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N-(Phosphonacetyl)-L-aspartate Synergistically Enhances the Cytotoxicity of 5-Fluorouracil/Interferon-α-2a against Human Colon Cancer Cell Lines

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SUMMARY

Recombinant interferon-α (IFN) enhances the cytotoxic effects of the fluorinated pyrimidine, 5-fluorouracil (5FU), against two human colon cancer cell lines. The aspartate transcarbamylase (ATCase) inhibitor, *N*-(phosphonacetyl)-L-aspartate (PALA), was studied in combination with 5FU/IFN to determine whether further anti-pyrimidine effects would result in greater cytotoxicity. By median effects analysis PALA synergistically augmented the cytotoxic effects of 5FU/IFN against both human colon cancer cell lines. This occurred in the absence of any effects of 5FU/IFN on ATCase and without further potentiation of the PALA-mediated inhibition of ATCase. To explore the mechanism by which this interaction occurred, detailed studies of pools of dNTPs were performed. Both 5FU/IFN and PALA/5FU/IFN treatments resulted in early (2–8 hr) depletion of pools of dTTP, but no effects on pools of dCTP. PALA had no effect on dTTP pools either alone or in the combination. In contrast, both PALA and PALA/5FU/IFN treatments resulted in later (12–24 hr) depletion of pools of dCTP. 5FU/IFN treatment had no effect on these pools. When pools of dCTP and dTTP were repleted by treatment with cytidine or thymidine, 20 μM, however, there was only partial reversal of cytotoxicity induced by 5FU/IFN + PALA, suggesting that the synergy observed did not result solely from a sequential anti-pyrimidine effect. The incorporation of 5FU into RNA was also studied; PALA enhanced the incorporation of [6-^{3}H]5FU into RNA by 83–150%, but not into DNA, suggesting an alternative mechanism of drug interaction.

The fluorinated pyrimidine, 5FU, is an effective inhibitor of pyrimidine synthesis. Nevertheless, clinically the vast majority of tumors are either resistant *de novo* or rapidly acquire resistance after exposure to drug. Administering higher doses of drug is generally ineffective as cells are capable of circumventing these effects through increased cellular levels of TS and/or dUMP, mutated forms of TS, "salvage" pathways, or decreased levels of cellular reduced folates and polyglutamylated forms of reduced folates (1–8).

The cytotoxic effects of 5FU are synergistically enhanced *in vitro* by IFN (9–12). The mechanism by which this occurs is not fully understood, but appears to result at least in part by potentiating the 5FU-mediated inhibition of its target enzyme, TS. This includes induction of thymidine phosphorylase activity which results in enhanced anabolism of 5FU to its active metabolite, FdUMP (13), inhibition of 5FU-induced induction of TS (14), and inhibition of thymidine salvage pathways (13). In the clinical setting, the combination of 5FU/IFN appears more active than 5FU alone, and has demonstrated clinical activity against carcinomas of the colon and rectum (15–17), the esophagus (18, 19), and the bladder (20).

PALA is a rationally designed transition state inhibitor of aspartate transcarbamylase, and thus, like 5FU, is an inhibitor of pyrimidine biosynthesis both *in vitro* and *in vivo* (21–24) and in the clinic (for a review, see 25). Like IFN, PALA synergistically enhances the cytotoxic effects of 5FU in experimental tumor models, although the mechanism appears to be different from that of 5FU/IFN.

The combination of 5FU/IFN, which results in depletion of thymidine pools, an PALA, which depletes pools of uridine and cytidine, could potentially result in greater than additive cytotoxic effects. In this study we have investigated the interaction of 5FU/IFN and PALA against two human colon cancer cell lines, and evaluated the possibility that the combination produces further augmentation of the antipyrimidine effects of the individual agents.

Materials and Methods

Cell culture. HT-29 and SW480 cells were the generous gift of Dr. Leonard Augenlicht. Cells were maintained in RPMI 1640 (GIBCO,

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ABBREVIATIONS: 5FU, 5-fluorouracil; TS, thymidylate synthase; IFN, interferon; FdUMP, fluorodeoxyuridylate; ATCase, aspartate transcarbamylase; PALA, *N*-(phosphonacetyl)-L-aspartate; FBS, fetal bovine serum; D-FBS, dialyzed FBS; D-PBS, Dulbecco's phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FUTP, 5-fluorouridine 5′-triphosphate; Fd, ferredoxin.

Grand Island, NY) with 10% FBS (GIBCO) and 1% penicillin-streptomycin at 37° in 5% CO_2. For drug exposures, cells were removed from standard culture medium 24 hr before drug exposure and incubated in folate-free RPMI 1640 (GIBCO) with 10% dialyzed FBS (D-FBS) and folic acid, 0.5 μM, without antibiotics.

Drugs and reagents. PALA was generously supplied by U.S. Bioscience, West Conshohocken, PA. Recombinant α-2a-interferon (IFN) was a gift of Hoffman-LaRoche (Nutley, NJ). 5FU was from Lyphomed (Rosemont, IL). DNA polymerase I, large fragment (Klenow fragment), and Dulbecco's phosphate-buffered saline (D-PBS) were from GIBCO. Oligonucleotides were from American Synthesis, Pleasanton, CA. All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Radioisotopes. [*methyl*-^{3}H]Thymidine 5′-triphosphate, tetrasodium salt,, 19 Ci/mmol, was from New England Nuclear (Boston, MA). [2,8-^{3}H]Deoxyadenosine triphosphate, 32 Ci/mmol, was from Moravek Biochemicals (LaBrea, CA). L-[U-^{14}C]Aspartic acid, 189 mCi/mmol, and [6-^{3}H]5-fluorouracil, 20 Ci/mmol, were from Sigma.

Measurement of cell growth. Cell proliferation was estimated using a slight modification of the sulforhodamine B assay (26). Results obtained with this assay were similar to those obtained with a clonogenic assay as reported previously (9). Cells were incubated with 5FU, 1–100 μM, with IFN, 500 units/ml, or with PALA, 20–2000 μM, or combinations of drug for 24 hr (approximately 1.5 doubling times). For experiments in which reversal of cytotoxicity was tested, cells were incubated with combinations of 5FU, IFN, and PALA with simultaneous exposure to cytidine, thymidine, or uridine, all at 20 μM. Preliminary tests demonstrated no significant loss of cells at 24 hr, and >95% of cells were viable by trypan blue exclusion after removal of drug. Drug-containing medium was removed and replaced with fresh medium. Cells were incubated in the absence of drug for 7 days and then were fixed and stained exactly as described (26). Correlation coefficients for standard curves comparing number of cells and absorbance were routinely >0.99.

Assessment of drug interactions. Drug interactions were quantitated by median effects analysis (27, 28) as described previously (9). When cells were incubated with 5FU/IFN and PALA, the PALA/5FU ratio was fixed at 20:1 in order to perform median effects analysis. IFN treatment resulted in no significant cytotoxicity; therefore, for the purposes of median effects analysis, it was employed in combination with 5FU at a fixed dose of 500 units/ml. Computer programs for the IBM-PC based on the median effect plot and combination index equation were employed for data analysis (29).

Measurement of ATCase activity. Cells were incubated in folate-free RPMI 1640 with 10% D-FBS and folic acid, 0.5 μM, with or without drug treatment for various time intervals. Cells were detached from the flasks with trypsin-EDTA, washed 3 times with D-PBS, then sonicated 3 times for 10 sec at 30% output in homogenization buffer (Tris-HCl, 20 mM, pH 7.5; EDTA, 0.1 mM; sucrose, 300 mM; β-mercaptoethanol, 50 mM; aprotinin, 500 units/ml; phenylmethylsulfonyl fluoride, 0.2 mM) on ice. Insoluble fragments were removed by centrifugation at 12,000 × *g* for 30 min at 4°, then the extracts were desalted and concentrated by centrifugation over a 10,000 NMWL ultrafiltration membrane (Millipore Products, Bedford, MA) for 30 min at 4°. The assay was a modification of the method of Porter *et al.* (30). In a total volume of 150 μl, cell-free extract was incubated with L-[U-^{14}C]aspartic acid (189 mCi/mmol), 3 mM; carbamoyl phosphate, 1 mM; and HEPES, 50 mM, pH 7.8 for 1 hr at 37°. The reaction was stopped by the addition of 1.5 ml of ice-cold acetic acid, 0.3 N, and carbamoyl-L-aspartate was separated from aspartate over Dowex 50-X8–400 columns, 400 mesh. Samples were dried under a vacuum, and counted by liquid scintigraphy. The assay was linear with respect to amount of protein and time.

Preparation of cell extracts for measurement of NTP and dNTP pools. Cells were incubated in folate-free RPMI 1640 with 10% D-FBS and folic acid, 0.5 μM, with or without 5FU and/or IFN for various time intervals in six-well Falcon tissue culture plates (Becton Dickinson, Lincoln Park, NJ). Media were rapidly poured off, and without washing (31) 200 μl of ice-cold PCA was added for 30 min at 4°. The extract was centrifuged for 2 min at 12,000 × *g*, then the supernatant was neutralized to pH 7.4 with 400 μl of alamine:Freon as described previously (13). Extracts were again centrifuged for 2 min, and the top layer removed for the assay.

Measurement of dNTP pools. The DNA polymerase assay, modified from that of Sherman and Fyfe (32), was employed. Oligonucleotides were modified to increase the sensitivity and decrease the background of the assay. Template sequences employed were:

dNTP	*Sequence*
dTTP	5′TTT ATT TAT TTA TTT ATT TAG GCG GTG GAG GCG GA3′
dCTP	5′TTT GTT TGT TTG TTT GTT TGA AAG GTG GAG GCG G3′

The primer sequence was:

dNTP	*Sequence*
dTTP dCTP	5′CCG CCT CCA CCT TT-3′

Oligonucleotides were dissolved in TE buffer (Tris-HCl, 10 mM, pH 7.4; EDTA, 1 mM), and their concentrations were confirmed by spectroscopy. Templates and primers were mixed in TE, vortexed, heated to 65° for 20 min, and cooled to room temperature. Samples were diluted in water to 0.8 μM and stored at −20°.

The assay mixture included: cell extract, 10–67 μl (depending on the particular nucleotide being measured); $MgCl_2$, 5 mM; HEPES, 100 mM, pH 7.4; [2,8-^{3}H]deoxyadenosine triphosphate or thymidine 5′-triphosphate, tetrasodium salt [*methyl*-^{3}H], 0.65 μCi; oligonucleotide, 4 pM; and Klenow fragment, 0.065 unit in a total volume of 100 μl. The mixture was vortexed for 10 sec, then incubated at 37° for 1 hr. Thirty microliters of assay mixture were spotted on DE81 filters (Whatman, Hillsboro, OR) prewetted with 1% sodium pyrophosphate, 5% trichloroacetic acid and washed with the same solution, 20 ml, three times. Filters were then dried and counted by liquid scintigraphy. Assays were performed in replicates of 6. dNTP standards were employed for each experiment; standard curves were linear to 0.125 pmol and correlation coefficients >0.99 were obtained routinely. The assay was linear with respect to time and concentration of dNTP. Isotopic dilution by unlabeled, endogenous nucleotide was estimated for each assay. This was considered negligible, as there was always at least >10-fold excess of labeled/unlabeled nucleotide per assay tube.

Measurement of NTP pools. Ribonucleoside triphosphates were separated and analyzed by HPLC on an HP1090 system using a Whatman Partisphere 5μ SAX column (4.7 × 150 mm), using a method as described previously (33). A mobile phase of 0.3 M NaH_2PO_4 (pH 5) was used with a flow rate of 1 ml/min. UTP, CTP, ATP, and GTP were detected by absorbance at 260 nm and quantified by comparison to standards run on the same day; they had typical retention times of 5.9, 9.0, 11.2, and 20.6 min, respectively. [^{3}H]FUTP had a retention time of 6.4 min, and was quantified by liquid scintillation counting after the collection of 0.5-min HPLC fractions.

Measurement of FUTP incorporation into total RNA. Cells were incubated with [6-^{3}H]5FU (2 μCi/ml), 20 μM, plus IFN, 500 units/ml, with or without PALA, 200 μM, for 24 hr. Cells were washed with D-PBS three times, and total RNA was isolated over a cesium chloride cushion, then precipitated with ethanol-acetate as described (34). RNA was resuspended, quantitated spectrophotometrically, then counted by liquid scintigraphy.

Measurement of FdUTP incorporation into DNA. Incorporation of FdUTP into DNA was performed as described previously (13). To ensure that trichloroacetic acid precipitation did not result in precipitation of [^{3}H]FdUMP-TS complex, a modification of the method of Nocentini (35) was employed. Cells were incubated with drug as described for incorporation of FUTP into RNA, washed three times with D-PBS, scraped from plates, and sonicated on ice three times for 5 sec. Extracts were adjusted to 800 μl in ice-cold 80 mM Tris (pH 8.0) to which DNase-free RNase (250 μg/ml) was added. The final pH was

7.8. After 2 hr of incubation at 37°, the solution was cooled on ice and trichloroacetic acid was added to a final concentration of 10%. DNA was allowed to precipitate at 4° for 1 hr, the pellet centrifuged at 4000 × *g*, and washed three times in ice-cold 5% trichloroacetic acid, then dissolved in NaOH, 1.25 M, overnight. The sample was neutralized with trichloroacetic acid, then counted by liquid scintigraphy.

Results

Effects of the combination of 5FU/IFN and PALA on cell growth. As shown in Fig. 1a, concentrations of PALA >100 μM, were necessary to achieve an IC_{50}. In combination with either IFN or PALA, the IC_{50} for 5FU was approximately 15–25 μM in the two cell lines. In contrast, when combined with PALA and IFN, treatment with 5FU at much lower concentrations, 3–6 μM, resulted in 50% cell kill. The addition of PALA to 5FU/IFN shifted the dose-response curves for both cell lines to the left. To determine whether these effects were synergistic, median effects analysis was performed. As shown in Fig. 1b, the interaction between 5FU/IFN and PALA was synergistic at all but the very highest concentrations of drug tested, whether assumptions of mutual exclusivity of action or nonexclusivity of action were made. Results are shown for HT-29 cells and were essentially identical for SW480 cells (not shown).

Effects of FU/IFN and PALA on ATCase activity. In HT-29 cells incubated with PALA, 200 μM, for 0.05–24 hr, inhibition of ATCase increased steadily over 24 hr reaching the maximal inhibition observed, 57%, at 24 hr. This most likely resulted from the slow uptake of PALA across cell membranes (36). When PALA was added directly to cell extract, there was nearly complete inhibition of ATCase activity within 1 hr.

As shown in Table 1, PALA inhibited ATCase activity in both HT-29 and SW480 cells by 57% and 47%, respectively. Treatment with 5FU and IFNα-2a resulted in only a 17% inhibition of ATCase activity in HT-29 cells and no change in SW480 cells. Treatment with the combination of 5FU/IFN and PALA was not different than that of PALA alone.

Effects of 5FU/IFN and PALA on pools of dNTPs. As shown in Fig. 2, the predominant effect of single agent PALA was depletion of pools of dCTP by 62–75% from 12 to 24 hr (HT-29) and by 54–69% from 8 to 24 hr (SW480). This pattern of depletion of dCTP pools at 8–24 hr was essentially the same as that observed for the combination of PALA + 5FU/IFN. In contrast, with the exception of a 40% decrease in dCTP pools at 8 hr, there was no significant effect of 5FU/IFN alone on pools of dCTP.

As shown in Fig. 3, treatment with PALA had lesser effects on pools of dTTP depleting them by 39% (HT-29) and 42% (SW480) beginning at 8 hr. In contrast incubation of cells with 5FU/IFN resulted in rapid early decreases in pools of dTTP by 93% (HT-29) and 95% (SW480) at 4–8 hr. When PALA was combined with 5FU/IFN, the effect on pools of dTTP was similar to that of 5FU/IFN alone with rapid depletion in dTTP pools at 4–12 hr, although the effect on HT-29 cells was somewhat smaller. There was no increase in dTTP depletion with the combination of three drugs as opposed to 5FU/IFN alone.

Effects of cytidine, uridine, and thymidine rescue on 5FU/IFN + PALA-induced cytotoxicity. As depletion of dCTP pools by PALA was associated with enhanced cytotoxicity, cells were incubated with or without 5FU/IFN or PALA

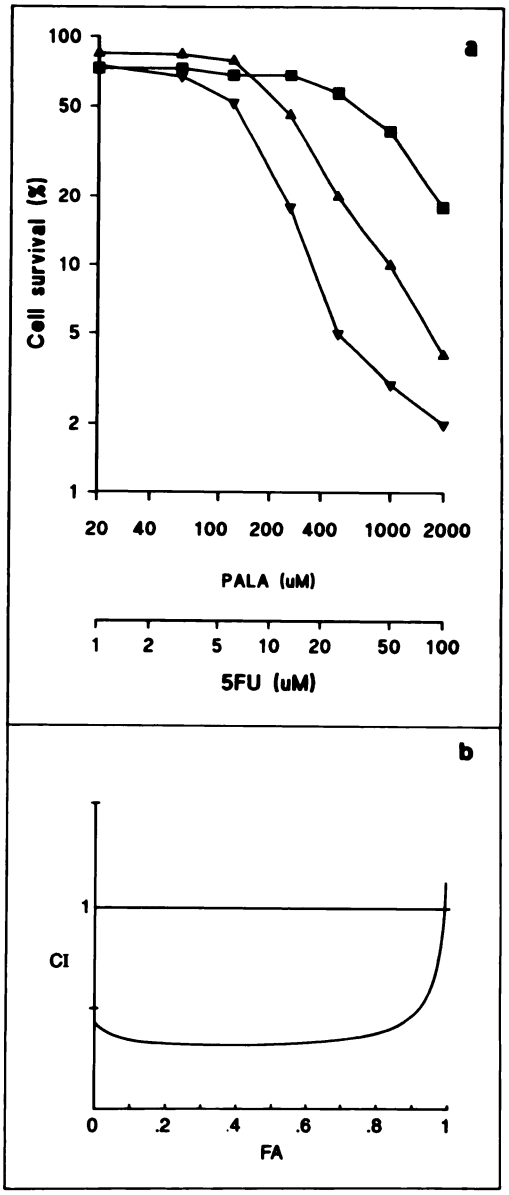

Fig. 1. The combination of PALA/5FU/IFN synergistically inhibits cell proliferation. a, HT-29 cells were treated with PALA (■), 5FU + IFN (▲), or the combination (▼) for 24 hr. The ratio of PALA:5FU was fixed as required for median effects analysis (27, 28); a 20:1 ratio was employed. The concentration of IFN was 500 units/ml. Incubation with the combination resulted in greater inhibition of cell growth than either PALA or 5FU/IFN employed alone. b, Median effects analysis was employed to assess the drug interactions. Points above the line indicate drug antagonism and those below the line drug synergy. Median effects analysis revealed synergy over the entire range of concentrations tested. Results are shown for HT-29 cells; results for SW480 cells, not shown, are essentially identical. *CI*, combination index; *FA*, fraction affected.

or the combination in the presence or absence of cytidine, 20 μM, for 2–24 hr. The effects of PALA were completely reversed at 20 and 24 hr when cells were simultaneously incubated with cytidine, and resulted in increases in dCTP levels to near or above baseline (Table 2). As shown in Fig. 4, despite complete reversal in dCTP depletion by cytidine incubation, there was only partial reversal of cytotoxicity resulting from incubation of cells with PALA + 5FU/IFN.

Cells were also incubated with 5FU/IFN + PALA in the presence or absence of uridine or thymidine, 20 μM, for various

TABLE 1

PALA, but not 5FU/IFN, inhibits aspartate transcarbamylase activity in colon cancer cells

Cells were plated in 25-cm² tissue culture flasks in folate-free RPMI 1640 with 10% D-FBS and folic acid, 0.5 μM, 24 hr before drug exposure. Cells were then treated either with 5FU, 20 μM + IFN, 500 units/ml, or PALA, 200 μM, or both for 24 hr. ATCase activity was measured at various times as described in Materials and Methods. Experiments were performed in triplicate and repeated twice; values are means ± standard error.

	HT-29	SW480
Control	61.1 ± 1.8 (100)[a]	82.4 ± 18.9 (100)
PALA	26.2 ± 2.6 (43)[b]	43.8 ± 7.2 (53)[b]
FU/IFN	50.8 ± 2.8 (83)	87.4 ± 12.7 (106)
PALA/FU/IFN	27.2 ± 4.8 (45)[b]	33.8 ± 3.8 (41)[b]

[a] Values stated are fmol/min/4 × 10⁶ cells (%).
[b] $p < 0.01$.

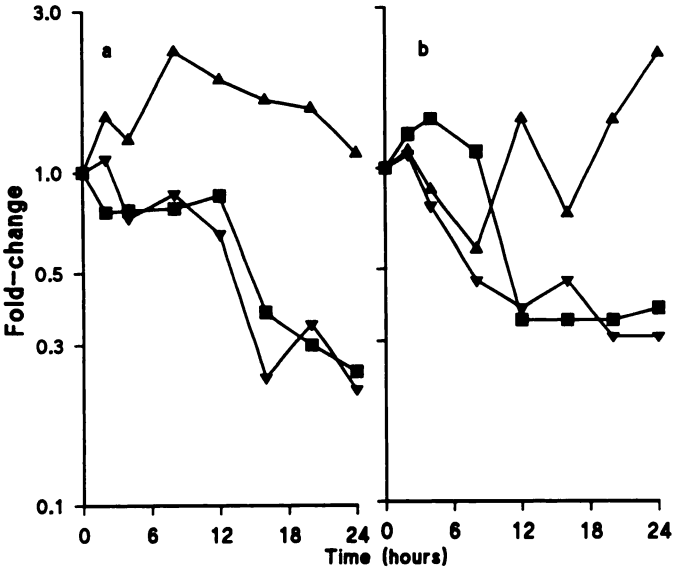

Fig. 2. Inhibitory effects of PALA, 5FU/IFN, or PALA/5FU/IFN on pools of dCTP. HT-29 (a) or SW480 (b) cells were incubated with PALA, 200 μM (■), with 5FU, 20 μM, + IFN, 500 units/ml (▲), or with PALA/5FU/IFN (▼) for 2–24 hr. Preparation of extracts and assay are as described in Materials and Methods. Data are expressed relative to baseline values. Each point represents the mean of 6 replicates. Standard error bars fall within each point.

times. At 8 hr thymidine completely reversed the 5FU/IFN + PALA depletion of dTTP and returned dTTP levels to baseline (Table 3). In contrast, incubation with uridine, 20 μM, had no effect on 5FU/IFN + PALA depletion of dTTP at 8 hr; dTTP levels were depleted by 58% and 33% in HT-29 and SW480 cells, respectively. As shown in Fig. 4, treatment of cells with uridine, 20 μM, for 24 hr partially reversed the excess cytotoxicity that resulted from the addition of PALA to 5FU/IFN. Thymidine, 20 μM, was incapable of reversing the cytotoxicity associated with 5FU/IFN + PALA.

Effects of 5FU/IFN and PALA on pools of NTPs. As shown in Table 4, treatment with 5FU alone or in combination with IFN, PALA,or both for 24 hr resulted in depletion of pools of UTP by up to 79% in both cell lines. Neither IFN nor PALA significantly augmented this effect, nor did either of these agents increase the ratio of FUTP to UTP pools. Effects on pools of ATP, GTP,and CTP were also measured after incubation with 5FU alone or in combination with IFN, PALA,or IFN + PALA. There were no significant effects on pool sizes of these NTPs.

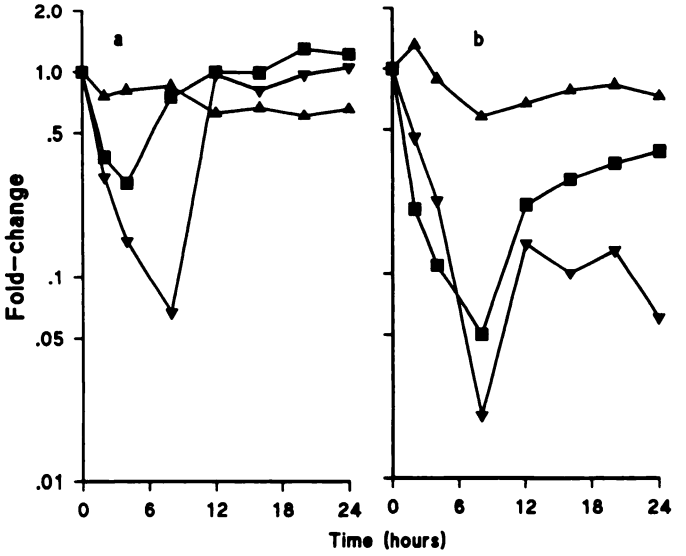

Fig. 3. Inhibitory effects of PALA, 5FU/IFN, or PALA/5FU/IFN on pools of dTTP. HT-29 (a) or SW480 (b) cells were incubated with PALA, 200 μM (▲), with 5FU, 20 μM, + IFN, 500 units/ml (■), or with PALA/5FU/IFN (▼) for 2–24 hr. Preparation of extracts and assay are as described in Materials and Methods. Data are expressed relative to baseline values. Each point represents the mean of 6 replicates. Standard error bars fall within each point.

Effects of PALA on incorporation of [6-³*H*]5FU into cellular RNA and DNA. As shown in Table 5, incubation of cells with [6-³H]5FU, 20 μM, and IFN, 500 units/ml, for 24 hr resulted in incorporation of [6-³H]FUTP into RNA. In HT-29 cells, co-incubation with PALA, 200 μM, significantly increased incorporation of FUTP by 83%, and in SW480 cells by 150%. In both HT-29 and SW480 cells, PALA failed to significantly increase incorporation of FdUTP into DNA (Table 5).

Discussion

In these studies PALA synergistically enhanced the cytotoxic effects of the combination of 5FU/IFN against two human colon cancer cell lines *in vitro*. Median effects analysis was employed; less stringest definitions of drug synergy have been shown to result in erroneous conclusions (37). These results suggest that the enhanced cytotoxicity observed for the combination did not simply reflect an additive effect on cell kill, but an interaction between the three drugs resulting in a greater than additive effect. Furthermore, synergy occurred across nearly the entire range of drug concentrations tested, suggesting that this is likely to be relevant to further use of these drugs in combination.

Modulation of the activity of 5FU by both IFN and PALA has previously been demonstrated *in vitro* and *in vivo*, and furthermore evidence suggests that these combinations have clinical activity. Thus, it would be important to attempt to define the mechanisms by which these drugs interact. The interaction of 5FU and IFN is at least in part attributable to enhancement by IFN of the inhibitory effects of 5FU against its target enzyme, TS. Evidence also suggests that effects "downstream" from TS, resulting from altered ratios of dNTPs, inhibition of DNA repair, or effects on DNA synthesis, may result in increased DNA strand breakage (38). The interaction of 5FU and PALA has been postulated in some studies to be secondary to enhancement of the effects at the level of TS. In

TABLE 2

Cytidine completely reverses depletion of dCTP pools

Levels of dCTP in HT-29 cells (top) and SW480 cells (bottom) were determined at various time points after treatment with 5FU/IFN or PALA. Cells, in folate-free RPMI 1640 + 10% D-FBS and 0.5 μM folic acid, were incubated with FI (5FU, 20 μM, + IFN, 500 units/ml); PALA, 200 μM; FIP (5FU + IFN + PALA), or FIPC (FIP + cytidine, 20 μM) for 8–24 hr. Levels of dCTP were measured in cell extracts as described in Materials and Methods. Results are expressed as pmol/10^6 cells (mean ± standard error of 6 replicates).

	Time, hr				
	0	8	12	20	24
Control	3.90 ± 0.22	–	–	–	–
FI	–	3.62 ± 0.42	3.65 ± 0.25	8.28 ± 0.66	2.97 ± 0.18
PALA	–	ND	ND	0.81 ± 0.15	0.93 ± 0.15
FIP	–	4.37 ± 0.23	2.08 ± 0.09	1.11 ± 0.09	1.20 ± 0.33
FIPC	–	ND	ND	6.57 ± 1.35	8.43 ± 0.30
Control	3.63 ± 0.22	–	–	–	–
FI	–	4.12 ± 0.29	3.05 ± 0.24	1.59 ± 0.26	4.64 ± 0.24
PALA	–	ND	ND	0.72 ± 0.09	1.29 ± 0.13
FIP	–	3.28 ± 0.25	1.01 ± 0.08	0.33 ± 0.07	0.90 ± 0.11
FIPC	–	ND	ND	2.19 ± 0.13	6.09 ± 0.37

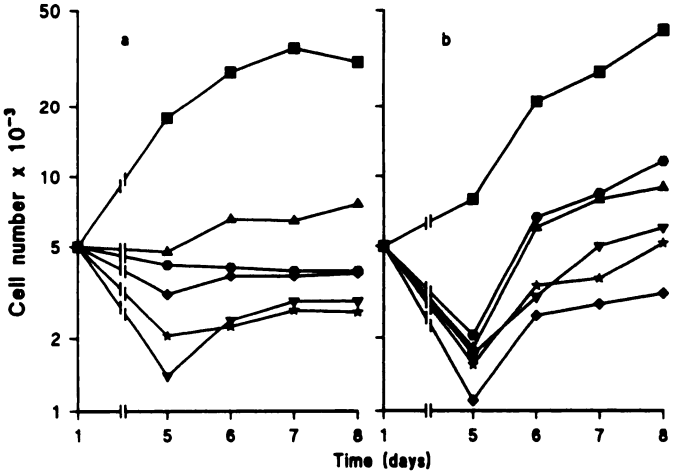

Fig. 4. Incubation with cytidine fails to rescue cells treated with 5FU/IFN + PALA. HT-29 (a) or SW480 (b) cells were incubated with either 5FU, 20 μM, and IFN, 500 units/ml, or PALA, 200 μM, or both in the presence or absence of cytidine, uridine, or thymidine, 20 μM. At 24 hr cells were washed with PBS three times, then resuspended in media. Cell proliferation was measured as described in Materials and Methods. ■, Control; ▲, 5FU/IFN; ♦, 5FU/IFN/PALA/cytidine; ★, 5FU/IFN/PALA/thymidine; ●, 5FU/IFN/PALA/uridine; ▼, 5FU/IFN/PALA. Experiments were performed in replicates of 6 at least twice.

TABLE 3

Thymidine, but not uridine, reverses depletion of dTTP by 5FU/IFN/PALA

Cells were incubated with 5FU, 20 μM, IFN, 500 units/ml, and PALA, 200 μM, with or without thymidine, 20 μM, or uridine, 20 μM. Control cells received no drug treatment. At 8 hr, dTTP was extracted and measured as described in Materials and Methods.

	HT-29	SW480
Control	1.69 ± 0.24 (100)[a]	2.09 ± 0.08 (100)
FIP	0.58 ± 0.09 (34)	1.27 ± 0.10 (61)
FIPT	2.58 ± 0.38 (154)	2.19 ± 0.11 (105)
FIPU	0.71 ± 0.03 (42)	1.39 ± 0.18 (67)

[a] Values stated are pmol/10^6 cells (% control).

murine sarcoma S180 cells, PALA augmented the inhibitory effects of 5FU on TS activity, including a decrease in catalytic activity and availability of free enzyme for binding, and increased formation of the ternary complex formed by FdUMP, TS, and N^5,N^{10}-methylenetetrahydrofolate (39). The mechanism reponsible for these effects was partial abrogation by

TABLE 4

Treatment with PALA fails to augment FUTP/UTP

Cells were incubated with [6-^{3}H]5FU for 24 hr. Extraction of ribonucleotides and analysis were performed as described in Materials and Methods. Numbers represent mean ± standard error of three to four experiments.

Cell line	Treatment	UTP[a]	FUTP[b]	FUTP/UTP (× 10^5)
HT-29	Control	8.45 ± 2.42	–	–
	5FU	3.38 ± 1.53	18.0 ± 16.0	5.3
	5FU/PALA	1.79 ± 1.41	7.0 ± 3.1	3.9
	5FU/IFN	2.82 ± 0.98	24.0 ± 9.5	8.5
	5FU/IFN/PALA	1.17 ± 0.78	11.0 ± 2.3	9.4
SW480	Control	7.03 ± 0.93	–	–
	5FU	2.06 ± 1.05	13.0 ± 5.4	6.3
	5FU/PALA	1.83 ± 0.49	10.0 ± 0.5	5.5
	5FU/IFN	3.52 ± 0.45	14.0 ± 4.3	4.0
	5FU/IFN/PALA	2.63 ± 0.72	15.0 ± 2.5	5.7

[a] Values stated are nmol/10^6 cells.
[b] Values stated are pmol/10^6 cells.

TABLE 5

PALA increases incorporation of [6-^{3}H]5FU into RNA, but not DNA

Cells were incubated with [6-^{3}H]5FU (2 μCi/ml), 20 μM, + IFN, 500 units/ml, with or without PALA, 200 μM, for 24 hr. RNA and DNA were isolated as described in Materials and Methods.

	HT-29	SW480
RNA		
–PALA	7.28 ± 0.55[a]	8.48 ± 0.92
+PALA	13.32 ± 1.13[b]	21.13 ± 2.56[b]
DNA		
–PALA	0.82 ± 0.07	0.71 ± 0.06
+PALA	1.15 ± 0.15[c]	0.87 ± 0.07[c]

[a] Values stated are pmol/10^6 cells.
[b] $p < 0.01$ by Student's t-test.
[c] p = NS.

PALA of a compensatory rise in dUMP levels in cells treated with 5FU, resulting from inhibition of *de novo* pyrimidine synthesis, and an increase in cellular levels of FdUMP, resulting from decreased competition of 5FU anabolites with competing endogenous metabolites and possibly from an increase in levels of a cofactor for FdUMP synthesis, phosphoribosylpyrophosphate. Other studies, however, have suggested that PALA enhances 5FU cytotoxicity by augmenting uptake of 5FU into RNA. Studies in two human breast carcinoma cell lines (MCF-7, MDA-MB-157), two human melanoma cell lines (IGR3, M5), and the hepatoma cell line, AS-30D, demonstrated

depletion of pools of UTP, increased incorporation of FUTP into RNA, decreased cell survival, and no inhibition of DNA synthesis when cells were pretreated with PALA before exposure to 5FU (40–44).

The current studies have attempted to further delineate the mechanism by which 5FU/IFN and PALA interact, with specific reference to effects of the combination on pools of nucleotides. Our initial hypothesis was that PALA and 5FU/IFN were synergistic because of either enhanced depletion of a single nucleotide pool or because of sequential depletion of pools of pyrimidines; however, several findings suggested an alternative mechanism of interaction. First, the effects of PALA appeared to occur at a later time interval than those of 5FU/IFN as reflected specifically in the early decreases in pools of dTTP and later decreases in pools of dCTP. Second, 5FU/IFN did not influence the effects of PALA on ATCase activity or on dCTP pools. Third, although specific effects on TS were not measured in these studies, it is unlikely that PALA substantially augmented the effects of 5FU/IFN against TS, as pools of dTTP were not further depleted when PALA was added to 5FU/IFN.

The studies with cytidine and thymidine rescue make it less likely that sequential depletion of dNTP pools was the basis for the synergy observed. Pools of dCTP and dTTP were thoroughly repleted to near baseline or above baseline by incubation with cytidine and thymidine, respectively, with only partial reversal of the cytotoxic effects of PALA. Thus, it is likely that a mechanism other than a combined anti-pyrimidine effect was responsible for the synergistic interaction.

Increased incorporation of FUTP into RNA was also observed in HT-29 and SW480 cells, despite an absence of an effect on the ratio of FUTP to UTP pools at 24 hr. This may reflect a difference in pool sizes occurring earlier than 24 hr. Alternatively, this may reflect compartmentalization of nucleotide pools within subcellular compartments which do not equilibrate readily (45).

Increased incorporation of FUTP into RNA represents a possible mechanism to account for the synergy between 5FU/IFN and PALA. The cytotoxicity of 5FU has been shown to correlate with levels of 5FU incorporated into RNA *in vitro* (46, 47) and *in vivo* (48). In various human and mouse cell lines, growth inhibition secondary to low dose 5FU (5–20 μM) was correlated with inhibition of TS, whereas the growth inhibition of high dose 5FU (50–200 μM) correlated with incorporation into RNA. Similar observations have been made with regard to duration of exposure: short term (4 hr) exposures to 5FU resulted in cell death through an RNA-mediated mechanism, whereas longer exposures (7 days) resulted in inhibition of TS with effects on DNA (49). Our studies suggest that, in combination with IFN and PALA, RNA effects of 5FU are observed at lower doses (20 μM) and relatively long exposures (24 hr) (50). Thus, it is possible that the synergy observed with the triple combination may result from a shifting of the mechanism of 5FU cytotoxicity toward an anti-RNA effect, resulting in inhibition of RNA splicing (51, 52) or translation. The inability of uridine treatment to completely reverse the cytotoxicity of the 5FU/IFN + PALA combination suggests that this shift may not be complete and that effects on DNA integrity may be a component of the cytotoxicity of this combination.

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