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Mechanisms of Action of 5-Fluorocytosine

ALAYN R. WALDORF†* AND ANNEMARIE POLAK

Pharmaceutical Research Division, F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland

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5-Fluorouracil and 5-fluorodeoxyuridine monophosphate levels were estimated in 75 isolates of *Candida albicans* to determine whether 5-fluorocytosine susceptibility could be ideally correlated with the intrafungal formation of both 5-fluorodeoxyuridine monophosphate and 5-fluorouridine triphosphate or a reciprocal formation of the two metabolites to prove the mechanism of 5-fluorocytosine activity. Using the results of four in vitro susceptibility tests, we separated isolates of *C. albicans* into susceptibility groups. For most strains, there was a positive correlation between the degree of 5-fluorocytosine susceptibility and the inhibition of biosynthesis of both RNA and DNA, incorporation of 5-fluorouracil into RNA, inhibition of ribosomal protein synthesis, and levels of 5-fluorodeoxyuridine monophosphate. However, in some strains with a similar degree of 5-fluorocytosine resistance, either reduced incorporation of 5-fluorouracil or reduced 5-fluorodeoxyuridine monophosphate levels occurred, suggesting that these two mechanisms are not necessarily linked to each other and that both may be responsible for 5-fluorocytosine activity.

The mechanism of action of the antifungal drug 5-fluorocytosine (5-FC) has been investigated in yeasts (7, 8, 12), aspergilli (12), and dematiaceous fungi (17). It has been suggested that the activity of 5-FC after uptake by the fungi and intracellular deamination is a consequence of intrafungal formation of two metabolites, 5-fluorodeoxyuridine monophosphate (FdUMP) and 5-fluorouridine triphosphate (FURTP) (11, 15).

FdUMP is a potent inhibitor of thymidylate synthetase (5), causing an impairment of DNA synthesis in bacteria and tumor cells. Furthermore, in one 5-FC-susceptible strain of *Candida albicans*, in the presence of 5-FC, FdUMP was formed, followed by a decrease in the thymidylate synthetase activity (2). Moreover, the addition of 5-FC to both hyphal and yeast phases of *C. albicans* cultures resulted in an immediate inhibition in the increase of DNA (12).

FURTP is incorporated into fungal RNA in place of uridylic acid (11, 15), which alters amino acylation of tRNA in vitro (4) and is believed to cause unbalanced internal pool and disturbed synthesis of proteins and carbohydrates (6, 13, 14). There is a statistical correlation between incorporation of the fluorinated metabolite into RNA and the antifungal activity of 5-FC, although there are considerable individual variations in incorporation in mutants with similar 5-FC susceptibility (1, 7, 10).

† Present address: Section of Infectious Diseases, Boston University Hospital, Boston, MA 02118.

Whether these two suggested mechanisms of 5-FC activity (FdUMP and FURTP) are linked or independent of each other is still unknown. The formation of FdUMP and decreased thymidylate activity may explain the antifungal 5-FC activity, the incorporation of FURTP in RNA being only incidental, or the converse. The purpose of this study was to determine on which of these mechanisms the activity of 5-FC is based.

MATERIALS AND METHODS

Organisms. A total of 75 isolates of *C. albicans* were used. Eleven isolates of known 5-FC resistance (10) were kindly supplied by S. Shadomy, Richmond, Va. The remaining 64 were chosen from 402 isolates obtained from R. Stiller, San Jose, Calif., so as to provide a broad spectrum of susceptible and resistant mutants, approximately half of which were of type A serotype (16). All cultures were maintained on Sabouraud dextrose agar.

Chemicals. The fluorinated pyrimidines 5-FC or [2-^{14}C]5-FC (1.4 μCi/mg) were synthesized by Roche Diagnostics, Nutley, N.J. FdUMP was obtained from Calbiochem Behring Corp., La Jolla, Calif. Thymidylate synthetase (specific activity, 136 μmol/h per ml) was obtained from New England Enzyme Center, Boston, Mass. 2-Deoxyuridylic acid and tetrahydrofolic acid were obtained from Sigma Chemical Co., St. Louis, Mo. L-[4,5-^{3}H]leucine (specific activity, 85 Ci/mmol) and [5-^{3}H]2-deoxyuridylic acid (specific activity, 14.8 Ci/mmol) were purchased from Amersham Radiochemicals, Amersham, England. All chemicals were of reagent grade quality.

Culture conditions. We incubated 24-h plate yeast cultures on Sabouraud dextrose agar overnight in

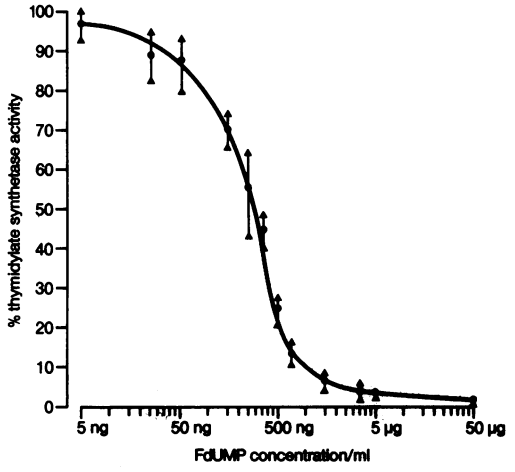

FIG. 1. FdUMP standard curve in thymidylate synthetase assay. The inhibitory effects of various concentrations of FdUMP on thymidylate synthetase of *Escherichia coli* (diluted 1:10) are shown. Each point is the average of five trials, each run in triplicate. The range of each point is given.

yeast nitrogen base (Difco Ltd.) containing 5% glucose (YNBG) at 37°C. If necessary, these cultures were diluted to yield a cell suspension of 1×10^6 to 3×10^6 cells per ml at the beginning of the experiment. Except in controls, 5-FC was added to the culture medium to obtain a final concentration of 100 µg/ml. All cultures were grown in YNBG and incubated with continuous agitation on a rotating shaker (100-rpm excentric shaker with a 2.5-cm throw).

In vitro susceptibility testing. Inocula were prepared from 24-h plate cultures on Sabouraud dextrose agar. Both agar and liquid dilution tests were used for determining minimal inhibitory concentration (MIC). Agar (20% [wt/vol]) was added to the YNBG, and cultures were examined for visible colonies in the MIC agar test. Disk diffusion studies were performed by using disks containing 1 and 100 µg of 5-FC each. Additionally, growth was measured spectrophotometrically during-log phase growth, and the 50% inhibitory concentration (IC_{50}) was calculated (3). On each day of testing and for each assay method, a known sensitive strain and a known resistant strain were included as controls.

The isolates were divided into three groups according to their susceptibility to 5-FC, using a modified scheme of Stiller et al. (16). Group 1 isolates had agar and liquid MICs after 7 days of less than or equal to 1 µg/ml. Group 2 (partially resistant strains) were divided into subgroups 2a and 2b; both subgroups were highly susceptible after 24 h (MIC < 1). However, after 48 h, the 2b group had MICs of >25 µg/ml. All group 2 isolates were resistant after 7 days of incubation. Group 3 isolates (fully resistant) had MICs of 100 µg/ml at 24 h. For six strains, agar disk diffusion and IC_{50} results justified their placement into a different susceptibility group. Agar and liquid MICs and IC_{50}s of 5-FC were expressed as the geometric means, and their 95% confidence levels were calculated. A value of 200 was used when a susceptibility test value was >100.

Cell growth, RNA, and DNA estimations. For each isolate, control and test cultures were run in parallel. Samples were taken at 1-h intervals during the first 8 h of incubation and at the end of the 24-h incubation period. The cells were counted, and the samples were frozen at −20°C for the determination of RNA and DNA. Nucleic acids were extracted with 0.5 M perchloric acid at 70°C (12). RNA was estimated from extinction measurements of dilutions of the extract (12), and the DNA was measured according to the method of Wain et al. (18), using diphenylamine reagent. RNA and DNA contents were expressed in milligrams per 10 ml of culture.

Uptake and distribution of [2-^{14}C]5-FC. Incorporation of pyrimidine into the RNA fraction after the addition of 100 µg of [2-^{14}C]5-FC per ml (1 to 2 µCi/ml) was measured after 6, 24, and 48 h of incubation and the amount of 5-fluorouracil (5-FU) was calculated in nanograms of [2-^{14}C]5-FC equivalent per milligram of RNA. After 48 h of culture, liquid media initially containing 100 µg of [2-^{14}C]5-FC per ml were assayed for 5-FU and 5-FC by thin-layer chromatography to ensure that resistance of isolates was not due to cytosine deaminase deficiency (11).

Ribosomal isolation. Labeled yeast cells (pulsed with [4,5-^{3}H]leucine [10 mg/ml, 1 µCi/ml for 90 min]) were disrupted in a Braun homogenizer for 2 to 3 min.

TABLE 1. 5-FC in vitro susceptibility in 75 selected strains of *C. albicans*

Susceptibility group no.	No. of strains in each serotype	Agar disk diffusion (mm)[a]				MIC[a] (µg/ml) in:	
		1 µg		100 µg		Agar	
		24 h	48 h	24 h	48 h	24 h	48 h
1	A = 12 B = 3	44.67 (±3.35)	40.3 (±5.73)	79.8 (±8.99)	76.2 (±7.92)	0.044 (0.037–0.06)	0.80 (0.074–0.1)
2a	A = 9 B = 14	37.36 (±5.28)	24.7 (±8.44)	68.32 (±6.48)	62.40 (±10.04)	0.151 (0.08–0.28)	3.197 (0.21–34.6)
2b	A = 5 B = 20	32.16 (±5.75)	8.23 (±12.49)	64.2 (±5.27)	50.2 (±17.78)	0.437 (0.25–0.75)	30.051 (2.5–346)
3	A = 4 B = 8	3.67 (±8.82)	0 (±0)	13.42 (±21.49)	1.67 (±5.77)	>100 (35–431)	>100

[a] Average (± standard deviation).
[b] Geometric mean (95% confidence limit of mean).

Monomeric 80S ribosomes were isolated by gradient ultracentrifugation from 24 selected strains of *C. albicans*, and the specific activity was calculated (17).

Thymidylate synthetase assay and estimation of FdUMP. Cells collected by centrifugation at 2,000 × *g* for 5 min were washed twice in cold phosphate-buffered saline (PBS) and disrupted in a Braun homogenizer for 2 to 3 min. For the thymidylate synthetase assay, homogenates of 10^{10} cells in PBS were used. For the FdUMP assay, an equal volume of cells cultured in the presence of 5-FC was homogenized in cold 1 M acetic acid.

The thymidylate synthetase activity of the PBS homogenates was measured as described by Diasio et al. (2).

The acetic acid homogenates of *C. albicans* were centrifuged at 100,000 × *g* for 30 min. In the precipitate, DNA was measured by using diphenylamine reagent (18). For FdUMP content analysis, supernatants were lyophilized to remove the acetic acid and were reconstituted in 0.05 M Tris-hydrochloride buffer (pH 7.4) (2).

The FdUMP levels in homogenates were measured indirectly by using the inhibitory effect of FdUMP on the purified thymidylate synthetase (New England Center), according to a modified method of Diasio et al. (2). The assay was carried out in a microcentrifuge tube. Each tube was given 50 μl of 2 × 10^3 M tetrahydrofolic acid in 2-mercaptoethanol buffer (2), 2.5 μmol of potassium fluoride in 50 μl, 50 μmol of potassium phosphate (pH 6.8) in 50 μl, 100 μl of water, 100 μl of thymidylate synthetase (New England Enzyme Center) diluted 1:10 in PBS, and 100 μl of the sample, thoroughly mixed and preincubated for 15 min at 37°C to facilitate inhibition. To initiate the reaction, 50 μl of 2-[5-^{3}H]deoxyuridylic acid (10^{-3} M, 1 μCi/ml) was added, and the reaction was run at 37°C for 10 min before being terminated with 100 μl of 1.0 M HCl. A charcoal slurry (200 μl) (2) was added, mixed thoroughly, and centrifuged at 8,000 × *g* for 5 min. The released ^{3}H was measured in 200-μl aliquots of the supernatant (9).

A standard curve of FdUMP in a concentration range of 5 ng to 50 μg/ml is shown in Fig. 1. With each determination, a positive control containing the enzyme in Tris-hydrochloride buffer was run and taken as 100% activity; a background control in the absence of enzyme was taken as 0% activity. Samples containing >100 mg of lyophilate per ml of buffer gave less than 100% activity. Therefore, all samples were run with concentrations of lyophilate less than 70 mg/ml. Each sample was run in triplicate, and the percent sample activity of the mean was calculated. Samples were diluted or concentrated as required so that the percent sample activity values were between 15 and 85%. To control the quantity of thymidylate synthetase in the assay, each sample was also run in the absence of exogenous enzyme, and if it contained >15% activity, the sample was diluted and reassayed. Quantities of FdUMP were expressed as picograms of FdUMP per milligram of lyophilate per microgram of total DNA in the sample.

RESULTS

Characteristics of the three susceptibility groups according to in vitro testing. The mean values obtained with the four in vitro susceptibility tests (MIC agar, MIC liquid, IC_{50}, and agar disk diffusion) are shown in Table 1. The results of group 1 and group 3 strains are statistically different from each other and from the results of group 2 strains. The values obtained for strains in group 2 were scattered within the boundaries of the group, and overlapping of individual values within the subgroups occurred.

Growth curves; RNA and DNA synthesis. Curves of mean values of cell number and RNA and DNA per 10 ml of cell culture at various incubation periods are shown in Fig. 2. A 100-μg/ml concentration of 5-FC caused an immediate inhibition in the exponential cell growth rate as well as RNA and DNA synthesis in the highly 5-FC-susceptible (group 1) isolates (Fig. 2a). Their inhibition remained throughout the 24-h incubation period. The resistant (group 3) isolates had only minimal reduction in cell growth and RNA and DNA synthesis in the first 8 h of incubation as compared with untreated controls. After 24 h of incubation, values for most isolates had reached control levels (Fig. 2b). All strains

TABLE 1—*Continued*

MIC[b] (μg/ml) in:						IC_{50} (μg/ml)
Agar		Liquid medium				
72 h	7 days	24 h	48 h	72 h	7 days	
0.165 (0.13–0.20)	0.48 (0.308–0.75)	0.029 (0.023–0.034)	0.082 (0.051–0.076)	0.152 (0.129–0.228)	0.334 (0.28–0.39)	0.009 (0.009–0.018)
14.767 (0.37–239)	57.712 (6.5–425)	0.144 (0.05–0.25)	1.310 (0.25–6.6)	30.71 (3.0–301)	>100 (7.4–400)	0.024 (0.014–0.044)
>100 (23–602)	>100 (67–321)	0.207 (0.03–1.2)	17.636 (1.0–289)	>100 (21.9–548)	>100 (102–309)	0.0331 (0.019–0.075)
>100	>100	16.360 (1.47–293)	>100	>100	>100	0.506 (0.023–12.7)

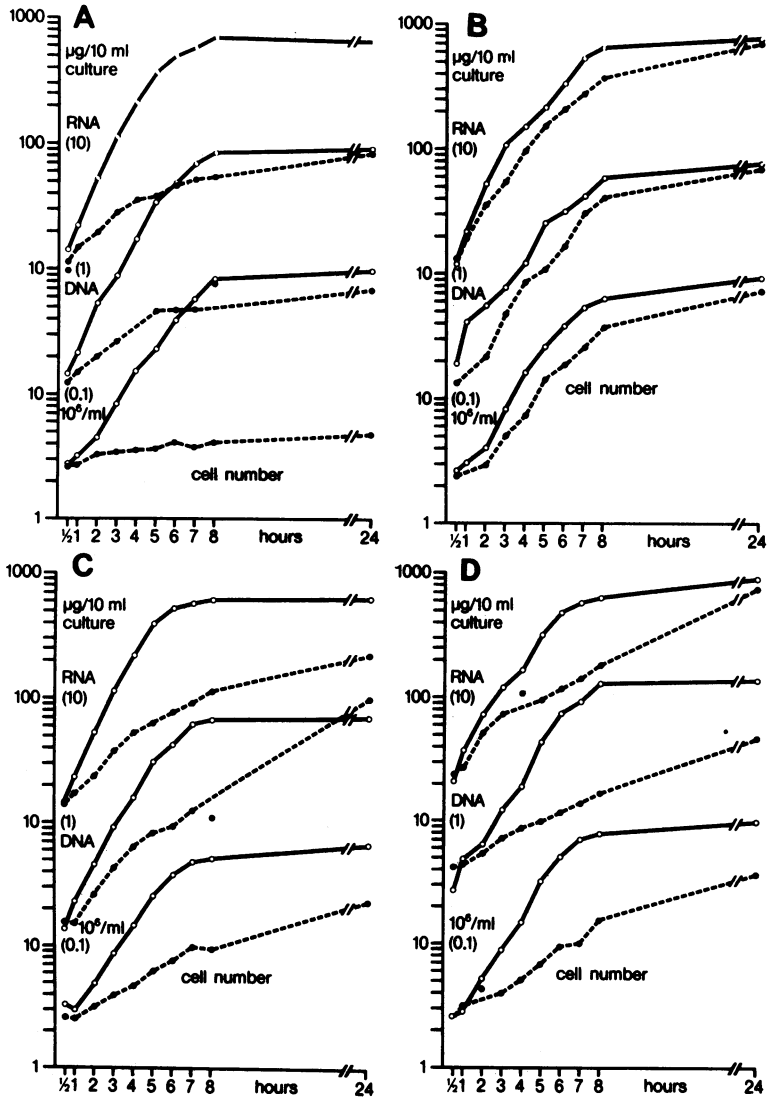

FIG. 2. Inhibition by 5-FC of cell growth and RNA and DNA synthesis in *C. albicans* during 24 h of incubation. 5-FC (100 μg/ml) was added to exponentially multiplying cultures at time zero. (A) Means of the susceptibility group 1. (B) Means of the resistant group 3. Panels C (10 isolates) and D (9 isolates) show the average values of unusual isolates from group 2 (for details, see the text). Symbols: O——O, control; ●------●, 5-FC.

in groups 1 (n = 15) and 3 (n = 12) followed the same pattern, whereas in group 2 (n = 48), important differences from the majority were observed. Twenty-nine partially resistant (group 2) strains followed an intermediary pattern between the group 1 and 3 strains (not shown in the figure) with a high standard deviation. However, two distinct patterns of RNA and DNA synthesis occurred among the remaining 19 isolates within this group. In both subgroups, the reduction in RNA and DNA synthesis in treated cultures approximated the reduced level of cell growth in the first 8 h of incubation. However, in 10 strains, the DNA (Fig. 2c) approached control levels at 24 h, and the cell number and the RNA levels were approximately one-third those of the controls. In another nine strains (Fig. 2d), the RNA approached control levels, whereas the DNA and the cell number were only one-third those of the controls.

Uptake and distribution of 5-FC. In the highly susceptible strains (group 1), the average incorporation of 5-FC-derived 5-FU into RNA was 81.1 μg/mg of RNA during log-phase growth

TABLE 2. Incorporation of 5-FU into RNA after exposure to 5-FC and inhibition of monomeric 80S ribosome synthesis in *C. albicans* isolates of various 5-FC susceptibilities

Susceptibility group no.[a]	[2-^{14}C]5-FU incorporation[b] into RNA after incubation for (h):			% Inhibition of 80S ribosome synthesis[c]
	6	24	48	
1 (n = 15)	81.1 ± 4.8	89.4 ± 5.5	105.5 ± 7.1	71.67 ± 2.6 (3)
2 (n = 48)	39.7 ± 5.0	33.5 ± 5.1	26.8 ± 6.3	43.01 ± 4.1 (15)
3 (n = 12)	9.1 ± 2.5	4.0 ± 2.3	3.6 ± 2.0	1.4 ± 1.6 (5)

[a] Group 1: highly susceptible; group 2: moderately susceptible ("partially resistant"); group 3: definitely resistant to 5-FC.

[b] Expressed as micrograms of [2-^{14}C]5-FC equivalent per milligram of RNA.

[c] Ribosomal protein synthesis was estimated by radiolabeled leucine incorporation during 90 min. Numbers in parentheses indicate the number of isolates examined.

(Table 2), and the average incorporation increased during the 48-h incubation period. In the strains with partial resistance (group 2), the average 5-FU incorporation was approximately half of that of group 1 at 6 h and decreased at 24 and 48 h of incubation. The resistant strains (group 3) distinguished themselves by an extremely low average incorporation (9 μg/mg of RNA) during log-phase growth. Moreover, incorporation decreased during the next 48 h of incubation. Marked variation among the individual values was observed, but there was no overlapping of individual values among the three groups.

Interestingly, the subgroup of isolates that had the unusually high RNA synthesis pattern (Fig. 2d) had a low 5-FU incorporation average (17 μg/mg of RNA) at 48 h of incubation, whereas the subgroup that had normal RNA synthesis but increased DNA synthesis (Fig. 2c) had average incorporation values for group 2 strains (27.1 μg/mg of RNA at 48 h).

Cytosine deaminase deficiency was never found in the 60 isolates with 5-FC resistance or partial resistance (groups 2 and 3). Statistically, there was no difference in 5-FU incorporation in RNA between serotype A and B isolates within each susceptibility group.

Effect of 5-FC on synthesis of 80S ribosomes. Table 2 lists the average percent inhibition of newly synthesized ribosomal proteins in the random sample of 23 isolates examined. In the three strains of group 1, the average inhibition was 72%. The 15 moderately resistant strains in group 2 had inhibition of ribosomal proteins of 50%, whereas in the 5 resistant group 3 isolates, there was no inhibition of ribosomal protein synthesis. The synthesis of ribosomal protein measured in 80S ribosomes correlated well both with the amount of 5-FU incorporated into the RNA and with the inhibition of RNA synthesis.

Thymidylate synthetase activity and estimation of FdUMP. The presence of thymidylate synthetase activity was demonstrated in all isolates of *C. albicans* tested. Preincubation of the samples with FdUMP inhibited the activity of the enzyme.

The average FdUMP values of the yeast cell homogenates obtained at 5 and 24 h for the three groups are given in Table 3. The subgroup of isolates that had the unusually high DNA synthesis pattern (Fig. 2c) did not have a lower mean FdUMP value in comparison with the group 2 average.

In six isolates, no FdUMP was measurable; unexpectedly, only two of these isolates were in the resistant group 3. The remaining four isolates were in group 2 (see Table 4).

Characteristics of isolates with abnormal patterns. In the highly 5-FC-susceptible group (group 1), all but one strain followed a homogenous pattern (Table 4). This strain had a significantly lower FdUMP concentration but was not distinguishable by any other parameters from the other susceptible strains. In the partially resistant group (group 2), 11 strains exhibited patterns different from the average in the corresponding group. In three strains showing high susceptibility to 5-FC after 24 and 48 h, the amount of 5-FU incorporated into RNA was significantly above average. Conversely, in nine strains showing various degrees of partial resistance and correspondingly low 5-FU incorporation at 24 h, unusually high FdUMP levels were found. From the totally resistant strains (group 3), one strain exhibited a high 5-FU incorporation, and four strains exhibited high levels of FdUMP.

DISCUSSION

The growth inhibition of fungi by antifungal drugs is due to inhibition or impairment of several vital functions. The sequence of these events, however, is not fully understood.

In the case of 5-FC, the antifungal action is believed to be a consequence of intrafungal formation of FURTP and FdUMP, with FURTP being incorporated into RNA and FdUMP inhibiting the DNA synthesis. Whether the two path-

TABLE 3. FdUMP levels in homogenates of *C. albicans* isolates of various 5-FC susceptibilities, exposed to 5-FC

Susceptibility group no.[a]	FdUMP level[b] after incubation for (h):	
	5	24
1 (n = 15)	19.0 (3.9–51.2)	14.2 (5.0–23.6)
2 (n = 48)	8.6 (0–22)	7.9 (2.8–19.2)
3 (n = 12)	5.2 (0–9.9)	3.5 (0.9–13.3)

[a] Group 1: highly susceptible; group 2: moderately susceptible ("partially resistant"); group 3: definitely resistant to 5-FC.

[b] Expressed as picograms of FdUMP per milligram of lyophilate per microgram of total DNA. Values are the average of each group. Numbers in parentheses indicate the range of individual values.

ways are necessarily linked to each other or only one is important, the other being only incidental, is not clear.

In the present paper, an attempt was made to clarify the importance of these pathways by measuring the 5-FU incorporation into RNA as a criterion for the first and by measuring the FdUMP levels as a criterion for the second mechanism.

In the majority of the tested strains (75%), a statistically significant positive correlation was found between the degree of 5-FC susceptibility and the incorporation of 5-FU into the RNA. Additionally, DNA and RNA synthesis and the ribosomal protein synthesis correlated well with both the degree of 5-FU incorporation and the susceptibility of the strains. The correlation between levels of FdUMP and the degree of antifungal activity existed but was less pronounced, and the variation among individuals was more marked. These results suggest that in most strains, both mechanisms are simultaneously active and contribute to the effect of 5-FC in vitro. Whether both pathways are equally important or one of them is decisive and the other only incidental remains unclear.

In 19 strains of group 2 (Fig. 2c and d), the inhibition of nucleic acid biosynthesis did not correlate with the inhibition of growth by 5-FC; the RNA approached control levels after 24 h, whereas the DNA and cell multiplication were inhibited. In these strains, the 5-FU incorporation into RNA was decreased. However, in other strains, the DNA approached control levels after 24 h, and the cell multiplication and RNA remained inhibited. In these strains, the FdUMP level was, surprisingly, not decreased in comparison with the average.

However, in 17 of the 75 strains examined (23%), either the 5-FU incorporation value or the FdUMP levels were inconsistent with the 5-FC susceptibility. In seven strains (9%), the 5-FC susceptibility or resistance in vitro was reflected by a high or low 5-FU incorporation, respectively, whereas the FdUMP levels were always low or absent, irrespective of whether the strain was susceptible or resistant. These results suggest that the incorporation of the

TABLE 4. Characteristics of isolates with abnormal patterns

Susceptibility group no.	MIC of 5-FC in liquid medium (μg/ml)		5-FU incorporation into RNA after 24 h of incubation (μg/mg of RNA)	% Inhibition of 80S ribosomes	FdUMP level after 5 h[a]
	24 h	48 h			
1	0.049	0.049	83		3.9
2	0.18	0.36	113		3.0
	0.09	0.78	52	36	1.9
	0.18	1.57	65		<0.2
	0.18	12.5	24	46	<0.2
	0.36	>100	14		<0.1
	0.09	0.78	13		14.2
	0.18	6.3	20	4	19.6
	0.18	6.3	3.4		13
	0.18	>100	17	73	20.1
	0.36	>100	12	65	12.8
	1.57	>100	6	66	10.8
3	>100		18		0.6
	>100		2	6	9.9
	>100		4	0	7.9
	>100		1.7		9.4
	>100		1.9		9.7

[a] Expressed as picograms of FdUMP per milligram of lyophilate per microgram of total DNA.

antimetabolite 5-FU into RNA was the decisive mechanism in these particular strains, whereas the formation of FdUMP was only incidental.

Moreover, on some occasions, even in totally resistant strains (5%), a high amount of FdUMP could be observed. Because the degree of 5-FU incorporation into RNA was low in these strains, the results suggest the importance of the 5-FU incorporation for the antifungal action of 5-FC.

Conversely, in seven strains (9%), the FdUMP levels correlated with the activity of 5-FC during the first 24 h, whereas the incorporation of 5-FU into RNA was low. In these strains, the FdUMP formation appeared to be responsible for the activity of the drug, at least during the first 24 h, and 5-FU incorporation appeared to be involved in the later (48 to 72 h) development of resistance.

The results suggest that in the majority of strains, both mechanisms of 5-FC activity were involved. The existence of resistant strains with high levels of either 5-FU or FdUMP indicate that in some strains, only one of the possible mechanisms of 5-FC activity may be of significance.

Thymidylate synthetase assay. The FdUMP level in *C. albicans* homogenates may be higher than the value estimated. In the assay of thymidylate synthetase, only free FdUMP within the cell was detectable, because the FdUMP which is irreversibly bound to the *C. albicans* enzyme would not be expected to dissociate. Although the range of measurable FdUMP concentrations was small, the values measured in our susceptible strains were similar to the concentration found for a susceptible strain of *C. albicans* (2) and were consistent with the concentration of FdUMP which was needed to block the endogenous thymidylate synthetase activity, as reported by Diasio et al. (2). The assay used to measure FdUMP in no way implies that the concentration of FdUMP that we measured is that which is needed to block thymidylate synthetase in intact cells—and therefore to have a sufficient impact on DNA synthesis—to explain the cessation of growth. However, because a small range of FdUMP concentrations was needed to block a highly specific activity of thymidylate synthetase, the small differences in FdUMP concentrations measured in susceptible and resistant mutants may be sufficient to decrease the thymidylate synthetase activity in the intact cell.

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