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Effects of 5-Fluorouracil and 2α-Methyldihydrotestosterone Propionate on the Growth of Human Breast Carcinoma MCF-7 *in Vitro**

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Abstract—*5-Fluorouracil (5-FU) and 2α-methyldihydrotestosterone propionate (MDTP) have effectively induced complete regressions of induced rat mammary carcinomas; in combination, regressions were additive and synergistic. Present aims were to determine whether similar antitumor effects were obtainable with a human mammary carcinoma, MCF-7, and to affirm the synergism of 5-FU and MDTP. After incubation* in vitro *for* 3 days *and exposure to drug for another* 2 days, *cell counts and/or determinations of total cell protein revealed growth inhibitions of* 16–87% *by 5-FU at* 130–1300 μg/ml *and* 16–94% *by MDTP at* 0.36–360.5 μg/ml. *Combinations of 5-FU and MDTP at the same inhibitory doses* (ID) *yielded approximately additive growth inhibitions. Algebraic and geometric (isobole) methods of analyses showed that these inhibitions were additive or synergistic, depending on the iso-effective dose used. Precursor incorporation into macromolecules also showed approximately additive effects for MCF-7 cells treated with 5-FU and MDTP, each at* ID_{15}. *These data demonstrate significant additive growth-inhibitory activity of 5-FU and MDTP in combination against MCF-7* in vitro, *thus affirming their antitumor effects* in vivo.

INTRODUCTION

CHEMOTHERAPY and hormone therapy act by different mechanisms and have different spectra of toxicities. Since both treatments are effective in clinical breast cancer, although to different degrees [1, 2], these characteristics make their combination potentially advantageous over therapy with each alone.

In a previous paper Teller *et al.* [3] reported effective antitumor activity against DMBA-induced rat mammary carcinomas by combinations of 5-FU and the androgen analog 2α-methyldihydrotestosterone propionate (MDTP). Growth inhibition and complete and partial regression of well-established tumors in rats occurred during and after therapy with each drug singly. When both agents were administered concurrently, the resulting antitumor effects were additive and synergistic, depending on the dose.

It was important to determine whether similar effective antitumor activity could be obtained in the case of human breast cancer. For this purpose human mammary carcinoma tissue culture cell line MCF-7 was selected. The DMBA-induced rat mammary carcinoma responds *in vivo* to hormone therapy, both additive and ablative [3–7]. The reported presence of receptors for various hormones in MCF-7 cells [8] suggested the possibility that this tumor would respond likewise to hormonal therapy. This paper reports the effects of 5-FU and MDTP, singly and in combination, on the growth of MCF-7 *in vitro* measured in terms of cell numbers and total

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protein, and on precursor incorporation into TCA-insoluble cellular macromolecules.

MATERIALS AND METHODS

Tumor culture

Human breast carcinoma cell line MCF-7 free of mycoplasma was generously provided by Dr. J. Fogh of the Sloan-Kettering Institute. The human and mammary tumor characteristics have been well substantiated [9, 10]. MCF-7 was maintained as monolayer cultures in T-25 and T-75 plastic flasks (Falcon) containing Eagle's MEM in HBSS (Gibco, Grand Island, NY) supplemented with L-glutamine (0.6 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), insulin (0.01 U/ml) and 10% calf serum (CS) (all purchased from Gibco). This culture fluid was designated medium A. The cells were incubated at 37°C in tightly closed flasks, in which growth was quantitatively similar to that in flasks maintained in a 5% CO_2–95% air mixture in a humidified atmosphere. The growth medium was renewed at 48-hr intervals or less. Cell growth reached approximately 75% confluency in about 7 days, at which time the cells were passaged. The cell layer was washed twice with saline A [11] and detached by incubation in trypsin (0.05%)–EDTA (0.02%) in HBSS (Gibco). After dilution with medium A and centrifugation at 125 *g* for 5 min, a cell suspension in medium A was prepared and distributed so that a T-25 flask contained 1×10^6 MCF cells/4 ml medium.

Measurements of MCF-7 cell growth are presented in terms of cell population or as total cell protein.

Therapy

For therapy experiments, MCF cells in the log phase of growth, usually day 7, were harvested, dispersed in duplicate or triplicate flasks per treatment at a density of 1×10^6 cells in 4 ml medium A and incubated at 37°C. At 48 hr the growth medium was replenished with medium B, similar to medium A but with CS reduced to 2%. At 72 hr the medium was replaced by medium B containing 5-FU and/or MDTP (both generously supplied by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, Bethesda, MD). The treated cells were washed and harvested 48 hr later. Effects of drugs were determined by cell counts in a hemocytometer and/or determinations of total protein by the method of Oyama and Eagle [12]. This procedure was designated as the standard protocol. 5-FU was dissolved in medium B; MDTP was prepared as a 1000-fold concentrate in ethanol and diluted with medium B so that the final concentration of ethanol was always <0.1%.

Precursor incorporation

Labeled precursor incorporation into TCA-insoluble cellular macromolecules was determined by a modification of Osborne *et al.* [13]. Groups of flasks seeded with MCF-7 were prepared in duplicate or triplicate per treatment. The cells were incubated in 5-FU and/or MDTP as for standard therapy. During the last 2 hr of incubation the cells were pulsed with [2-^{14}C]-thymidine (56 mCi/mmol), [2-^{14}C]-uridine (55 mCi/mmol) (Moravek Biochemicals, City of Industry, CA) or [carbonyl-^{14}C]-leucine (54 mCi/mmol) (Schwarz Mann, Orangeburg, NY), 0.2 μCi/ml medium final concentration. Zero-time samples were processed immediately to determine non-specific adsorption of label. These values were consistently low (28.2 ± 2.7 dpm/flask, mean of 4 experiments) and were subtracted from experimental counts.

At the end of the 2-hr pulse the cells were washed with cold Dulbecco's phosphate-buffered saline, suspended by treatment with trypsin–EDTA and precipitated with 10% TCA. The precipitate was collected on a glass fiber filter (No. 934AH, Reeve Angel, Clifton, NJ) to which 2 cold 5% TCA rinses were added. The precipitate was then washed 3 times with cold 5% TCA and the radioactivity of the filter in 4 ml Biofluor (New England Nuclear, Boston, MA) was evaluated in a liquid scintillation spectrophotometer (Packard Tri-Carb Spectrometer, Model 544). Labeled precursor incorporation was expressed as dpm/μg protein. Determinations of total cell protein/flask were carried out for duplicate flasks per treatment.

Sterility was maintained throughout, except for the last 2 hr in those experiments requiring pulse labeling.

Statistical significance was determined by Student's *t* test.

Synergism, additiveness and antagonism of 5-FU and MDTP in combination were determined as described by Berenbaum [14]. In the algebraic method, the following formula was used. For any ID (ID_x)

$$\frac{\text{dose of } A}{A_a} + \frac{\text{dose of } B}{B_a} = (<1, 1, >1),$$

where A and B are different drugs, 'dose of A' in combination with 'dose of B' produces ID_x, and A_a and B_a are doses of the drugs which produce ID_x when used alone. The drugs are synergistic, additive or antagonistic when the equation yields, respectively, <1, 1, >1. Isobolograms were prepared as described by Berenbaum [14].

RESULTS

Cell growth

The protocols for therapy experiments and for measuring precursor incorporation comprise cell growth in medium A containing 10% CS for 48 hr followed by growth in medium B containing 2% CS. Cell proliferation under this protocol was compared with that in medium A throughout to determine the effect of serum concentration on growth of MCF-7. Two groups of T-25 flasks containing medium A were seeded with 1×10^6 MCF-7 cells and incubated for 2 days, after which one group was continued on medium A and the other on medium B for the remainder of the experiment. Cells in flasks were harvested in triplicate at various intervals of time and total cell protein/flask determined. Results are illustrated in Fig. 1.

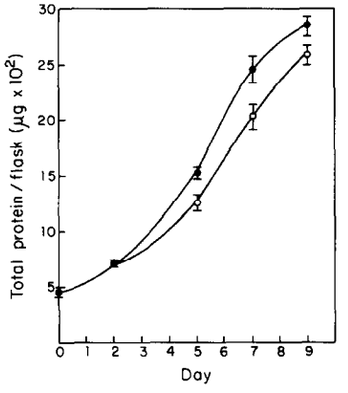

Fig. 1. Growth of MCF-7 in medium with 10% calf serum continuously ●——● compared with 2% calf serum O——O beginning on day 3. Cells were plated in flasks at a density of 1×10^6 cells in 4 ml medium which was replenished at the end of days 2, 5 and 7. Cells were harvested at the end of days 2, 5, 7 and 9 and total cell protein/flask determined. Results are expressed as means ± S.D. (bars).

The growth rate of MCF-7 decreased when the concentration of CS in the medium was reduced to 2%, so that at the end of day 5 the amount of cell protein/flask (1245 ± 74.8 μg) was about 19% less than that in medium A. During the next 4 days the rate of cell growth in medium B approximately paralleled that in medium A, but the amount of cell protein/flask on day 9 (2575 ± 90.5 μg) still differed from that in medium A by 9.25%. Amount of protein/flask in the 2 groups differed significantly ($P < 0.001$) on days 5 and 7 and also ($P < 0.005$) on day 9.

Dose response to 5-FU and MDTP

The effects of 5-FU and MDTP on the growth of MCF-7 were determined to provide a guideline for selecting inhibitory dose (ID) levels for combination therapy. The standard protocol was used in these experiments, and duplicate or triplicate flasks were used for each dose of compound. Control flask media after 48 hr contained 0.1% ethanol, the solvent for MDTP. This concentration of ethanol had no effect on cell growth; the amount of cell protein/flask was approximately the same as in control media without ethanol.

Preliminary experiments showed that growth-inhibitory effects against MCF-7 were obtainable with 5-FU at doses between 130 and 1300 μg/ml. At the latter dose the inhibitory effect approached a plateau. With MDTP no growth-inhibitory effect occurred below 0.36 μg/ml, but the percentage inhibition approached a maximum at 360.5 μg/ml. Figure 2 shows the effects of each drug on growth of MCF-7 at the doses indicated.

5-FU at concentrations of 130, 162.5, 325, 650 and 1300 μg/ml inhibited the growth of MCF-7 16.3 ± 7.9, 46.8 ± 1.9, 76.0 ± 2.3, 85.2 ± 0.4 and 87.0 ± 3.1% respectively, the means of pools of 3–7 experiments at each dose level. No inhibition was seen at 13 μg/ml. With respect to MDTP, doses of 0.36, 3.61 and 36.1 μg/ml inhibited growth 16.1 ± 2.8, 44.4 ± 4.2 and 87.3 ± 1.4% for pools of 3–4 experiments at each dose level. The inhibitory effects at the highest and lowest doses tested were 94.2 ± 0.9% at 360.5 μg/ml and 2.2 ± 3.3% at 0.36 μg/ml (not shown).

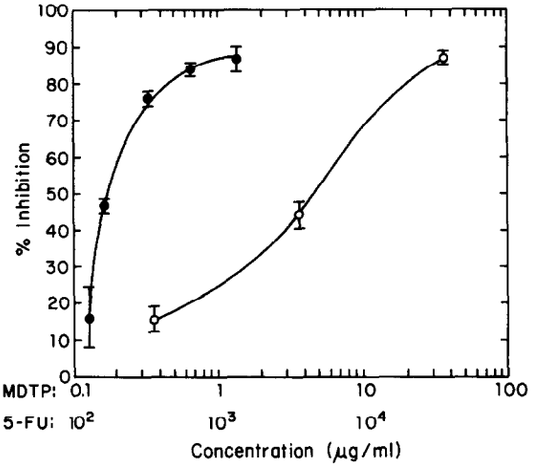

Fig. 2. Repsonse of MCF-7 to various concentrations of 5-FU (●----●) and MDTP (O——O) in vitro. Flasks were plated with 1×10^6 cells in medium A (10% calf serum), which was changed to medium B (2% calf serum) after 48 hr. The flasks were replenished at the end of day 3 with fresh medium B containing 5-FU and MDTP. The cells were harvested 2 days later and total protein determined. Each point is the mean ± S.D. (bars) of duplicate or triplicate flasks at each drug concentration expressed as percentage inhibition based on total protein in control flask cultures.

Combination therapy

The effects of 5-FU and MDTP in concurrent combinations on the growth of MCF-7 cells *in*

vitro were ascertained. Doses of these drugs expected to produce growth inhibitions of approximately 35, 25 and 15% (ID_{35}, ID_{25}, ID_{15}) were derived from the dose-response curves shown in Fig. 2. The concentrations of drugs in the medium for producing ID_{35}, ID_{25} and ID_{15} were, for 5-FU: 142.5, 135.0 and 127.5 μg/ml respectively; and for MDTP: 2.1, 1.0 and 0.35 μg/ml respectively. Experiments were carried out using duplicate flasks per treatment, and growth of MCF-7 was measured in terms of number of cells/flask harvested at the end of day 5. Results are illustrated in Fig. 3.

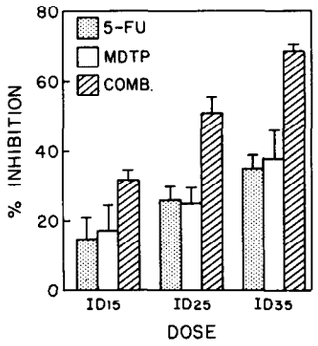

Fig. 3. Effects of various inhibitory doses (ID) *of 5-FU and MDTP, singly and in combination, on proliferation of MCF-7* in vitro. *MCF-7 cells were cultured and harvested as described in Fig. 2 and the number of cells per flask was determined. Means* (±S.D.) *of cell counts of duplicate flasks are expressed as percentage inhibition based on mean number of cells in control flasks.*

At ID_{15} the growth of MCF-7 was inhibited 14.4 ± 6.48% by 5-FU and 16.4 ± 6.96% by MDTP. In combination at the same doses the inhibition was 31.3 ± 2.43%. The combination of each drug at ID_{25} and ID_{35} likewise yielded approximately double the percentage of inhibition induced by each alone (Fig. 3). The growth-inhibitory effects of the combinations are significantly different from the effects induced by each drug alone ($P < 0.01$).

The number of MCF-7 cells diminished during incubation in medium containing both 5-FU and MDTP, each at ID_{35}. Cell death between day 3 and harvest on day 5 was 57.6%. For combinations of each drug at ID_{25} and ID_{15} cell deaths were, respectively, 32.8 and 6.3%. Except for the latter combination, the data suggest a direct effect of the drug combinations on MCF-7, resulting in cell death.

Analysis of drug interaction

The algebraic and geometric (isobole) methods described by Berenbaum [14] were used to analyze the effectiveness of combinations of drugs with the effectiveness of their constituents. For both methods the required data consist of doses of 2 drugs which singly and in combination induce the same degree of growth inhibition. The doses which induce 30, 50 and 70% growth inhibition when used singly were derived from the dose-response curves of Fig. 2. They were, for 5-FU and MDTP respectively, ID_{30}: 137.5 and 1.5 μg/ml; ID_{50}: 170 and 4.6 μg/ml; and ID_{70}: 270 and 11 μg/ml. The doses of 5-FU and MDTP inducing growth inhibitions of 30, 50 and 70% when in combination were those used in the previous combination therapy experiment (Fig. 3).

Substitution of these doses in the equation described in Materials and Methods enabled the determination of synergy, additivism and antagonism for 5-FU and MDTP in combination. Three inhibition levels were analyzed:

$$\text{for } ID_{30}, \quad \frac{127.5}{137.5} + \frac{0.35}{1.50} = 1.1 \ (\sim 1, \text{ borderline additive});$$

$$\text{for } ID_{50}, \quad \frac{135.0}{170.0} + \frac{1.0}{4.6} = 1.0 \ (=1, \text{ additive});$$

$$\text{for } ID_{70}, \quad \frac{142.5}{270.0} + \frac{2.1}{11.0} = 0.7 \ (<1, \text{ synergism}).$$

Identical conclusions were reached by analysis of the isoboles prepared from these doses and illustrated in Fig. 4.

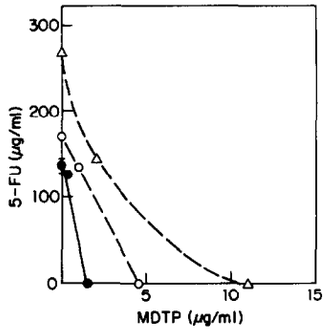

Fig. 4. Isobolograms showing additive and synergistic effects against MCF-7 by combinations of 5-FU and MDTP. For doses of 5-FU and MDTP yielding growth-inhibitions of 70% (ID_{70}) *singly and in combination the curve is concave, indicating synergism* [1]. *For doses yielding* ID_{50} (O----O) *and* ID_{30} (●——●) *the plots are straight lines, indicating additivism.*

Precursor incorporation

In the previous experiments the effects of 5-Fu and MDTP on cell growth and inhibition were measured in terms of surviving cell numbers or total cell protein. It was desirable, for additional

confirmation, to determine whether macromolecular syntheses were inhibited likewise.

Incorporation of radiolabeled precursors into acid-soluble macromolecules in MCF-7 cells, as affected by 5-FU and MDTP, was measured. The protocol used was as described in Materials and Methods. Determinations were also made of total cell protein of duplicate cultures growing in the absence of precursor. Percentage inhibition of precursor incorporation, measured as dpm/μg protein, was calculated based on control values.

Concentrations of drugs used in these experiments were: 5-FU, 1300 (ID_{85}), 137.5 (ID_{30}) and 127.5 μg/ml (ID_{15}); MDTP, 1.5 (ID_{30}) and 0.35 μg/ml (ID_{15}). The highest concentration of 5-FU was included to test whether an anomalous effect on precursor incorporation might take place, compared to effects at lower doses.

Table 1 shows that, in general, incorporation of the labeled precursors, thymidine, uridine and leucine, into, presumably, DNA, RNA and protein, respectively, were all inhibited roughly to the same degree by the same dose of compound. For example, 5-FU at ID_{30} inhibited all precursor incorporations into cellular macromolecules by 27.3–32.0%, and inhibitions with MDTP at ID_{30} were 26.5–34.9%. Inhibition of protein synthesis followed closely that of precursor incorporation: for 5-FU at ID_{30} inhibition of total protein ranged between 27.7 and 30.9% and for MDTP at ID_{30}, between 26.0 and 31.1%. In addition, the data, including that for 5-FU at ID_{85}, show a dose–response relationship between concentrations of 5-FU and MDTP in the medium and precursor incorporation.

At ID_{15} measurements of precursor incorporation and total cell protein show inhibitions of roughly 15% for each compound separately. In combination the resulting inhibition was roughly double that at ID_{15}. Table 1 also shows that the inhibition provided by combining the 2 drugs, each at ID_{15}, may also be obtained by using each drug separately at ID_{30}. However, there may be an advantage in using combinations of lower doses of each compound in that decreased host toxicity may result with no diminution of antitumor effect.

DISCUSSION

Our specific aims were to determine whether the antitumor effects produced by combinations of 5-FU and MDTP against rat mammary carcinomas were demonstrable with a human mammary cancer, specifically MCF-7 carcinoma, and to affirm the additive and synergistic effects of the agents in combination. These experiments were carried out *in vitro* rather than *in vivo* to permit a greater degree of control over experimental conditions. Drug therapy of heterotransplanted MCF-7 could have been essayed but was not considered because of possible host interaction immunologically with drug effect. Such interactions occur even when tumor and host are syngeneic [15, 16].

Like most DMBA-induced rat mammary carcinomas [3–7], MCF-7 is hormone-responsive [8]. Therefore serum was used to supplement the defined medium so that metabolic processes might proceed more 'normally' than in its absence and to avoid the growth-limiting condition of serum-free medium [17]. In the experiments described here the serum content of the medium was reduced from 10 to 2% after 48 hr of growth to decrease the possibility of alteration of drug effect on target cells by high concentrations of nucleosides in the serum [18, 19]. This decrease in serum resulted in a difference in cell proliferation of <20% (Fig. 1) but little change in drug effect on cell growth. Growth inhibition of MCF-7 by 5-FU in medium containing 10 vs 2% CS (not shown)

Table 1. Effects of 5-FU and MDTP, singly and in concurrent combination, on incorporation of radiolabeled thymidine, uridine and leucine into macromolecules in MCF-7 cells in vitro

Dose (μg/ml)		% inhibition [2-^{14}C]-Thymidine		[2-^{14}C]-Uridine		[^{14}C]-Leucine*	
5-FU	MDTP	Incorporation	Total protein	Incorporation	Total protein	Incorporation	Total protein
1300	—	92.3	82.7	83.2	83.1	85.9	85.5
ID_{30}	—	28.2	27.7	27.3	29.6	32.0	30.9
	ID_{30}	34.9	26.0	26.5	29.9	30.4	31.1
ID_{15}	—	16.2	12.9	11.9	15.2	15.4	14.9
	ID_{15}	13.0	14.5	12.5	16.8	17.1	15.6
ID_{15}	ID_{15}	28.6	27.7	22.5	32.8	28.7	33.6

MCF-7 cells were cultured as described in Fig. 2. Two hours before termination of the experiment, cultures in duplicate flasks were pulsed with radiolabeled precursor. Incorporation into macromolecules was determined by count of label in acid-insoluble cell precipitate. Similar flasks in duplicate were harvested and cells counted. Percentage inhibition was calculated based on values for control cultures (dpm/mg protein).
*[Carbonyl-^{14}C]-leucine.

was non-significantly greater by 3.5% in the medium with 2% CS.

Weichselbaum *et al.* [20] reported that growth retardation of MCF-7 cells *in vitro* by 10^{-7} M 17β-estradiol led to a decrease in the proportion of cells in the S phase and consequently a reduction in the efficacy of the S phase-specific chemotherapeutic drug 1-β-D-arabinofuranosylcytosine. Growth inhibition by the drug was greater when applied after incubation of MCF-7 in 10^{-9} estradiol-17β, a stimulatory dose. They suggested that such changes caused by hormones may have important implications in clinical therapy by altering the efficacy of cycle-active agents. In the experiments described here the growth-inhibitory effects induced by MDTP did not appear to affect noticeably the efficacy of the S-phase-specific 5-FU at the pharmacologic dose levels used in the combination. The additive inhibitions of growth and of precursor incorporation confirm the absence of change in efficacy. It is possible that experimental differences such as growth media and serum content, the use of hormone and cytotoxic drug concurrently (rather than sequentially after a lapse of 24 hr [20]) and the use of an androgen rather than estrogen were the causes of the dissimilar results.

As seen in Table 1, the inhibitory effect on precursor incorporation by 5-FU or MDTP at ID_{30} may be obtained also with 5-FU at ID_{15} in combination with MDTP at ID_{15}. In the case of 5-FU with its severe toxicity [21] the use of this combination of agents, each at ID_{15}, would lead to a reduction in overall toxicity to the host yet maintain the same level of inhibition. At the lower dose of MDTP, reduction in at least virilizing activity would also be a reasonable expectation.

Additive growth- and precursor incorporation-inhibitory effects were found for 5-FU and MDTP in combination (Fig. 3, Table 1). These were confirmed by algebraic and isobole methods except in one instance, at ID_{70}. The data used in these methods are the iso-effective doses for each compound (ID_{70} in this case) and the doses (ID_{35}) for each drug in the combination yielding 70% growth inhibition. All doses were derived from the dose–response curves of Fig. 2. The algebraic calculations and the isobologram (Fig. 4), which used the same data, both revealed synergy, i.e. the calculation $=<1$ and the isobologram is a concave curve. Additional experimentation is needed to clarify this dissimilarity with the additive effect derived experimentally.

Lippman *et al.* [17] suggested that death of MCF-7 cells in media containing $>10^{-5}$ M 5α-dihydrotestosterone may be a non-specific effect. Similarly, in our precursor incorporation experiments, data based on cell population provide evidence that 5-FU and MDTP in combination at high ID levels do not merely retard growth but actually reduce the numbers of cells in the culture. The cell count at termination of the experiment on day 5, 2 days after addition of 5-FU and MDTP at ID_{35}, showed a decline of 57.6% from that on day 3. Smaller declines occurred at lower doses. The specificity of this cell kill has not been determined.

Finally, the data demonstrate significant growth-inhibiting activity of 5-FU and MDTP against human mammary carcinoma MDF-7 *in vivo*. The effects for combinations of the 2 agents in pharmacologic doses are at least additive, and cytocidal. These activities were manifest in all 5 parameters measured: cell proliferation, total cell protein, incorporation of radiolabeled thymidine, and uridine and leucine into acid-insoluble macromolecules. The results do not suggest a specific effect on any one parameter measured. The therapeutic effects of 5-FU and MDTP on DMBA-induced mammary carcinomas in rats were affirmed in the MCF-7 human mammary carcinoma *in vitro* assays.

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