Biochemical Pharmacology, Vol. 30, No. 15, pp. 2045–2049, 1981.
Printed in Great Britain.

0006-2952/81/152045-05 $02.00/0
Pergamon Press Ltd.

SYNERGISTIC EFFECT OF 5-FLUOROURACIL AND *N*-(PHOSPHONACETYL)-L-ASPARTATE ON CELL GROWTH AND RIBONUCLEIC ACID SYNTHESIS IN A HUMAN MAMMARY CARCINOMA

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(*Received* 27 *September* 1980; *accepted* 22 *December* 1980)

Abstract—The biological effects of *N*-(phosphonacetyl)-L-aspartate (PALA) and 5-fluorouracil (5-FU) were examined singly, and in combination, on the growth of a human mammary carcinoma (MDA) cell line in culture. All combinations of 5-FU (2.5×10^{-7} to 1.5×10^{-5} M) and PALA (6.0×10^{-5} to 3.6×10^{-3} M) resulted in synergistic inhibition of cell growth as revealed by a 50 per cent isobologram. To examine the biochemical basis for the synergism, measurements of the incorporation of [^{3}H]-5-FU into total non-poly(A)- and poly(A)-RNA, and of the simultaneous incorporation of [^{14}C]deoxyguanosine and [^{3}H]deoxyuridine into DNA, were determined. The combination of 3.7×10^{-5} M PALA and 1×10^{-6} M 5-FU produced 65–85 per cent inhibition of cell growth after continuous treatment for 1–3 days. Treatment of the cells for 3 or 24 hr with the same drug regimen produced approximately a 170 per cent increase in the incorporation of 1×10^{-6} M [^{3}H]-5-FU into poly(A)RNA in comparison to [^{3}H]-5-FU treatment alone; exposure for 24 hr to 3.7×10^{-5} M PALA and 1×10^{-6} M [^{3}H]-5-FU resulted in a 285 per cent increase in the incorporation of [^{3}H]-5-FU into non-poly(A)RNA. The incorporation of either [^{14}C]deoxyguanosine or [^{3}H]deoxyuridine into DNA was not inhibited by this drug regimen; however, the incorporation of [^{3}H]deoxyuridine into DNA was elevated significantly upon 12 or 24 hr of exposure to PALA alone. PALA and 5-FU treatment resulted in a 75 per cent reduction in the concentration of UTP and no change in the concentration of 5-fluorouridine-5′-triphosphate (5-FUTP) versus 5-FU treatment alone. Thus, the proportion of 5-FUTP in the total 5-FUTP + UTP pool was enhanced more than 3-fold by the combination regimen. These results indicate that the synergistic effect of the combination of PALA and 5-FU on the growth of MDA cells correlates with an increased proportion of 5-FUTP in the pyrimidine nucleotide pool and, consequently, with an enhanced incorporation of 5-FU into RNA, but not with inhibition of DNA synthesis.

N-(Phosphonacetyl)-L-aspartate, a transition state inhibitor of aspartate transcarbamylase, significantly inhibits a variety of experimental and human solid tumors [1–4]. The pharmacological activity of PALA† is believed to be the result of its ability to inhibit pyrimidine biosynthesis *de novo*. Marked reductions in UMP have been observed in sensitive murine tumors treated with PALA [4]. Limited clinical activity in recent phase I trials has also been observed [5, 6].

5-Fluorouracil, a widely used antimetabolite with limited clinical efficacy [7–9], acts by inhibition of thymidylate synthetase via its metabolite, 5-dFUMP [10–12], or via interference with RNA synthesis by incorporation of 5-FUTP into nascent RNA [13–16], or both.

Inhibition of pyrimidine biosynthesis by PALA would be expected to lead to depletion of, possibly, both UTP and dUMP within the cell. Because generation of 5-FUTP and 5-FdUMP from 5-FU via salvage routes during pyrimidine nucleotide deprivation from the *de novo* pathway would increase the proportion of these metabolites contributing to the total pyrimidine nucleotide pool, PALA and 5-FU might produce complementary inhibitory effects on tumor growth. To evaluate this hypothesis, combinations of PALA and 5-FU were tested, using the human mammary carcinoma (MDA) cell line, for their effects on cell growth and the relationships of these effects to the perturbation of RNA and DNA synthesis.

MATERIALS AND METHODS

Chemicals. PALA and 5-FU were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health,

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† Abbreviations: PALA, *N*-(phosphonacetyl)-L-aspartate; 5-FU, 5-fluorouracil; 5-FdUMP, 5-fluoro-2′-deoxyuridylic acid; 5-FUTP, 5-fluorouridine-5′-triphosphate; dGR, 2′-deoxyguanosine; IC, inhibitory concentration; poly(A), polyriboadenylic acid; poly(U), polyribouridylic acid; and dUR, 2′-deoxyuridine.

Bethesda, MD. The MDA-MB-157 cell line was derived originally from ascites cells obtained from patients with infiltrating ductal carcinoma of the breast and was a gift from Dr. Marc Lippman, Medicine Branch, NCI. 5-Fluoro[6-^{3}H]uracil (1.4 Ci/mmole), 5-fluoro[2-^{14}C]uracil (50 mCi/mmole), deoxy[6-^{3}H]uridine (24 Ci/mmole), and deoxy[8-^{14}C]guanosine (54.4 mCi/mmole) were obtained from Amersham/Searle, Arlington Heights, IL.

Cell growth. MDA cells were grown in minimum essential media [17] containing 5% (v/v) dialyzed fetal calf serum, 0.06% (w/v) L-glutamine, penicillin (62 mg/l), and streptomycin (135 mg/l) at 37° in an atmosphere of 5% CO_2–95% air. For the initiation of cell growth experiments, logarithmically growing cells were harvested with Dulbecco's phosphate-buffered saline containing 0.05% trypsin and 0.02M EDTA and were suspended in medium plus serum; 5×10^5 cells were plated in 75 cm^3 plastic tissue culture flasks. After allowing the cells to adhere to the flask for 1 day, the medium was decanted and replaced with fresh medium containing 5% dialyzed fetal calf serum and the chemotherapeutic drug being evaluated. Three days after drug treatment, the cells were washed once with Dulbecco's phosphate-buffered saline, trypsinized, and counted in an F_N Coulter counter. Results are expressed as a percentage of the control cell count.

Incorporation of [^{3}H]*5-FU into non-poly(A)- and poly(A) RNA.* Exponentially growing MDA cells (1 day after plating 5×10^5 cells) were incubated for 3 or 24 hr with either 1×10^{-6} M [6-^{3}H]-5-FU (1.4 Ci/mmole) or 3.7×10^{-5} M PALA and 1×10^{-6} M [6-^{3}H]-5-FU. At the end of 3 or 24 hr, cells were trypsinized, washed with Dulbecco's phosphate-buffered saline, and dissolved directly in 3 ml of 0.1% sodium dodecylsulfate : 0.1 M Tris–HCl (pH 9.0) : 7 M urea. After the addition of 1.5 ml of phenol mixture (phenol–*m*-cresol–H_2O, 7:2:2, by vol.), the emulsion was continuously vortexed for 1 min after which 1.5 ml chloroform was added and vortexing was continued for 5 min. After centrifugation of the emulsion at 12,000 *g* for 10 min, the aqueous phase was removed and precipitated at −20° with 3 vol. of 2% potassium acetate in 95% ethanol. RNA was digested for 30 min with 10 μg of electrophoretically pure DNase I in 0.01 M Tris–HCl (pH 7.2) : 0.01 M $MgCl_2$: 0.5 M NaCl, and precipitated at −20° with 3 vol. of 2% potassium acetate in 95% ethanol. Non-poly(A)- and poly(A)RNA were isolated by poly(U)Sepharose affinity chromatography [15].

Incorporation of [^{14}C]*dGR and* [^{3}H]*dUR into DNA.* Exponentially growing MDA cells (1 day after plating 5×10^5 cells) were incubated with either 0.9% NaCl, 3.7×10^{-5} M PALA, 1×10^{-6} M 5-FU, or the combination of 3.7×10^{-5} M PALA and 1×10^{-6} M 5-FU for 1, 3, 6, 12 and 24 hr. [8-^{14}C]dGR (0.5 μCi; 54.4 mCi/mmole) and [6-^{3}H]dUR (10 μCi; 24 Ci/mmole) were added to all flasks, and incubation was continued for 1 hr. At the appropriate times, cells were trypsinized and washed three times with Dulbecco's phosphate-buffered saline, and DNA was extracted from the cell pellet as described previously [18].

Measurement of UTP and [6-^{14}C]*-5-FUTP concentrations.* Exponentially growing MDA cells were incubated with either 0.9% NaCl, 1×10^{-6} M [2-^{14}C]-5-FU (1.5 μCi; 50 mCi/mmole) or 3.7×10^{-5} M PALA and 1×10^{-6}M [2-^{14}C]-5-FU for 24 hr. Cells were trypsinized and centrifuged, and the cell pellet was homogenized in 5% (w/v) ice-cold perchloric acid. Following centrifugation at 12,000 *g* for 1 min in an Eppendorf centrifuge, the supernatant fluid was neutralized with 4 N KOH, kept on ice for 20 min, and centrifuged at 10,000 *g* at 4° to remove $KClO_4$. Neutralized extracts were stored at −80° for several days until analyses were performed. UTP and [^{14}C]-5-FUTP were separated by high pressure liquid chromatography using an Altex model 420 system (Altex Instruments, Berkeley, CA) equipped with a Partisil-10 SAX column (0.45 × 25) (Whatman, Inc., Clifton, NJ). Separation was accomplished by elution for 10 min at 1 ml/min with 0.005 M KH_2PO_4, pH 2.8, after which the flow rate was increased to 2 ml/min, and a linear gradient of 0.005 M KH_2PO_4 (pH 2.8) to 0.5 M KH_2PO_4 (pH 4.8) was maintained over 20 min. Absorbance was monitored at 254 nm, and fractions of eluate were collected every 30 sec. UTP and [^{14}C]-5-FUTP co-eluted 17.9 min after the start of the gradient.

RESULTS

Cell growth. The effect of a 3-day treatment with either 5-FU or PALA on MDA cells in culture is shown in Fig. 1. The IC_{50} values of 5-FU or PALA alone were 3×10^{-6} M and 3×10^{-4} M respectively. In combination (Fig. 2), the drugs synergistically reduced cell number, at concentrations which alone had little or no effect. With 1×10^{-6} M 5-FU (IC_{20}) and 3.7×10^{-5} M PALA (IC_0), a 65 per cent reduction in cell growth was observed during a 24-hr exposure, and almost a one log reduction in cell growth was obtained with this drug regimen after a 3-day exposure. The synergistic effect of combinations of PALA

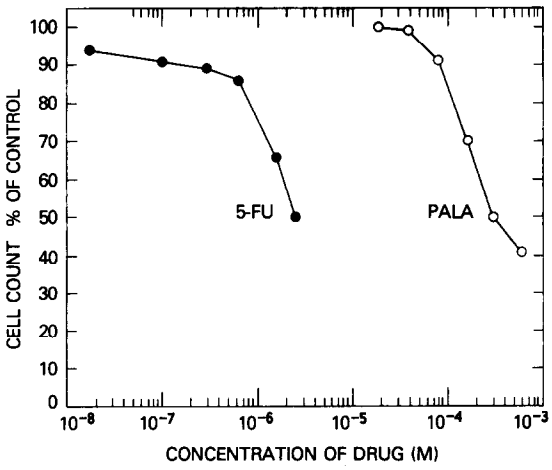

Fig. 1. Effect of 5-FU or PALA on the growth of MDA cells. Logarithmically growing cells were exposed continuously to the indicated concentration of drug, and the cell number was determined after 3 days.

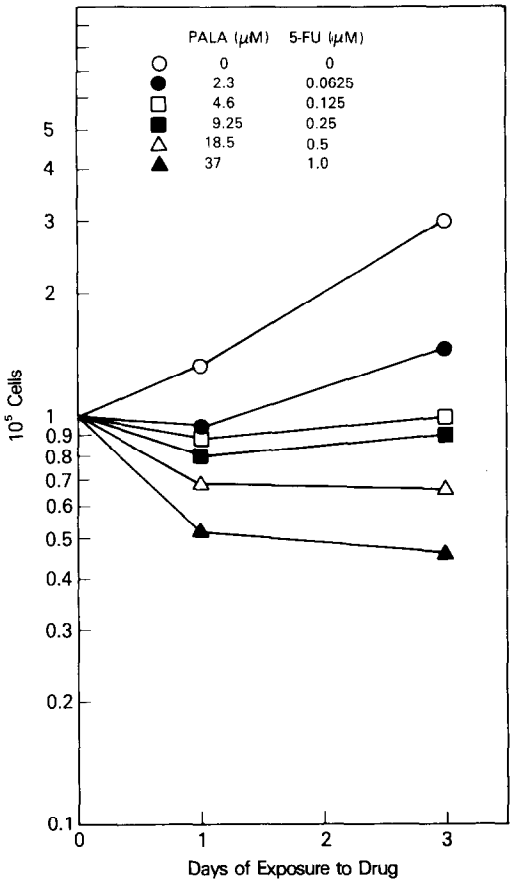

Fig. 2. Growth responses of MDA cells to combinations of 5-FU and PALA. Logarithmically growing cells were exposed continuously to the indicated concentrations of drugs, and the cell number was determined after 1 and 3 days.

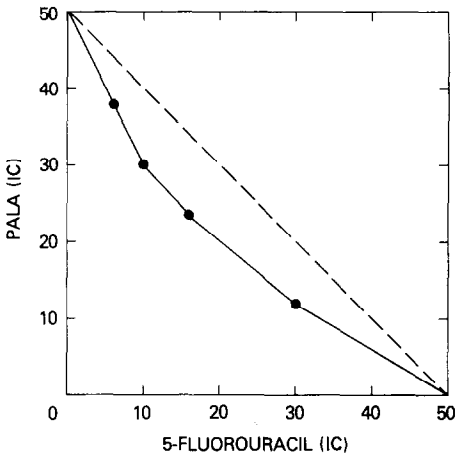

Fig. 3. Effect of combinations of 5-FU and PALA on the growth of MDA cells. The inhibitory concentrations (IC values) of combinations of 2.5×10^{-7} to 1.5×10^{-5} M 5-FU and 6×10^{-5} to 3.7×10^{-3} M PALA are plotted as an isobologram [19]. Synergism is indicated by the downward concave shape of the graph; the dashed line indicates summation of individual effects.

and 5-FU is presented in Fig. 3 as an isobologram. The downward concavity of the curve indicates a more than additive effect at the drug concentrations tested.

RNA synthesis. Measurements of the incorporation of 1×10^{-6} M [^{3}H]-5-FU into non-poly(A)RNA (rRNA and tRNA) and poly(A)RNA (mRNA) in the presence and absence of 3.7×10^{-5} M PALA are presented in Table 1. The two drugs in combination enhanced significantly the incorporation of [^{3}H]-5-FU into poly (A)RNA measured at 3 hr of treatment, whereas marked enhancement of incorporation of [^{3}H]-5-FU by PALA into both non-poly(A)- and poly(A)RNA was achieved by a 24-hr exposure to both drugs.

DNA synthesis. To assess the effects of PALA and 5-FU on DNA synthesis and thymidylate synthetase simultaneously, MDA cells were treated with the drugs alone and in combination for various periods of time, and DNA was pulse-labeled for 1 hr with [^{14}C]dGR and [^{3}H]dUR (Fig. 4). Although 1×10^{-6} M 5-FU did not reduce the incorporation of [^{14}C]dGR into DNA, approximately a 20 per cent reduction in the incorporation of [^{3}H]dUR was noted. PALA at a concentration of 3.7×10^{-5} M also did not affect [^{14}C]dGR incorporation into DNA, but it significantly elevated the incorporation of [^{3}H]dUR during 12 and 24 hr of drug exposure. The combination of 5-FU and PALA had no significant

Table 1. Effect of PALA on the incorporation of [^{3}H]-5-FU into total non-poly(A)- and poly(A)RNA*

Treatment	Incubation time (hr)	Specific radioactivity† (dpm/A_{260})	
		Non-poly(A)RNA	Poly(A)RNA
5-FU	3	15,900 ± 1,000 (100 ± 6)	7,100 ± 1,100 (100 ± 20)
5-FU + PALA	3	21,400 ± 2,200 (135 ± 15)	12,300 ± 1,500 (173 ± 22)‡
5-FU	24	22,200 ± 4,400 (100 ± 20)	24,400 ± 1,700 (100 ± 7)
5-FU + PALA	24	62,000 ± 8,400 (285 ± 31)§	39,300 ± 2,800 (164 ± 12)§

* Analyses were performed as described under Materials and Methods.

† Each result is the mean ± S.E. of four determinations; the numbers in parentheses represent percentages of 5-FU treatment alone equal to 100 per cent.

‡ Statistically significant difference ($P < 0.05$) vs 3-hr 5-FU treatment.

§ Statistically significant difference ($P < 0.01$) vs 24-hr 5-FU treatment.

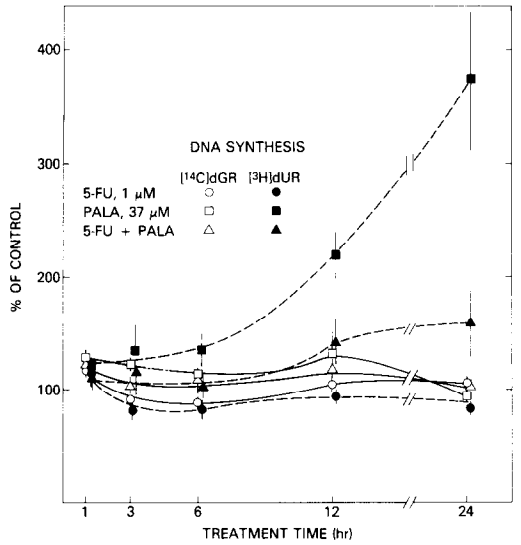

Fig. 4. Effects of 5-FU and PALA on the incorporation of [^{3}H]dUR and [^{14}C]dGR into DNA. Logarithmically growing MDA cells were exposed to 1×10^{-6} M 5-FU and 3.7×10^{-5} M PALA singly, or in combination, for the indicated times. [^{3}H]dUR and [^{14}C]dGR were then added, and incubation was continued for 1 hr. Incorporation of [^{3}H]dUR and [^{14}C]dGR into DNA was measured as described under Materials and Methods. Each value is the mean ± S.E. of six determinations. Values (dpm/A_{260}) for untreated cells for the 1-hr incorporation of [^{3}H]dUR and [^{14}C]dGR into DNA at 1, 3, 6, 12 and 25 hr after treatment were: 8,700, 15,300, 17,400, 19,200 and 18,100, and 34,000, 44,000, 44,000, 51,300 and 53,000 respectively.

effect on DNA synthesis as measured by the incorporation of either [^{14}C]dGR or [^{3}H]dUR.

UTP and [^{14}C]-5-*FUTP concentrations.* To assess whether the enhanced incorporation of 5-FU into RNA and the increased incorporation of [^{3}H]dUR into DNA by the combination of PALA and 5-FU truly reflected changes in the pyrimidine nucleotide pool, the concentrations of UTP and 5-FUTP were measured. Exponentially growing cells treated with 1×10^{-6} M [^{14}C]-5-FU and 3.7×10^{-5} M PALA for 24 hr had 75 per cent less UTP than cells treated with 5-FU alone (Table 2). Cells treated with PALA and [^{14}C]-5-FU had the same amount of [^{14}C]-5-FUTP as cells treated with 1×10^{-6} M [^{14}C]-5-FU alone; however, the proportion of [^{14}C]-5-FUTP in the total UTP + [^{14}C]-5-FUTP pool was 10 per cent with [^{14}C]-5-FU treatment alone and 33 per cent with the PALA + [^{14}C]-5-FU regimen.

DISCUSSION

We have demonstrated that the combination of 5-FU and PALA is clearly biologically synergistic in inhibiting tumor cell growth. This synergism correlates with the incorporation of 5-FU into RNA and not with its interference with thymidylate synthetase or DNA synthesis. This phenomenon can be explained by the following hypothesis. PALA, a potent inhibitor of aspartate transcarbamylase, leads to a reduction in the formation *de novo* of UMP and, hence, of UTP. Since 5-FU can also be anabolized to 5-FUTP, a reduction in the concentration of UTP would augment the percentage of 5-FUTP contained in the UTP + 5-FUTP pool and, hence, result in greater utilization of 5-FUTP in RNA synthesis. 5-FU-substituted RNA can lead to interference with its synthesis and function, as well as with cell growth [13–16, 20–22]. The reduced concentration of UTP produced by treatment with PALA, the enhanced proportion of 5-FUTP in the pyrimidine nucleotide pool, and the elevated incorporation of 5-FU into RNA confirm this supposition.

5-FU is also a potent inhibitor of thymidylate synthetase via its metabolite 5-FdUMP [13]. Therefore, the possibility existed that enhanced inhibition of this enzyme was responsible for the synergism expressed by the combination of PALA and 5-FU. It is evident, however, that DNA synthesis was not perturbed by either drug alone or in combination as assessed by the incorporation of [^{3}H]dUR via thymidylate synthetase or by the incorporation of [^{14}C]dGR (via a thymidylate synthetase-independent pathway). Enhanced incorporation of [^{3}H]dUR at 12 and 24 hr of exposure to PALA alone could be a reflection of a diminution of the dUMP pool through a reduction in the synthesis of pyrimidine nucleotides *de novo* that would result in enhanced specific radioactivity of [^{3}H]dUR. Indeed, UTP levels were reduced by 75 per cent in PALA + 5-FU-treated cells versus 5-FU treatment alone and, therefore, a similar reduction in dUMP would be expected via *de novo* pathways utilizing uridine nucleotides. The fact that DNA synthesis was not reduced (as measured by [^{14}C]dGR incorporation) indicates that thymidylate synthetase was not a limiting factor under these conditions.

Table 2. Effect of PALA on the concentrations of UTP and [^{14}C]-5-FUTP*

Treatment	UTP† (a)	[^{14}C]-5-FUTP† (b)	$\frac{(b)}{(a)+(b)} \times 100$
	(nmoles/10^6 cells)		
Saline	3.6 ± 0.3 (100)		
[^{14}C]-5-FU	3.6 ± 0.4 (100)	0.40 ± 0.10 (100)	10
[^{14}C]-5-FU + PALA	0.9 ± 0.1‡ (25)	0.45 ± 0.10 (113)	33

* Analyses were performed as described under Materials and Methods.

† Each result is the mean ± S.E. of three determinations; numbers in parentheses represent percentages of saline treatment equal to 100 per cent.

‡ Statistically significant difference ($P < 0.01$) vs 5-FU treatment.

Recent studies with experimental solid tumors of the colon, ovary, and lung have shown that the combination regimen of 5-FU and PALA is clearly more effective than either drug alone [23]. A 4-fold enhancement by PALA of the incorporation of 5-FU into total RNA of a mouse mammary tumor was related to a proportional reduction in tumor mass [24]. An enhanced incorporation of 5-fluorouridine in the presence of PALA was also observed in rat ascites hepatoma cells *in vitro* [25]. These results and those of the present study suggest a rationale for further therapeutic trials with this drug combination.

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