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MODE OF ACTION OF 5-FLUOROCYTOSINE

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Abstract—A 5-fluorocytosine-sensitive isolate of *Candida albicans* was examined for the presence of thymidylate synthetase activity and 5-fluoro-2′-deoxyuridylic acid in the presence and absence of a growth-inhibiting concentration of 5-fluorocytosine. Initial studies demonstrated thymidylate synthetase activity in a crude homogenate prepared from this isolate. This activity could be inhibited by preincubating the homogenate with 5-fluoro-2′-deoxyuridylic acid. Evidence is presented suggesting that the inhibition of this enzyme is irreversible. Later studies measured thymidylate synthetase activity and 5-fluoro-2′-deoxyuridylic acid levels in homogenates prepared from harvested *C. albicans* after varying intervals of exposure to 100 μg/ml of 5-fluorocytosine. Thymidylate synthetase activity was observed to be 20 per cent of control after 1 hr of exposure to 5-FC, remaining decreased through the remainder of the 24 hr. 5-fluoro-2′-deoxyuridylate was measurable at all of the time points examined in the fungal cells exposed to 5-fluorocytosine in levels from 2 to 40 pmoles/μg of DNA while no evidence was found for this compound in the controls. In light of these findings, it is suggested that inhibition of thymidylate synthetase activity by endogenously synthesized 5-fluoro-2′-deoxyuridylic acid is a possible mechanism by which 5-fluorocytosine exerts its antifungal effect.

5-fluorocytosine (Flucytosine; 5-FC) can effectively inhibit the growth of several clinically important fungi, both *in vitro* and *in vivo* [1]. The mode of action of this drug has been investigated with several hypotheses offered to explain its effectiveness. Previous studies with mutant fungal isolates have demonstrated that 5-FC is ineffective when cytosine deaminase is absent [2, 3], suggesting that the deamination of 5-FC to 5-fluorouracil (5-FU) is an initial important step. Once formed within the fungal cell, 5-FU may be converted to a nucleotide compound by those enzymes which normally act upon uracil.

The existence of ribonucleotide metabolites of 5-FU has previously been demonstrated with evidence that as much as 50 per cent of the uracil in fungal RNA may be replaced by 5-FU [3, 4]. It is widely believed that the RNA that is formed is defective, resulting in possible alterations in protein synthesis [2–6]. These findings, coupled with an apparent positive correlation between the incorporation of 5-FU into RNA and the antifungal activity of 5-FC, have led some investigators to claim that incorporation into RNA is a prerequisite to antifungal activity [4].

Incorporation of 5-FU into RNA is known to occur in bacterial cells and mammalian cells exposed to 5-FU [7, 8]. The major site of action, however, is now thought to be interruption of DNA synthesis caused by formation of 5-fluoro-2′-deoxyuridylic acid (FdUMP) with resultant inhibition of thymidylate synthetase (EC 2.1.1.b) [6]. In these cells this enzyme is utilized to form thymidylate needed for DNA synthesis. Previous studies have demonstrated that FdUMP is a very potent inhibitor of thymidylate synthetase isolated from cells as diverse as *Escherichia coli* and Ehrlich ascites tumor cells [7, 8]. Evidence supporting such a mechanism of action for 5-FC in fungal cells is lacking. Some investigators have discounted this as a possibility, based on the lack of effect of exogenous FdUMP on growing fungal cells [4].

The purpose of the present study was first to determine whether thymidylate synthetase isolated from a 5-FC-sensitive fungal isolate could be inhibited by FdUMP, and second to ascertain whether this isolate growing in the presence of 5-FC would produce detectable quantities of FdUMP associated with concomitant decreased thymidylate synthetase activity.

MATERIALS AND METHODS

Chemicals. 5-FC was a gift from Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, NJ. FdUMP was obtained from Terra-Marine Bioresearch, LaJolla, CA. Thymidylate synthetase from dichloromethotrexate-resistant *Lactobacillus casei* used for the nucleotide assays was obtained from New England Enzyme Center, Tufts University Medical School, Boston, MA. 2′-Deoxyuridylic acid (dUMP) and tetrahydrofolate (FH_4) were obtained from Sigma Chemical Co., St. Louis, MO. [5-^{3}H]dUMP (sp. act. 11 Ci/nmole) was obtained from Amersham Radiochemicals. Other chemicals used were of analytical grade.

Isolate. The isolate of *Candida albicans* used in this study was obtained from the blood of a patient. The minimal inhibitory concentration of 5-FC with this organism was 2.5 μg/ml. The isolate was maintained by serial subculture on Sabouraud's agar at 30°.

Culture medium. Growth from Sabouraud's agar after a 24 hr incubation was suspended in saline and inoculated onto agar containing yeast nitrogen base (Difco) and 1% glucose (YNB). After overnight incu-

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bation at 34°, growth on YNB was harvested in saline and washed twice. Cells obtained in this way were then used either for enzyme-inhibitor studies or as the inocula for growth studies evaluating 5-FC effect.

Growth in 5-FC medium. Growth from YNB agar was suspended in saline and adjusted by hemocytometer count to 5×10^8 cells/ml. An L-shaped glass rod was used to spread 1.0 ml of this inoculum into each of twenty 32-oz bottles, in which was layered 100 ml YNB containing either 5-FC (100 μg/ml) or water (control). At stated intervals growth was harvested. This was done by washing cells from agar repeatedly with ice-cold phosphate-buffered saline solution (pH 7.4). A cell count was made by hemocytometer on an appropriate dilution. The remaining cells were collected by centrifuging at 2000 *g* for 5 min in a refrigerated centrifuge. The supernatant was discarded, and the cell pellet was then used to prepare the homogenate.

Preparation of homogenates. Harvested cells (approximately 10^{10} cells) were homogenized in one of two ways depending on whether the nucleotide or enzyme assay was being performed. For the nucleotide assay, cells were suspended in 4 vols. of ice-cold 1 M acetic acid prior to homogenizing. For the enzyme assay, prior to homogenization, cells were suspended in 4 vols. of ice-cold sodium phosphate buffer, pH 7.4. Acetic acid was added to maximize nucleotide recovery as described by Nazar *et al.* [9].

In either case, 0.5-mm glass beads were added in a volume of 1:2 to the total volume of cell suspension contained in an ice-chilled 60 cm^3 Pyrex homogenizing bottle (Braun). A Braun homogenizer cooled by a siphon CO_2 spray was used for homogenization. The process was preformed in five cycles of 45 sec each with 15-sec rest intervals in between. In this way, the temperature of the suspension was maintained below 10°, as measured by periodic examination with a thermocouple probe. Efficiency of cytoplasmic release was periodically assessed by examination of a smear prepared from the homogenate and stained with lactophenol cotton blue stain. Release of cytoplasmic contents was greater than 90 per cent by this method.

Thymidylate synthetase assay. The homogenate obtained was centrifuged at 100,000 *g* to remove particulate material. The supernatant remaining was then examined for thymidylate synthetase activity by utilizing the tritium-release assay developed by Lomax and Greenberg [10] with modifications by Dunlap *et al.* [11] and Kawai and Hillcoat [12]. The assay was carried out in a 1.5-ml microcentrifuge tube in a total volume of 0.55 ml, and included the following: 0.05 ml of 2×10^{-3} M *dl*, L-5, 10-methylenetetrahydrofolate (made up by dissolving 5 mg of *dl*, L-tetrahydrofolate in 5.0 ml of a solution containing 0.05 M $NaHCO_3$, 0.07 M HCHO, and 0.25 M *B*-mercaptoethanol), 2.5 μmoles potassium fluoride, 50 μmoles potassium phosphate (pH 6.8), 0.1 ml of H_2O, and enzyme extract to be tested. The reaction was initiated by adding 0.05 ml of 10^{-3} M [5^{-3}H]dUMP and allowed to incubate at 37° for 10 min before terminating with 0.1 ml of 1.0 M HCl. After thorough mixing by vortex agitation, 0.2 ml of a charcoal slurry was added (Norit A, 10 g/100 ml; bovine serum albumin, fraction V, 2.5 g/100 ml; and high molecular weight dextran, 0.1 g/100 ml; all from Sigma Chemical Co.). Each sample was vortexed again and then centrifuged at 8000 *g* for 5 min. A 0.2-ml aliquot of the supernatant was mixed with 10 ml Aquasol (New England Nuclear, Boston, MA). and the released tritium was counted in a Packard scintillation counter (tritium efficiency = 25–31 per cent). Because of the problem of possible non-enzymatic exchange of tritium previously alluded to in the literature [12], controls were examined in the absence of enzyme with every determination.

FdUMP and dUMP assay. The acetic acid homogenates obtained from the growth experiments were centrifuged at 100,000 *g* for 30 min. The precipitate was saved for DNA analysis. The method of Schneider [13] was used to extract the DNA, while the method of Burton [14] was used to measure DNA content.

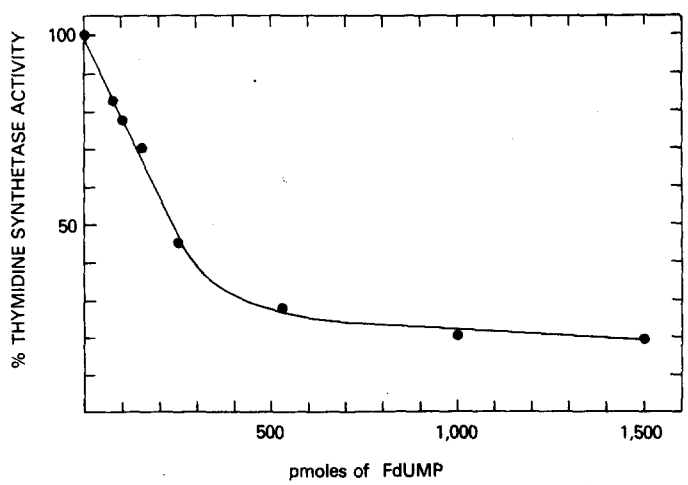

Fig. 1. Thymidylate synthetase activity was examined after preincubating a constant volume of *C. albicans* extract (0.1 ml) with increasing concentrations of FdUMP. FdUMP concentrations shown correspond to a range from 1.15×10^{-7} to 2.3×10^{-6} M (1500 pmoles FdUMP). The ordinate depicts the relative amount of activity remaining after exposure to FdUMP, where 100 per cent activity represents activity in the absence of FdUMP.

The supernatant was examined for dUMP and FdUMP content. The acetic acid was removed by lyophilization, leaving a residue that was then reconstituted in 0.05 M Tris–HCl buffer (pH 7.4). The measurement of dUMP and FdUMP was determined by spectrophotometric assay as described in previous reports from this laboratory [15, 16].

RESULTS

Demonstration of thymidylate synthetase activity in C. albicans *isolate and inhibition by FdUMP.* The presence of thymidylate synthetase activity was first demonstrated in the isolate of *C. albicans* being studied. Activity could be easily detected in the supernatant of a crude homogenate prepared from 10^{10} cells. The previously reported displacement of tritium by non-enzymatic causes [12] was found to be negligible as assessed by controls.

Figure 1 illustrates the effect of preincubating the above enzyme extract for 15 min with increasing concentrations of FdUMP. Thymidylate synthetase activity was seen to decrease with increasing concentrations of FdUMP in a fashion suggesting irreversible inhibition. Figure 2 indicates that the inhibition was indeed irreversible. Titration of a constant amount of FdUMP with varying amounts of enzyme gave a line parallel with the uninhibited line, behavior diagnostic of irreversible inhibition [17]. These results are consistent with the pattern of inhibition FdUMP causes in thymidylate synthetase from mammalian and bacterial sources [15, 18].

Effect of 5-FC on C. albicans *growth; simultaneous measurements of dUMP, FdUMP, and Thymidylate Synthetase activity.* Figure 3 demonstrates the effect of 100 μg/ml of 5-FC on the growth of 2.5×10^8 organisms on solid YNB medium. It can be seen that growth ceased almost immediately and did not resume during the 24-hr period of observation. In contrast, the control cells were seen to undergo a period of rapid logarithmic growth during the first 8 hr, with a leveling off of growth occurring as the cells began to overgrow the agar surface.

These results demonstrate that the effects of 5-FC on fungal metabolism are quite rapid. To evaluate whether FdUMP synthesis from 5-FC represents a reasonable cause for this cessation of growth, we monitored both formation of this inhibitor and disappearance of thymidylate synthetase activity. As can be seen in Fig. 4, thymidylate synthetase activity decreased markedly to less than 20 per cent of the 0 hr

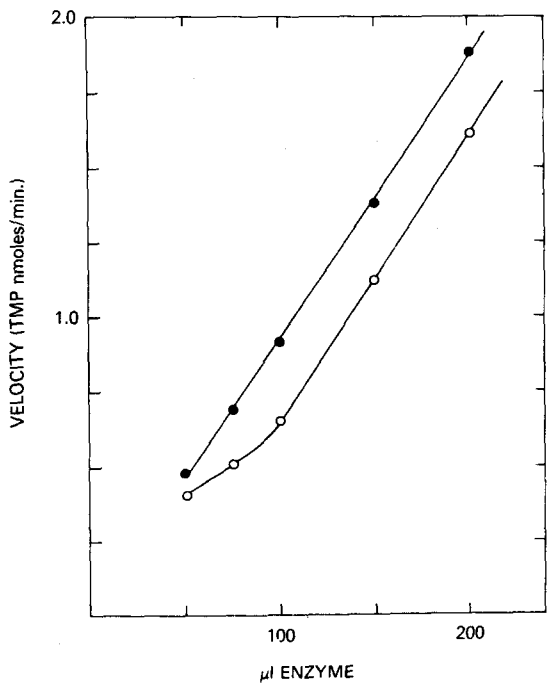

Fig. 2. Ackermann–Potter plot for FdUMP with *C. albicans* enzyme extract. Effect of increasing enzyme concentration on velocity of thymidylate formation after preincubation with FdUMP, 150 pmoles (●——●) and with water control (○——○).

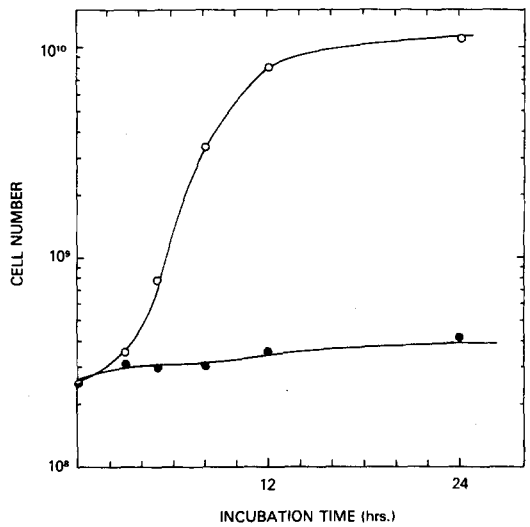

Fig. 3. Effect on cell growth of incubation in YNB medium containing 100 μg/ml 5-FC (●——●) as compared with control containing water (○——○). The starting concentration was 2.5×10^8 cells/bottle for each time point.

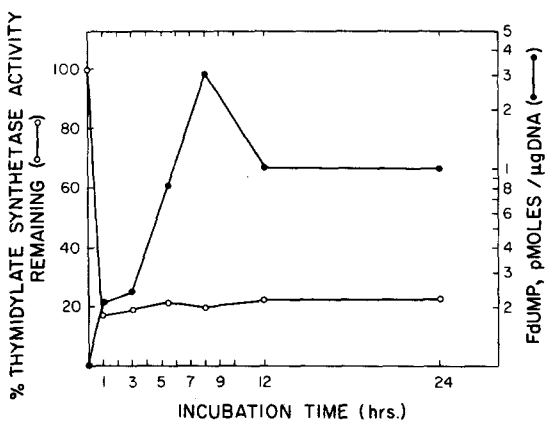

Fig. 4. Results of parallel studies conducted in YNB medium containing 100 μg/ml of 5-FC. Thymidylate synthetase activity (○——○) is expressed as a percentage of activity remaining (i.e. dis./min/mg of protein for each time point as a per cent of dis./min/mg of protein at zero time point before exposure to 5-FC). FdUMP levels (●——●) were assayed from each time point and are expressed as pmoles/μg of DNA.

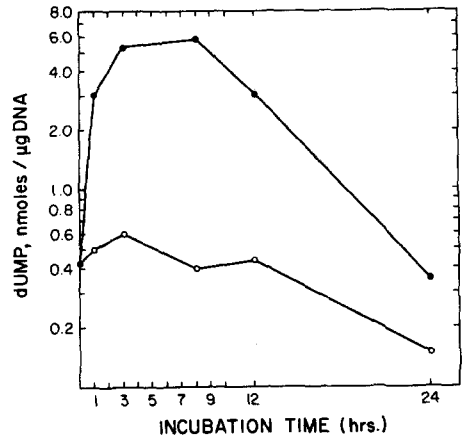

Fig. 5. The dUMP concentration is expressed as nmoles/µg of DNA and is noted at each time point for control cells (O——O) and those incubated in 100 µg/ml of 5-FC (●——●).

(before exposure to 5-FC) value at the earliest time point of 1 hr, and remained depressed throughout the 24-hr period. Free FdUMP was detectable as early as 1 hr, with peak levels occurring at 8 hr. Examination of the control cells revealed no evidence of FdUMP, at any time point. Thymidylate synthetase activity in the control cells was found to be approximately equal to the 0 hr value, with some decrease after 8 hr, possibly representing decreased enzyme activity secondary to cells entering a postlogarithmic growth stage.

If inhibition of thymidylate synthetase is of sufficient magnitude to block growth, one would expect accumulation of dUMP, the substrate for this enzyme. This was confirmed; as can be seen in Fig. 5, 5-FC exposure results in a 1.5 log expansion in the dUMP pool. In contrast, dUMP concentration did not dramatically change in the control.

It should be stressed again that the assay used here is capable of measuring only free FdUMP and not that which is already bound. The results of the Ackermann–Potter plot demonstrate that FdUMP which is bound to the *C. albicans* enzyme would not be expected to disassociate.

Evidence suggesting that the inhibitor we have called free FdUMP is indeed that, and not some other compound, is provided by the co-migration of this inhibitor with known FdUMP on thin-layer chromatography. After determination of dUMP and FdUMP in the homogenate prepared from cells exposed to 5-FC, 100 µg/ml for 8 hr, the remainder was streaked on cellulose plates and chromatographed in a solvent system containing tertiary amyl alcohol–formic acid–water (3:2:1, v/v). An FdUMP standard was run in parallel. The plate was scraped into five fractions, prepared horizontal to the solvent front, and each fraction was then examined for FdUMP. Known FdUMP had an R_f of 0.56 ± 0.04 in this system. FdUMP activity was found primarily in the fraction extending from an R_f of 0.5 to 0.6. The unknown was also chromatographed in a solvent system containing ethyl acetate–acetone–water (7:4:1, v/v). In this system, presumed FdUMP activity remained at the origin as did the FdUMP standard.

DISCUSSION

Previous investigators have focused almost exclusively on the effects of 5-FC on fungal RNA synthesis, function and stability and have largely ignored the possible formation of FdUMP with subsequent inhibition of thymidylate synthetase. In the present study, we have demonstrated, for the first time, the existence of thymidylate synthetase in *C. albicans* and have shown that this enzyme is irreversibly inhibited by FdUMP. In addition, we have shown that a 5-FC-sensitive isolate of *C. albicans* exposed to 5-FC forms FdUMP with a simultaneous decrease in the thymidylate synthetase levels. The metabolic significance of this inhibition is strengthened by the observed simultaneous expansion of the endogenous dUMP pool. We propose that such a block in *de novo* thymidine synthesis may have sufficient impact on DNA synthesis to explain the cessation of growth observed in Fig. 3.

Two of the observations from this study deserve further comment. The first is that thymidylate synthetase activity, as can be seen in Fig. 5, never completely disappears despite the presence of free FdUMP. We feel that this finding is not artifact based on our observation that heat-inactivated extract had no measurable thymidylate synthetase activity. The second is the dUMP pool fluctuation initially increasing 1.5-fold after exposure to 5-FC, then decreasing with time despite the continued presence of free FdUMP. The initial increase in the dUMP pool in the fungal cells exposed to 5-FC is similar in magnitude to the increase in the dUMP pool seen in both P1534 ascites tumor cells and in host (CDF_1 mouse) bone marrow and duodenal mucosal cells exposed to 5-FU [15, 16]. The expansion in the dUMP pool in the fungal cells as in the murine cells referred to above may reflect accumulation of substrate (dUMP) secondary to enzymatic block. The expansion of the dUMP pool in the fungal cells exposed to 5-FC was not sustained, however, as it was in the murine tumor and host cells. The decrease in the dUMP pool occurred after 8 hr in spite of the continued presence of decreased thymidylate synthetase activity and detectable levels of free FdUMP. The lack of studies of pyrimidine metabolism in *C. albicans* or related fungi allows for little more than speculation at this time.

In light of the present study, reconsideration of the affect of 5-FC on RNA must be undertaken. There exists at least one reported exception to the hypothesis of Polak and Scholer [4] regarding the degree of incorporation of 5-FU into RNA as being the determinant of fungal sensitivity to 5-FC. Block *et al.* [19] reported on a 5-FC-resistant isolate of *Cryptococcus neoformans* which was noted to incorporate large amounts of 5-FU into a trichloroacetic acid (TCA)-insoluble fraction, when the organism was exposed to 5-FC. Evaluation of the 5-FC-sensitive, parent isolate revealed essentially no difference in the amount of 5-FU incorporated into the TCA-insoluble fraction. Among the possible explanations for the organism's resistance to 5-FC must be an inability to inhibit thymidylate synthetase due to insufficient synthesis of FdUMP. Thus, since many of the pathways are common in the formation of pyrimidine ribo- and deoxyribonucleotides, it can be seen that

the previously observed 5-FC-resistant mutants [2–4, 20] could derive their resistance to 5-FC not only because 5-FU was not incorporated into RNA, but also perhaps more importantly, because FdUMP was not synthesized.

The results of the present study demonstrate that inhibition of thymidylate synthetase by FdUMP must be considered an alternative mechanism by which 5-FC exerts its fungistatic effect. Determination of the relative importance of this effect and the effect on RNA may have significance in the rational design of future antifungal therapy.

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