Cancer Chemother Pharmacol (1982) 8: 87–91

Cancer Chemotherapy and Pharmacology

Modulation of 5-FU Metabolism in Human MCF-7 Breast Carcinoma Cells

Pierre P. Major*, E. Michael Egan, Lalla Sargent, and Donald W. Kufe

Divisions of Pharmacology and Medicine, Sidney Farber Cancer Institute – Harvard Medical School, Boston, Massachusetts 02115, USA

Summary. *We have previously demonstrated a highly significant relationship ($P < 0.0001$) between the incorporation of 5-fluorouracil (5-FU) into total cellular RNA and loss of clonogenic survival of the human MCF-7 breast carcinoma cell line. The present studies explore the applicability of this relationship to MCF-7 cells exposed to 5-FU and modulting agents such as PALA, MTX, and MMPR. PALA treatment produces a minimal increase in the absolute amount of 5-FU incorporated into total cellular RNA, but it results in a three-fold enhancement of the $[^3H]FU/^{32}P$ ratio, which measures 5-FU misincorporation into newly synthesized RNA. MTX and MMPR increase intracellular PRPP levels up to four-fold; nevertheless these agents result in only minimal increases in absolute (5-FU)RNA formation. In contrast, the relative incorporation of 5-FU into newly synthesized RNA of MTX- or MMPR-treated cells is increased 2.5-fold. The combination of PALA/MMPR results in a two-fold absolute increase in (5-FU)RNA formation and a nine-fold enhancement of the $[^3H]FU/^{32}P$ ratio. Combinations of modulating agents with 5-FU result in more than additive decreases in MCF-7 clonogenic survival. The relationship between 5-FU incorporation into RNA and loss of clonogenic survival was highly significant ($P < 0.0002$) when corrected for newly synthesized RNA, while the correlation with absolute amounts of (5-FU)RNA formation was less significant ($P < 0.05$). These studies demonstrate that the relationship previously established between (5-FU)RNA formation and loss of clonogenic survival should be corrected for the amount of newly synthesized RNA when 5-FU is combined with modulating agents that alter rates of RNA synthesis.*

Reprint requests should be addressed to D. W. Kufe

* Fellow of the Medical Research Council of Canada

Introduction

5-Fluorouracil (5-FU) is an effective agent in the treatment of human breast carcinoma [1–4]. We have recently demonstrated a highly significant relationship ($P < 0.0001$) between the incorporation of 5-FU in total cellular RNA and loss of clonogenic survival of the MCF-7 human breast carcinoma cell line [6]. The extent of 5-FU incorporation into RNA was concentration- and time-dependent. Further, the cytotoxicity associated with 5-FU exposure was not S-phase-specific, and identical results were obtained when thymidine was employed to by-pass the block of thymidylate synthetase and reverse inhibition of DNA synthesis. These studies suggest that the incorporation of 5-FU into RNA is a major mechanism of cytotoxic action in this human cell line.

Various approaches have been employed to enhance the incorporation of 5-FU into cellular RNA [2, 5]. *N*-(Phosphonacetyl)-L-aspartate (PALA) reduces uracil nucleotide pools [10] and increases (5-FU)RNA formation [7, 9, 11]. This effect has been associated with increased cell lethality. However, no attempt has been made to establish a correlation between increased incorporation of 5-FU into RNA and cytotoxicity. The incorporation of 5-FU into RNA has also been enhanced by increasing intracellular levels of phosphoribosyl-l-pyrophosphate (PRPP) with either methotrexate (MTX) or 6-methylmercaptopurine riboside (MMPR) [2, 5]. An increase in (5-FU)RNA formation with these agents is also associated with increased cell kill [2, 5].

Agents that modulate 5-FU metabolism may also alter rates of RNA synthesis. Thus, the correlation between 5-FU incorporation in MCF-7 RNA and loss of clonogenic survival may vary in dependence upon the degree of inhibition of RNA synthesis. Results presented here demonstrate that modulating agents such as PALA, MTX, and MMPR increase the

0344-5704/82/0008/0087/$ 01.00

incorporation of 5-FU in newly synthesized MCF-7 cellular RNA and that this effect is associated with increased loss of clonogenic survival. The absolute amount of 5-FU incorporated into RNA increases with cell kill; however, a more significant relationship is obtained when the amount of 5-FU incorporated into newly synthesized RNA is correlated with cytotoxicity. These findings support the conclusion that the incorporation of 5-FU in RNA is a relevant parameter in evaluation of the antitumor activity of 5-FU used in combination with a modulating agent.

Materials and Methods

Cell Culture. The MCF-7 cells (Michigan Cancer Foundation, Detroit, MI, USA) were grown in MEM (Grand Island Biological Company, Grand Island, NY, USA) supplemented with 10% heat-inactivated and dialyzed fetal calf serum (FCS), 1% L-glutamine, 100 μg penicillin/ml, and 100 units streptomycin/ml.

The MCF-7 cells were in log growth phase at the time of drug exposures. PALA (Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA) was employed at concentrations of 0.01, 0.10, and 1.00 mg/ml. MTX (Lederle Laboratories, Pearl River, NY, USA) and MMPR (Sigma Chemical Company, St Louis, MO, USA) were prepared at concentrations of 10^{-4} *M*, sterilized by millipore filtration, and used at concentrations ranging from 10^{-5} *M* to10^{-7} *M*.

Measurement of [^{3}H]FU and ^{32}P Incorporation into MCF-7 Nucleic Acids. MCF-7 cells at a density of 1×10^6 cells per 100 × 20 mm tissue culture dish (Falcon Plastics, Oxnard, CA, USA) were washed and incubated in 5 ml medium with 10^{-5} *M* [^{3}H]FU (Amersham, Arlington Heights, IL, USA; specific activity, 2, 8 Ci/mmole) and 10 μCi ^{32}P/ml (New England Nuclear, Boston, MA; carrier-free) for 3 h at 37° C. The nucleic acids were then purified and analyzed by cesium sulfate gradient centrifugation [7].

Measurement of Intracellular PRPP Levels. The PRPP levels were measured by a previously described technique [8].

Clonogenic Survival of MCF-7 Cells. The effects of PALA, MTX, and MMPR alone and in combination with 5-FU were studied on cells in logarithmic growth phase. 5-FU (Roche Laboratories, Nutley, NJ, USA) exposures were performed at 10^{-5} *M* for a period of 3 h. Following drug exposure, the cells were harvested by mild trypsinization, washed, and resuspended in culture dishes (100 × 20 mm) at a concentration of 10^3 cells per plate. Viability was determined after 21 days by scoring colonies greater than 50 cells. The percentage of viable cells was determined by the ratio of colonies formed by 5-FU-treated cells to colonies formed by untreated cells × 100. Cloning efficiency of the untreated MCF-7 cells ranged between 20% and 25%.

Results

The effect of PALA on incorporation of [^{3}H]FU into RNA is illustrated in Fig. 1. In these experiments, ^{32}P incorporation into RNA is used to monitor relative rates of RNA synthesis, and the ratio of [^{3}H]FU/^{32}P in the RNA fraction permits a comparison of the relative incorporation of [^{3}H]FU into newly synthesized RNA [11]. The [^{3}H]FU/^{32}P ratio increases following exposure to 0.01 mg PALA/ml for 19 h and reaches a plateau at concentrations of 1.0 mg/ml.

The MCF-7 cells were also exposed to MTX and MMPR at concentrations ranging from 10^{-5} *M* to 10^{-7} *M* for periods of up to 9 h. Increases in PRPP were maximal at a concentration of 1 μ*M* for each agent, achieving levels approximately five times the

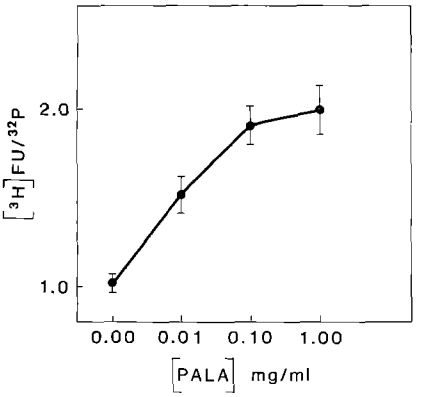

Fig. 1. Effect of PALA on the incorporation of [^{3}H]FU into newly synthesized MCF-7 RNA. Cells were exposed to varying concentrations of PALA for 19 h/prior to the addition of 10^{-5} *M* [^{3}H]FU and 10 μCi/ml ^{32}P for an additional 3 h. The ratio of [^{3}H] FU to ^{32}P in RNA for cells not exposed to PALA was 2.0. Results are expressed as the mean ± standard deviation for three determinations, normalized to the control ratio

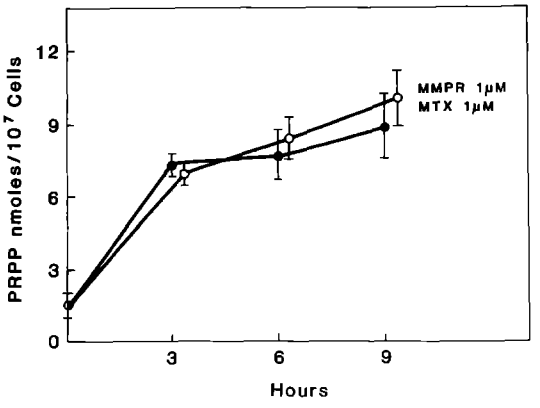

Fig. 2. Effect of MMPR and MTX on PRPP levels in MCF-7 cells. Cells were exposed to 10^{-6} *M* MTX or MMPR for 3, 6, or 9 h and assayed for PRPP as previously described [8]. Results are expressed as the mean ± standard deviation for at least three determinations

control level after 3–9 h of exposure (Fig. 2). The PRPP levels following exposure to MTX and MMPR alone and in combination with PALA are listed in Table 1.

The effects of PALA (0.1 mg/ml, 19 h), MMPR (1 μ*M*, 6 h), and the combination of these agents on the incorporation of [^{3}H]FU and ^{32}P into RNA were monitored by Cs_2SO_4 gradient analysis (Fig. 3). In these experiments, PALA alone resulted in approximately a three-fold increase in the [^{3}H]FU/^{32}P ratio, while the absolute amount of [^{3}H]FU incorporated into RNA was similar to that incorporated for control cells. Exposure to MMPR also resulted in minimal increase in [^{3}H]FU incorporation, while the [^{3}H]/^{32}P ratio increased by up to 2.5 times that of control. The combination of PALA/MMPR resulted in a nine-fold

Table 1. Effect of various drug combinations on MCF-7 intracellular PRPP levels, incorporation of [^{3}H]FU into RNA, and clonogenic survival[a]

Drug combination	PRPP	Amount of 5-FU Incorporated into RNA (pmoles)	Ratio ([^{3}H]FU/^{32}P)	Percent survival	
				−5-FU	+5-FU (10^{-5}*M*)
Control	1.0	5.85 ± 0.01	1.0	100	95.6 ± 5.7
MTX	3.2 ± 0.34	6.76 ± 1.15	1.7 ± 0.1	78.0 ± 2.6	58.0 ± 4.8
MMPR	4.3 ± 0.65	6.86 ± 0.17	2.5 ± 0.5	98.0 ± 3.4	61.5 ± 12.0
MTX/MMPR	1.7 ± 0.15	6.22 ± 0.11	2.6 ± 0.2	64.0 ± 2.4	51.0 ± 7.8
PALA	2.9 ± 0.19	6.78 ± 1.31	3.4 ± 1.5	100.0 ± 2.8	37.0 ± 9.3
PALA/MTX	3.8 ± 0.60	7.56 ± 0.81	7.2 ± 1.0	48.0 ± 2.8	17.2 ± 4.9
PALA/MMPR	7.4 ± 0.23	11.25 ± 3.89	9.0 ± 1.9	65.3 ± 6.0	20.2 ± 7.1
PALA/MTX/MMPR	2.6 ± 0.96	10.74 ± 3.14	9.6 ± 2.8	27.0 ± 7.1	11.7 ± 4.0

[a] Treatment with the various modulating agents was as follows: PALA, 0.01 mg/ml for 19 h; MTX, 1 μ*M* for 6 h; MMPR, 1 μ*M* for 6 h. 5-FU was then added at 10^{-5} *M* for an additional 3 h of drug exposure. The results are expressed as the mean ± standard deviation for at least three determinations. Control PRPP level was 1.5 nmoles/10^7 cells and the control [^{3}H]FU/^{32}P ratio was 2.6. The absolute incorporation of 5-FU in RNA is expressed as pmoles/2 × 10^5 cells

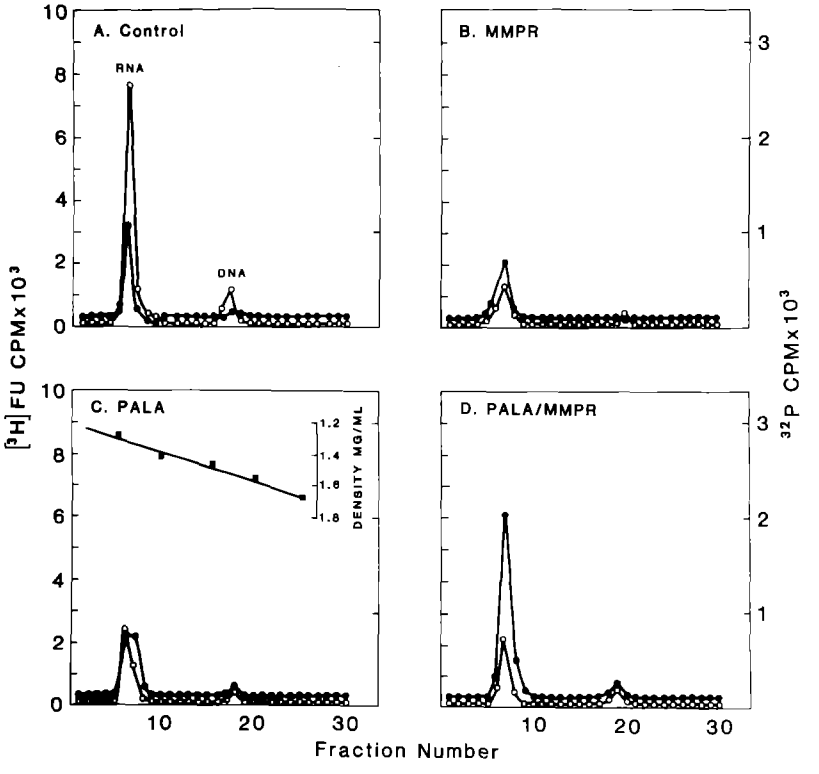

Fig. 3A–D. Incorporation of [^{3}H]FU (○) and ^{32}P (●) into MCF-7 nucleic acids after treatment with PALA, MMPR, or PALA/MMPR, as described in the footnote to Table 1. The total cellular nucleic acids was purified and analyzed by Cs_2SO_4 gradient-density centrifugation [7]

increase in the [^{3}H]FU/^{32}P ratio, while the absolute amount of [^{3}H]FU incorporated was less than twice that observed for the control. The results obtained with these drugs used alone and in combination are listed in Table 1.

The effects of 5-FU alone and in various combinations with PALA, MTX, and MMPR on the clonogenic survival of MCF-7 cells are also listed in Table 1. Exposure to 10^{-5} *M* 5-FU for 3 h resulted in little, if any, cytotoxicity, while combinations with PALA, MTX, and MMPR all yielded greater than additive effects.

The relationship between the loss of clonogenic survival following combination drug treatment and either the absolute amount of 5-FU incorporated in RNA or the ratio of [^{3}H]FU/^{32}P was studied by probit analysis [3]. The relationship between the log picomoles of 5-FU incorporated in RNA and loss of clonogenic survival (coefficient [R] = −0.767, $P < 0.05$) was not as significant as that obtained between the log [^{3}H]FU/^{32}P ratio and the loss of clonogenic survival (coefficient [R] = 0.953, $P < 0.0002$).

Discussion

We have previously demonstrated a highly significant relationship between the incorporation of 5-FU in RNA and loss of clonogenic survival of the MCF-7 human breast carcinoma line [6]. These studies demonstrated a concentration (C)- and time (T)-dependent incorporation of 5-FU into RNA and showed that the loss of clonogenic survival also followed a CXT relationship. The dose-response relationship was demonstrated over a drug concentration range between 10^{-6} and 10^{-4} *M*. The present studies were undertaken to determine whether this relationship was maintained in the presence of a modulating agent. Agents such as PALA, MTX, and MMPR have been employed in the present studies, based upon our previous work which demonstrated that these drugs modulate the metabolism of 5-FU and enhance the formation of (5-FU)RNA [5].

The results in the present study demonstrate that PALA, MTX, and MMPR result in increased intracellular levels of PRPP in the MCF-7 cells. Further, each of these agents results in little if any significant increase in the absolute amount of 5-FU incorporated into RNA. The combination of PALA/MMPR resulted in the greatest absolute increases in (5-FU)RNA formation, which was nearly twice that of control. However, these modulating agents inhibit RNA synthesis and therefore the absolute amount of 5-FU incorporated would not reflect the frequency of 5-FU substitution for uracil.

The relative amount of 5-FU incorporated in newly synthesized RNA can be monitored by simultaneous labeling with ^{32}P and by calculation of the [^{3}H]FU/^{32}P ratio [11]. The correction for relative rates of RNA synthesis results in enhanced 5-FU incorporation of up to 3.4-fold with PALA, MTX, or MMPR. Further, the PALA/MMPR combination increases the [^{3}H]FU/^{32}P ratio nine-fold. The results demonstrate that in the presence of modulating agents, the [^{3}H]FU/^{32}P ratio correlates better with loss of MCF-7 clonogenic survival than does the absolute amount of 5-FU incorporated in RNA. The [^{3}H]FU/^{32}P ratio, therefore may provide a relevant in vitro parameter to monitor the effects of other modulating agents on the metabolism of 5-FU.

These studies thus demonstrate that several modulating agents can be employed to increase the incorporation of 5-FU into newly synthesized RNA and decrease the clonogenic survival of MCF-7 cells. However, despite the use of optimal dose scheduling of these modulating agents to maximize the increase in the frequency of 5-FU misincorporation in RNA, the increase in cytotoxicity observed in cells treated with combinations of 5-FU and modulating agents was small compared with the cytotoxic effects obtained by treating with modulating agents alone. It is unclear whether such increases in 5-FU incorporation will result in clinically significant responses, and in fact trials with the combination PALA/5-FU have thus far demonstrated activity similar to previous experience with single agent 5-FU [12]. It will now be necessary to expand these initial phase I–II studies and to include MTX or MMPR in the drug combinations to increase intracellular levels of PRPP and perhaps obtain more meaningful clinical results.

Acknowledgements. The authors appreciate the excellent technical assistance of David Herrick. Miss Carolyn Klebart's secretarial support is gratefully acknowledged.

This work was supported by grant CA-28488 from the National Cancer Institute and by a Junior Faculty Research Award from the American Cancer Society (DWK).

References

1. Ansfield F, Klotz J, Nealon T, Ramirez G, Minton J, Hill G, Wilson W, Hugh D (1977) A phase III study comparing the clinical utility of four regimens of 5-fluorouracil. Cancer 39: 34–40
2. Cadman E, Heimer R, Davis L (1979) Enhanced 5-fluorouracil nucleotide formation following methotrexate: biochemical explanation for drug synergism. Science 205: 1135–1137

3. Cox D (1969) Analysis of binary data. Barnes & Noble, New York, pp 76–99
4. Horton J, Olson KB, Sullivan J, Reily C, Schnider B (1970) 5-Fluorouracil in cancer: an improved regimen. Ann Intern Med 73: 897–900
5. Kufe DW, Egan EM (1981) Enhancement of 5-fluorouracil incorporation into human lymphoblast ribonucleic acid. Biochem Pharmacol 30: 129–133
6. Kufe DW, Major PP (1981) 5-FU incorporation into human breast carcinoma correlates with cytotoxicity. J Biol Chem 256: 9802–9805
7. Kufe DW, Major PP, Egan EM, Beardsley GP (1980) Correlation with incorporation of ara-C into DNA. J Biol Chem 255: 8997–9000
8. Kornberg A, Lieberman I, Simms E (1955) Enzymatic synthesis of 5-phosphoribosylpyrophosphate. J Biol Chem 215: 389–402
9. Martin D, Stolfi R, Sawyer R, Nayak S, Speigelman S, Young C, Woodcock T (1980) An overview of thymidine. Cancer 45: 1117–1128
10. Moyer J, Handschumacher R (1979) Selective inhibition of pyrimidine synthesis and depletion of nucleotide pools by *N*-(phosphonacetyl)-L-aspartate. Cancer Res 39: 3089–3094
11. Speigelman S, Nayak R, Sawyer R, Stolfi R, Martin D (1980) Potentiation of the anti-tumor activity of 5-FU by thymidine and its correlation with the formation of (5-FU)RNA. Cancer 45: 1129–1134
12. Weiss GR, Ervin TJ, Meshad MW, Kufe DW (to be published) A phase II trial of combination therapy with continuous infusion *N*-(phosphonacetyl)-L-aspartate and bolus infusion 5-fluorouracil. Cancer Treat Rep

Received August 3/Accepted September 15, 1981