchronized by dThd were prepared for FCM by PBS wash and trypsinization. Cells analyzed for DNA content were fixed in 10% formalin and stained with acriflavin as previously described [5]. After washing of formalin-fixed cells in PBS, cells were resuspended in 4 *N* HCl for 20 min, centrifuged, washed in distilled water, and recentrifuged to a pellet containig approximately 10^7 cells. Cells were then suspended in 2 ml of a filtered acriflavin solution (20 g/100 ml acriflavin in 0.05 *N* HCl) for 20 min, washed in acid-alcohol (0.1 *N* HCl in 70% ethanol) twice, and resuspended in distilled water. Stained cells were filtered through a 37-mm nylon mesh, wrapped in foil, and stored refrigerated until analysis by FCM. Cell cycle analysis by FCM was performed on a Becton-Dickinson FACS IV instrument (Mountain View, CA). Two-dimensional DNA histograms were recorded on an X-Y chart recorder. Histograms were graphically analyzed by time sequencing DNA content through 'windows' selected to monitor the fraction (% control untreated cells) of cells flowing through $G_{0/1}$, S, and G_2/M phases, a technique described by Zietz [61].

Results

Treatment of HCT-8 cells with 0.5 m*M* dThd for 18 h resulted in 75% (± 10%) of control clonogenic growth, while treatment for 24 h reduced growth to 27% (± 4%) of control. After 18- or 24-h exposure to 0.5 m*M* dThd, the remaining viable cells were growth-arrested at the G_1-S boundary, and when dThd was washed out, these cells appeared to progress through the cell cycle in a partially synchronized fashion. Figure 1 demonstrates this effect on synchronized HCT-8 cells analyzed by FCM; the synchronized cells pulsed through S-phase within the first 6 h after dThd release, returning to G_1 phase by 12−24 h.

Sensitivity of HCT-8 cells to either MTX or FUra has been shown to be dependent on drug concentration and exposure time [4, 6]. The sensitivity of synchronized HCT-8 to a 6-h and 24-h MTX dose was compared with similar exposure of an asynchronous HCT-8 population. Figure 2 shows that dThd synchronization reduced growth after 6 h MTX from 93% to 57% of control, and growth after 24 h from 74% to 52% of control. Synchronization by dThd rendered the HCT-8 cells as sensitive to 6 h MTX as to 24 h MTX, which is consistent with the results in Fig. 1, showing a large pulse of synchronized cells passing through S-phase within 6 h. In an asynchronous population of HCT-8, 18−24 h would be necessary to observe the entire cell population pass through S-phase.

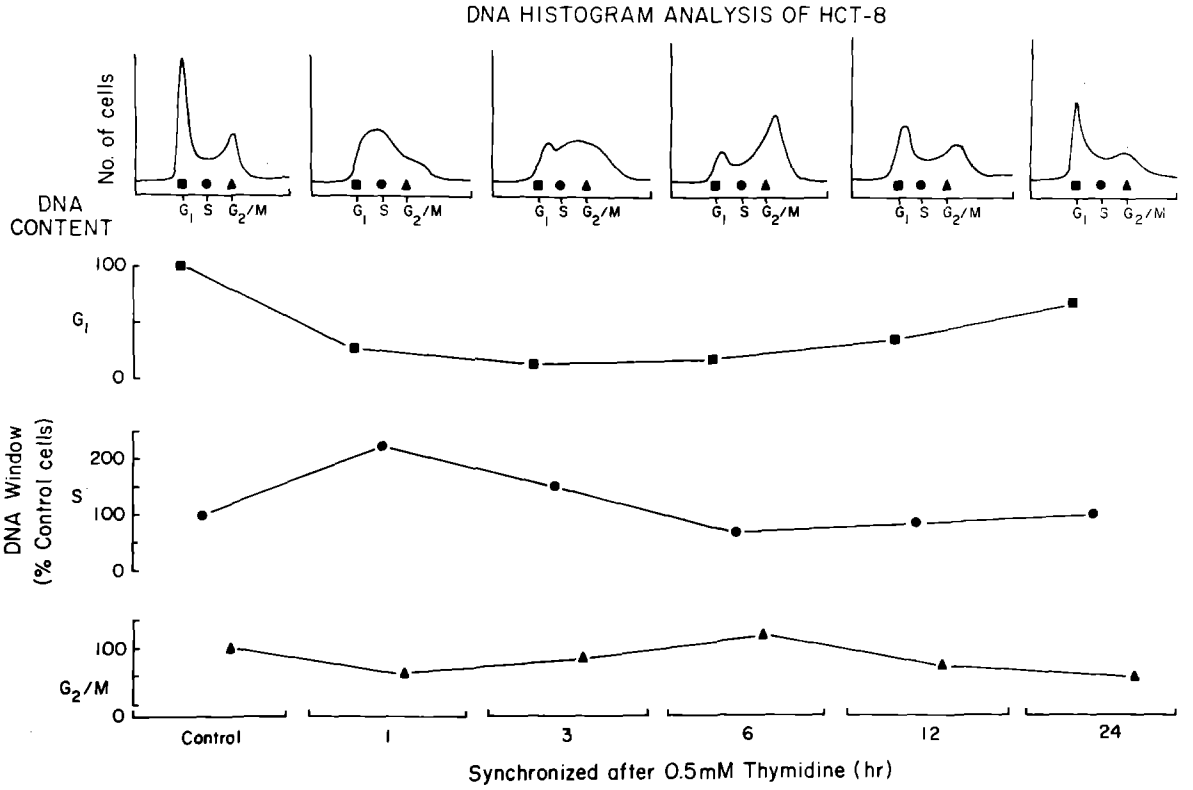

Fig. 1. Asynchronous log phase cells and HCT-8 cultures growth arrested by 0.5 m*M* dThd × 18 h were given fresh media and followed for 1–24 h before being stained with acriflavin and analyzed for DNA content by flow cytofluorometry. As described in *Methods*, DNA histograms of control and dThd released cells (*top panel*) were monitored through 'windows' into G_1 (■), S (●), and G_2M (▲) phases. The phase alterations in dThd-synchronized cells were recorded as percent of asynchronized control cells counted

FUra is generally recognized as a cycle-active but not phase-specific drug; thus dThd synchronization might not be expected to alter FUra toxicity. From a biochemical point of view, however, FUra toxicity can be classified as DNA-directed or RNA-directed according to the lethal effects of its intracellular metabolites FdUMP and FUTP [33, 56]. FdUMP, which inhibits synthesis of thymidylate and DNA, might be expected to show S-phase specificity. Table 2 correlates intracellular FUra accumulation, FUra deoxynucleotide (FdUMP) formation, and RNA incorporation with the number of cells in each DNA histogram window at five different intervals after release from a 24-h (0.5 m*M*) dThd block. As can be seen, $G_{0/1}$ phase cells correlated best with total drug accumulation and incorporation into RNA, while formation of FUra deoxynucleotide showed a strongly negative correlation with cells in G_2/M-phase. These measurements suggested that G_2/M-phase HCT-8 cells should be more resistant than G_1 or S-phase cells. Figure 3 shows that hourly FUra exposure following dThd synchronization produced a pattern of toxicity appearing to spare cells between 3–12 h after dThd release, corresponding to the pulse of cells through G_2/M observed in Fig. 1. Taken together, however, the survival values over 18 h had a mean value and standard deviation that were not significantly different from the calculated additive toxicity of FUra and dThd alone. When this experiment was repeated using 2-h exposures of FUra after dThd synchronization, an identical pattern of FUra toxicity resulted.

The effect of dThd synchronization on the schedule-dependent synergy between MTX and FUra was also measured. Figure 4 shows that when a 6-h FUra dose was administered to

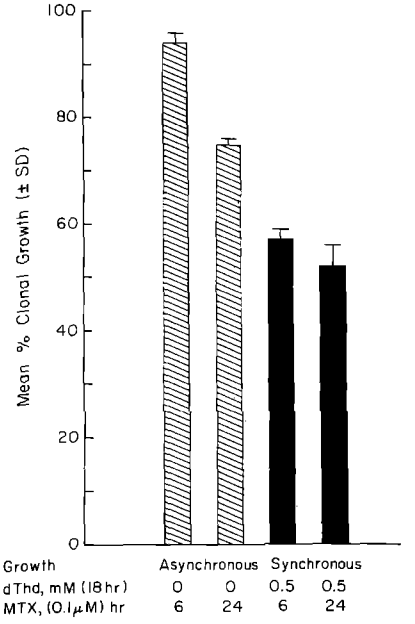

Fig. 2. Monolayer HCT-8 colonies were set up in triplicate and 0.5 m*M* dThd added at 24 h to half of the flasks. After 18 h of dThd treatment, all flasks were rinsed with fresh media, resulting in control (asynchronous) and synchronized cultures. MTX (0.1 μ*M*) was then added for either 6 h or 24 h to synchronous *(solid bars)* and asynchronous *(hatched bars)* cultures. After MTX treatment, the cell cultures were again rinsed with fresh media and allowed to grow for another 5 days before counting. Clonogenic survival was expressed as the mean percent of control growth (± SD)

Table 2. Phase-specific correlation of FUra metabolism[a]

dThd release (hr)	Cells in DNA Window $G_{0/1}$	S	$G_{2/M}$	FUra Accumulation (nmol/10^6 cells/hr)	FUra deoxynucleotide (pmol/10^6 cells/hr)	RNA incorporation (pmol/10^6 cells/hr)
1-3	291	481	246	0.09	5.7	21.4
3-6	144	338	357	0.07	4.1	19.7
6-9	190	161	512	0.10	1.3	24.1
12-15	367	206	217	0.13	6.5	36.6
24-27	698	239	221	0.18	7.8	44.4
Correlation coefficient, r				-0.49	-0.97*	-0.56
				-0.46	+0.24	-0.48
				+0.95*	+0.79	+0.92*

[a] HCT-8 cells synchronized by dThd (0.5 mM × 24 h) were treated with [^{3}H]FUra (100 μM × 3 h) at different intervals after dThd release. Biochemical measurements of total intracellular FUra accumulation, FUra deoxynucleotide (FdUMP) formation, and RNA incorporation were individually correlated with the number of cells in each DNA window, recorded by flow cytofluorometry at the beginning of each time interval. Linear correlation was determined by Pearson's coefficient, r; significant values ($P \leq 0.01$) are indicated by asterisks

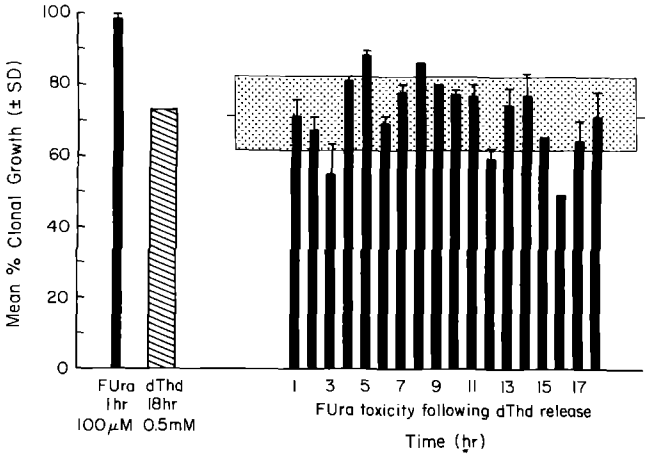

Fig. 3. Clonogenic survival after 1 h FUra (100 μM) treatment was measured in control HCT-8 and cultures synchronized by dThd (0.5 mM × 18 h). FUra was added to triplicate flasks during each of 18 intervals of 1 h following dThd release. After each 1-h FUra exposure, flasks were rinsed with fresh media and colonies allowed to grow for another 5 days before counting. Clonal growth was recorded as the mean percent (± SD) colony count of asynchronously growing controls. The *stippled area* represents the average survival for all hourly FUra exposures; this was not significantly different than the additive toxicity of FUra and dThd alone

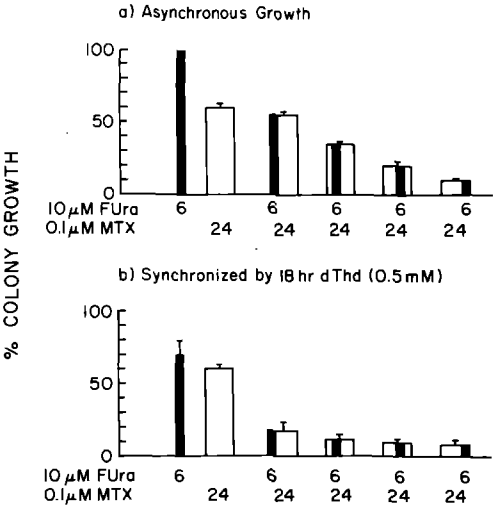

Fig. 4a and b. Clonogenic survival from combined MTX (0.1 μM × 24 h) and FUra (10 μM × 6 h) treatments in asynchronous **(a)** and synchronous **(b)** cultures of HCT-8. Cultures were set up, treated, and counted as described in *Methods*. FUra was added during the first through fourth 6-h intervals of a continous 24-h exposure to MTX. Results were recorded as the percent of control (± SD) colony growth

asynchronous HCT-8 cells during a 24-h exposure to MTX, synergistic cytotoxicity increased with time of exposure to MTX, becoming greatest after 12–18 h of MTX pretreatment. In dThd synchronized cells, however, synergy between MTX and FUra was no longer dependent on MTX pretreatment (Fig. 4b); combined MTX-FUra toxicity during the first 6 h of MTX exposure was similar to that observed during the final 6 h of MTX exposure.

Biochemical modulation by dThd was measured in FUra-treated HCT-8, independent of MTX pretreatment. Figure 5 shows that combined dThd-FUra cytotoxicity ranged from less than additive to synergistic, depending on the schedule of administration. FUra and dThd doses were designed to simulate in vivo administration of these drugs [3, 12, 15, 23, 34, 36, 40, 55, 58]. When FUra was given during the first 6 h of a 24-h dThd exposure, less than additive toxicity was observed. The greatest toxicity was observed when FUra was given during the final 6 h of dThd exposure. Figure 6 shows the averaged results of four experiments in which [^{3}H]FUra (100 μM × 3 h) was used to measure intracellular drug

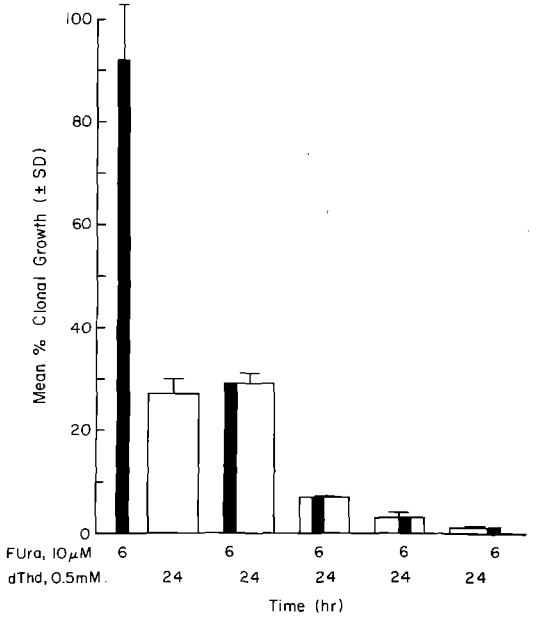

Fig. 5. Clongenic survival from combined dThd (0.5 mM × 24 h) and FUra (10 μM × 6 h) treatments in asynchronous cultures of HCT-8. Triplicate cultures were set up, treated and counted as described in *Methods*. FUra was added during the first through fourth 6-h intervals of a continuous 24-h exposure to dThd. Results were recorded as the percent of control (± SD) colony growth

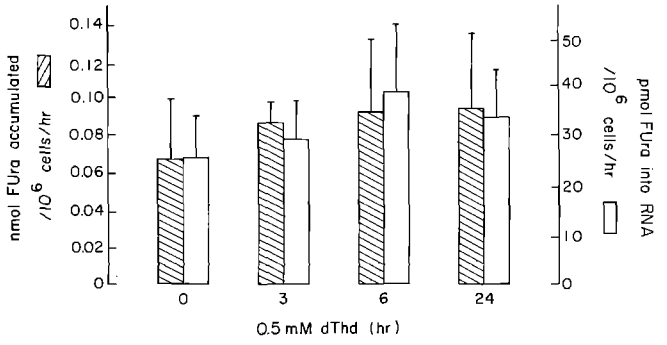

Fig. 6. Intracellular accumulation and RNA incorporation of FUra in dThd pretreated HCT-8. Monolayer cells had [^{3}H]-FUra (10 μM) added at the indicated times following dThd (0.5 mM) pretreatment, and except for control (0 h) cells, dThd remained in the cultures during the 3-h exposures to [^{3}H]FUra. Results were expressed as the mean (± SD) values from four separate experiments

accumulation and RNA incorporation during dThd exposure. The differences were not significant between control (0 h) and dThd-treated (3–24 h) cells. The 30%–40% variation in mean values might be attributed to. The 30% increase in cell size and 25% increase in total RNA content observed in cultures treated with dThd for 6–24 h, reflecting dThd-induced 'unbalanced growth' [32]. When specific FUra incorporation into RNA was measured, treated and control values showed no difference, with a mean value of 14.5 ± 1.3 pmol FUra/ μg RNA. Soluble FdUMP formation was also measured in a single experiment; after 0, 3, 6, and 24 h of dThd (0.5 mM) treatment there was 3.2, 2.7, 11.6 and 5.6 pmol FdUMP formed/10^6 cells/h, respectively. This increase in FdUMP formation measured between 6 and 24 h of dThd exposure in HCT-8 is consistent with increases in FdUrd formation

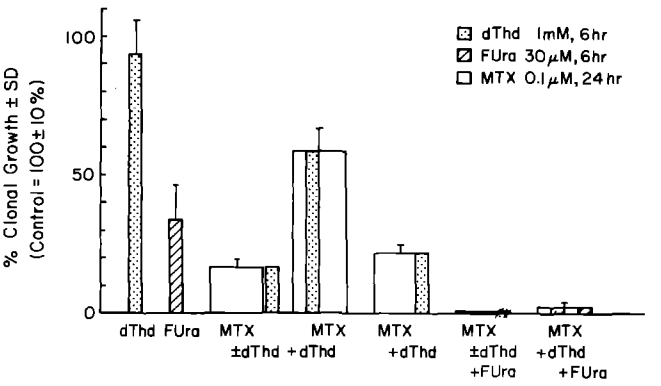

Fig. 7. Clonogenic survival of HCT-8 treated with combinations of MTX (0.1 μM × 24 h), FUra (30 μM × 6 h), and dThd (1 mM × 6 h). Triplicate cultures were set up and treated, and mean clonal growth (± SD) was recorded as described in *Methods*. FUra *(hatched bars)* was combined during the fourth 6-h interval of a continuous 24-h exposure to MTX *(open bars)*. dThd *(stippled bars)* was added alone after washout of 24-h MTX, resulting in no additional toxicity. dThd was added in combination with MTX during the second and fourth intervals of continuous 24-h MTX exposure, which also included FUra exposure as indicated. Only 1% of nearly 2,000 control colonies were counted in the condition showing maximum toxicity

measured in cultured SK-L7 cells [44] and inhibition of thymidylate synthetase by FdUMP measured in human tumor extracts [57]. In addition, HCT-8 cells appear to possess modest amounts of the nucleoside phosphorylase necessary to catalyze this conversion [2].

Although dTTP levels were not quantitated, the following results assessing the cytotoxic effects of combining dThd during sequentially administered MTX-FUra indicated that significant dTTP levels were formed within 6 h of treating HCT-8 cells. Figure 7 shows that dThd (6 h) exposure within the first 12 h of a 24-h MTX treatment partially prevented MTX toxicity, although it did not entirely prevent the synergistic toxicity occurring when FUra was added during the final 6 h. Administration of dThd during the final 6 h of MTX exposure, or for 6 h after MTX exposure, did not alter 24-h MTX toxicity, which is consistent with the inability of delayed dTTP formation to reverse the antipurine effects of MTX [24, 25]. FUra with or without dThd during the final 6 h of MTX treatment produced maximum toxicity in HCT-8.

Discussion

Concentrations of dThd approaching 1 mM can be used to modulate MTX and FUra toxicity by exploiting either cytokinetic or biochemical mechanisms, as listed in Table 1. Inhibition of ribonucleotide reductase by dTTP is believed to be the reversible mechanism responsible for dThd-induced G_1-S block and subsequent cell synchronization [8, 22, 41, 59]. HCT-8 cells synchronized by dThd show increased sensitivity to 6-h MTX exposure and earlier synergistic cytotoxicity with sequentially combined MTX-FUra. By itself, FUra toxicity in synchronized cells shows no significant evidence for G_1, S, or G_2/M phase specificity, although there is a pattern of relative G_2/M resistance which correlates with the biochemical evidence for decreased intracellular accumulation of FUra, formation of FdUMP, and incorporation into RNA.

The reversible inhibition of other enzymes necessary for pyrimidine synthesis and catabolism contribute to the modu-

lating effects of dThd on FUra toxicity (Table 1). HCT-8 cells, known to possess pyrimidine nucleoside phosphorylase and kinase activities, show increased formation of soluble FdUMP which peaks 6 h after dThd exposure. Total FUra accumulation and RNA incorporation, on the other hand, show no significant change during 24 h of dThd exposure. None of these measurments appears to correlate with the pattern of dThd-enhanced FUra toxicity which is maximal after 18 h of dThd pretreatment. Other antimetabolites, including MTX, that are known to inhibit purine and pyrimidine pathways, influence FUra toxicity in HCT-8 cells by regulating total intracellular accumulation of FUra [4]. Inhibition of FUra catabolism in dThd-treated HCT-8 cells, not measured in these studies, would also be expected to enhance total drug accumulation and incorporation into RNA. These results suggest that dThd may modulate FUra toxicity in HCT-8 by some other, less well studied biochemical mechanism. The cytotoxicity studies with MTX-pretreated HCT-8 demonstrate that concurrent administration of dThd and FUra does not reduce the synergistic interaction between MTX and FUra, although earlier administration of dThd may abrogate this synergy. Longer (12–24 h) dThd and FUra exposures in HCT-8 pretreated with MTX would be optimal to determine whether dThd can further augment combined MTX – FUra toxicity in vitro, as Martin et al. have shown in vivo [40].

The reported effects of dThd on RNA metabolism are possible examples of other mechanisms for biochemically modulating FUra toxicity. In vivo studies showing dThd-enhanced incorporation of FUra into tumor RNA [3, 49, 50] can be explained by the reduction in plasma FUra clearance measured in dThd-treated mice and men [3, 45, 58]. Rate of FUra entry into RNA may be a more important determinant of RNA-directed FUra toxicity than absolute amount of RNA incorporation [1], and this may partially explain why in vivo dThd-enhanced RNA incorporation is not always associated with increased antitumor activity [3]. Exposing HeLa cells in culture to dThd inhibits DNA synthesis 12 h before any inhibition in cellular RNA synthesis can be detected biochemically [32]. However, autoradiographic and histochemical techniques have shown that high-dose dThd completely inhibits nucleolar RNA synthesis just subsequent to inhibition of DNA synthesis, while inhibition of extranucleolar (chromatin) RNA synthesis occurs independently of DNA inhibition, becomes progressively more pronounced 6 h after dThd administration, and never reaches completion [18, 31]. Selective and rapid entry of FUra into this latter species of RNA, further compromising its synthesis or processing, would correlate with the pattern of dThd-enhanced FUra toxicity observed in HCT-8 without necessarily requiring enhanced total incorporation of FUra into RNA. Further studies of the influence of dThd on RNA-directed FUra toxicity in cultured HCT-8 may provide more insight into this interesting possibility.

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