ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1986, p. 756–762
0066-4804/86/110756-07$02.00/0

Vol. 30, No. 5

Noninvasive and Quantitative ^{19}F Nuclear Magnetic Resonance Study of Flucytosine Metabolism in *Candida* Strains

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Received 12 May 1986/Accepted 11 August 1986

^{19}F nuclear magnetic resonance was used for a noninvasive and quantitative study of flucytosine (FC; 5-fluorocytosine) metabolism in two strains of *Candida albicans* and one strain of *Candida tropicalis* with various susceptibilities to FC. Three intracellular fluorinated metabolites were detected in the highly susceptible strain, F-nucleotides (Fnt), F-nucleosides, and 5-fluorouracil (5FU). Fnt were partially converted into 5FU when the spectra of the yeasts were recorded at 37°C without perfusion, but the intensities of the signals were not modified at 4 or 37°C if the cells were perfused. In the acid extract, the Fnt signal was resolved into three distinct peaks; none of them was attributable to 5-fluoro-2′-deoxyuridine-5′-monophosphate. The same signals were detected in the partially resistant strain, but only 5FU was observed in the highly resistant strain; the resistance of the latter strain therefore was primarily due to a defect in UMP pyrophosphorylase. At the end of the incubation period, only FC and released 5FU were present in the culture media. The concentration of the intracellular fluorinated metabolites was increased if the strain was susceptible to FC. The total amount of metabolized FC was very similar for the highly susceptible and the partially resistant strains, but the percentage of Fnt was much higher in the former (38%) than in the latter (8%); the mild resistance of the partially resistant strain therefore was attributed to the decreased activity of UMP pyrophosphorylase.

Flucytosine (FC; 5-fluorocytosine) has gained an important place in the systemic treatment of deep-seated fungal infections in humans, in particular against candidiasis, cryptococcosis, and chromomycosis (16). Investigation of the intrafungal pathway of the drug is an important key to the understanding of the mechanisms of drug action and drug resistance. Previous studies of the FC mode of action have involved invasive techniques requiring extraction procedures and, most often, the use of labeled drugs (5, 15, 19, 20).

We used ^{19}F nuclear magnetic resonance (NMR) to study the mode of action of FC. This noninvasive technique allows a direct analysis of heterogeneous biological samples without prior extraction and does not require a labeled drug, as the observed probe, the ^{19}F nucleus, is an inherent part of the parent drug and its metabolites. Moreover, this technique permits simultaneous identification and quantification of all the fluorinated metabolites present in the analyzed sample (1). We have already applied this methodology to metabolism studies of antineoplastic fluoropyrimidines, 5-fluorouracil (5FU), and 5′-deoxy-5-fluorouridine in human biofluids and cultured tumor cells (12, 13; M. C. Malet-Martino, F. Faure, J. P. Vialaneix, C. Palevody, E. Hollande, and R. Martino, Cancer Chemother. Pharmacol., in press).

Here, we report a quantitative study of FC metabolism in three strains of *Candida* with various susceptibilities to FC by ^{19}F NMR. Since the completion of this study, a similar study has been published by Di Vito et al. (6), although it was directed only toward a qualitative approach. Our data are complementary and generally in agreement with those of Di Vito et al. (6).

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MATERIALS AND METHODS

Chemicals. FC, 5-fluorouridine (FUR), and 5-fluoro-2′-deoxyuridine (FUdR) were generously supplied by Hoffmann-La Roche, Basel, Switzerland. 5FU and 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) were products of Sigma Chemical Co., St. Louis, Mo. Sodium 4-fluorobenzoate (FBEN) was used as an external standard for quantification. It was prepared by titrating 4-fluorobenzoic acid (Fluka, Buchs, Switzerland) with an NaOH solution. The relaxation reagent, chromium(III) acetylacetonate [$Cr(acac)_3$] was a product of Spectrométrie Spin Techniques, Paris, France.

Organisms. The three *Candida* strains used in this study were recent clinical isolates. One *C. albicans* strain (FC MIC, 0.25 μg ml^{-1}; serological type A) was considered highly susceptible, the other *C. tropicalis* strain (MIC, 32 μg ml^{-1}) was considered partially resistant, and the other *C. albicans* strain (MIC, 128 μg ml^{-1}; serological type B) was considered fully resistant (20). MICs were determined by the agar dilution method after 48 h of incubation.

The isolates were routinely maintained on yeast nitrogen base (Difco Laboratories, Detroit, Mich.) containing 0.5% glucose (YNBG) agar at 37°C.

Culture conditions. The growth from YNBG agar after an overnight incubation at 37°C was suspended in 10 ml of YNBG liquid medium to obtain an inoculum size of 10^8 cells ml^{-1}. This inoculum was placed in a 500-ml Erlenmeyer flask containing 240 ml of YNBG liquid medium. The growth was monitored spectrophotometrically at 640 nm. At the beginning of the logarithmic phase, FC was added to the medium to give the desired final concentrations (10 μg of FC ml^{-1} for the highly susceptible *C. albicans* strain, 20 or 200 μg of FC ml^{-1} for the partially resistant *C. tropicalis* strain, and 200 μg of FC ml^{-1} for the highly resistant *C. albicans* strain), and

the culture was incubated at 37°C under slight agitation for an additional 16 h. The cultures used consisted of the pure yeast phase of the *Candida* strains.

Preparation of samples for ^{19}F NMR analysis. At the end of the culture, cells were harvested by centrifugation (10 min, 1,500 × *g*, 20°C). A fraction of the culture medium was retained for a subsequent ^{19}F NMR analysis. Cells were washed twice with a citrate-phosphate buffer (citric acid, 1.8×10^{-2} M; $Na_2HPO_4 \cdot 2H_2O$, 1.65×10^{-1} M [pH 7.0]), and the cell pellet obtained after centrifugation (10 min, 1,500 × *g*, 4°C) was immediately placed into an NMR tube.

In some experiments, an internal perfusion system that maintains cell viability was used. Culture medium was put into a 50-ml syringe (Glenco; Techmation, Paris, France) and positioned on an infusion pump (Precidor; Infors AG, Basel, Switzerland). It was injected at a rate of 0.03 ml min^{-1} at the bottom of the NMR tube through a polyethylene catheter (inside diameter, 0.6 mm; Biotrol Pharma, Paris, France). Excess medium was removed under suction through a similar catheter capped with a Millipore membrane filter (0.45 μm pore size) to retain the cells and positioned outside the region detected spectroscopically. The cell viability was determined by the CFU method (see below).

In some experiments, prior to the ^{19}F NMR analysis, perchloric acid (PCA) extracts of highly susceptible *C. albicans* yeasts were prepared. The cell pellet was stirred in 4 ml of 1.3 M PCA for 30 min at 4°C; the mixture was then centrifuged (10 min, 2,000 × *g*, 4°C), and the deposit was extracted with 2 ml of 1.3 M PCA; the latter procedure was repeated five times. The six pooled supernatants were neutralized by the addition of 4 M KOH–0.4 M KH_2PO_4. After 5 min at 4°C to achieve the precipitation of $KClO_4$, the suspension was cleared by centrifugation and the supernatant was lyophilized. For the ^{19}F NMR analysis, the lyophilysate was dissolved in 1 ml of citrate-phosphate buffer. The PCA-insoluble fraction was also collected and studied by ^{19}F NMR.

Yeast counts. To assess the efficiency of the perfusion system and for ^{19}F NMR quantification (see below), the *Candida* cells were counted by the CFU method. The number of viable cells was determined by plating an appropriate dilution of the cell pellet obtained after centrifugation on YNBG agar and then counting the colonies after incubation for 72 h at 37°C. This counting was done immediately before and after the ^{19}F NMR analysis to check the validity of the perfusion system and only before the NMR experiment for quantification.

Analytical method. ^{19}F NMR spectra were recorded at 250 MHz on a Cameca 250 FT spectrometer interfaced with a 16-kilooctet memory Nicolet 80 computer. Spectra were run without proton decoupling and with no frequency field lock in 5-mm-diameter NMR tubes. The magnetic field was shimmed by using the ^1H NMR resonance of water observed in the continuous wave mode. The signals were referenced to the resonance peak of an external reference, CF_3COOH (5 g $liter^{-1}$ aqueous solution, 25°C). For any biological sample studied and for the CF_3COOH reference, the H_2O proton signal was always positioned at the same frequency to obtain reproducible chemical shifts. The instrumental settings were established as follows: probe temperature, 4, 25, or 37°C; sweep width, 33,333 or 11,111 Hz; pulse width, 1.6 μs (i.e., flip angle $\alpha \simeq 40°$); recycle time, 2 s; computer resolution, 4.1 or 1.4 Hz per point; line broadening caused by exponential multiplication, 20 Hz for spectra of cells or 2 Hz for spectra of culture media and PCA extracts; receiving filter, out. The spectra usually were acquired in blocks of 3,000 scans.

In the studies in nonperfused systems, quantification of the intracellular metabolites was done (quantification and perfusion were impossible to perform together because of the small diameter of the tubes accepted in the NMR probe). The spectrometer was modified to obtain a sufficient spectral density of the radiofrequency pulse and a correct receiver bandwidth (2). For this quantitative purpose, a capillary containing the FBEN standard [aqueous solution of FBEN (7.4×10^{-2} M) and $Cr(acac)_3$ (2.7×10^{-3} M)] was inserted coaxially in the NMR tube. The spin-lattice relaxation time of FBEN in this aqueous solution was 1.4 s. Using a pulse width of 1.6 μs, we verified that a recycle time of 2 s allowed a good quantification of this compound (2). We did not measure the spin-lattice relaxation time of the different intracellular fluorinated metabolites but, in an experiment with *Candida* yeasts at 4°C (the temperature at which there is no change in the intensities of the signals), we verified that a value of the recycle time of >2s does not modify the intensity of the F-nucleotide (Fnt) and F-nucleoside (Fns) signals. It was previously reported (Malet-Martino et al., in press) that this recycle time was also sufficient for the estimate of 5FU in tumor cells. The recording conditions of our ^{19}F NMR study therefore allowed a correct quantification of the various intracellular fluorinated metabolites in the analyzed cellular suspension.

The different fluorinated metabolite concentrations were determined from the intensities of their respective NMR signals. These intensities were estimated by comparing the expanded areas of the NMR signals (expanded scale, 65 Hz cm^{-1}) with that of FBEN. The areas were determined after the different signals were cut out and weighed. The concentration of an intracellular fluorinated metabolite x (C_x) is given by:

$$C_x = \frac{N_{st}}{V} \times \frac{A_x}{A_{st}}$$

where A_x and A_{st} are the areas of x and standard (st) (FBEN) signals, respectively; N_{st} is the number of moles of the standard (FBEN) present in the detection zone of the NMR probe; and V is the volume of the cellular pellet in this zone. The capillary calibration, i.e., the determination of the ratio of N_{st}/V, was done from earlier NMR analyses of 5FU solutions of known concentrations (concentration range, 10^{-3} to 10^{-4} M), into which the capillary was inserted. The value of the ratio of N_{st}/V was constant to ±5%. We verified that the total concentration of the intracellular fluorinated metabolites did not change during the NMR recording. This indicates that there was no decantation in the NMR tube. The concentrations of the different intracellular fluorinated metabolites determined by NMR (millimoles per gram of cellular suspension) were expressed in picomoles per 10^6 cells, considering the number of cells per gram of cell pellet.

For the quantification of the extracellular 5FU, a solution of $Cr(acac)_3$ (80 μl, 2.7×10^{-3} M) was added into a fraction (800 μl) of the culture medium after 16 h of incubation with FC. We verified that the recycle time used (2 s) was sufficient to obtain a good estimate of FC and 5FU, the only fluorinated compounds detected in the culture media. The percentage of 5FU was estimated by integration. The amount of released 5FU was thus determined from the amount of FC and 5FU in the culture medium (initial amount of FC − amount of intracellular fluorinated metabolites). It was ex-

pressed in picomoles per 10^6 cells, considering the total number of cells at the end of the culture.

The accuracy of the NMR assays was 5 to 10%, depending on the signal-to-noise ratio of the peaks.

RESULTS

Highly susceptible *C. albicans* strain: nonperfused studies. In Fig. 1 is shown the ^{19}F NMR spectrum obtained when the highly susceptible *C. albicans* strain was treated with FC (10 μg ml^{-1}) for 16 h and recorded at 4°C for 6 h without perfusion. Three peaks corresponding to intracellular fluorinated metabolites were observed, one located at a ^{19}F chemical shift (δ) of −93.8 ppm, which is consistent with 5FU, another peak located at a δ of −90.4 ppm corresponding to Fns (FUR or FUdR), and the major peak at a δ of −89.4 ppm corresponding to Fnt. Peak assignments were obtained from an experiment in which the untreated yeasts were disrupted with a French press, and the cell lysate obtained (>90% broken cells) was spiked with small amounts of standard compounds (FC, 5FU, FUR, FUdR, and FdUMP). The δ for FUR and FUdR are identical (11; Malet-Martino et al., in press), and those of the various Fnt are close (11, 18). It is therefore only possible to assign the resonances at a δ of −90.4 ppm and a δ of −89.4 ppm to Fns (and not to phosphorylated derivatives of 5FU as reported by Di Vito et al. [6]) and Fnt, respectively. In two of the six experiments, a fourth low signal (δ = −92.4 ppm) corresponding to FC was detected. During the NMR recording at 4°C, we found no significant change in the proportions of the various fluorinated metabolites.

When the spectrum of the same strain of *C. albicans*, similarly treated with FC, was recorded at 37°C without perfusion, the same signals were observed (Fig. 2a and b). In all the spectra recorded at 37°C, the chemical shifts of the different intracellular fluorinated metabolites were deshielded by 0.3 to 0.5 ppm with regard to the δ observed for the spectra recorded at 4°C (this value must be ≈0.2 ppm higher due to the deshielding of the H_2O proton signal when the probe temperature is raised from 4 to 37°C). During NMR recording at 37°C, a rapid change in the metabolite proportions was noticed; the Fnt peak decreased in favor of the 5FU signal, and the Fns resonance was constant. 5FU represented 22% of the fluorinated metabolites between 0 and 1.5 h of NMR recording (Fig. 2a), as opposed to 41% between 1.5 and 3 h (Fig. 2b).

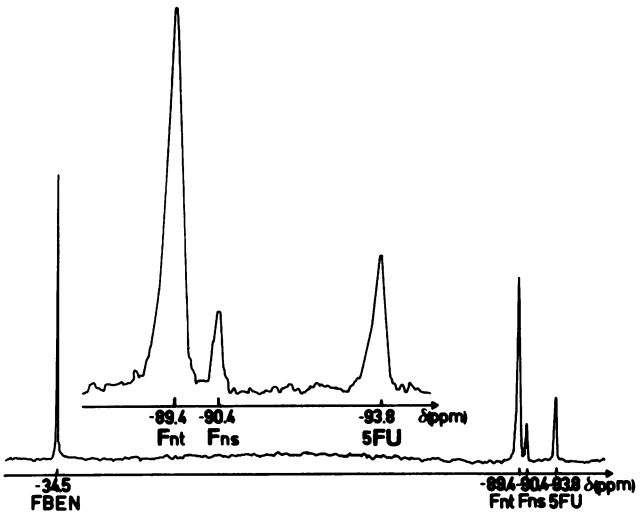

FIG. 1. ^{19}F NMR spectrum of a highly susceptible *C. albicans* strain treated with FC (10 μg ml^{-1}) for 16 h and recorded at 4°C without perfusion for 6 h (number of scans, 12,000). FBEN was the external standard for quantification. The resonance positions are referenced to an external reference, CF_3COOH (5-g $liter^{-1}$ aqueous solution), resonance peak.

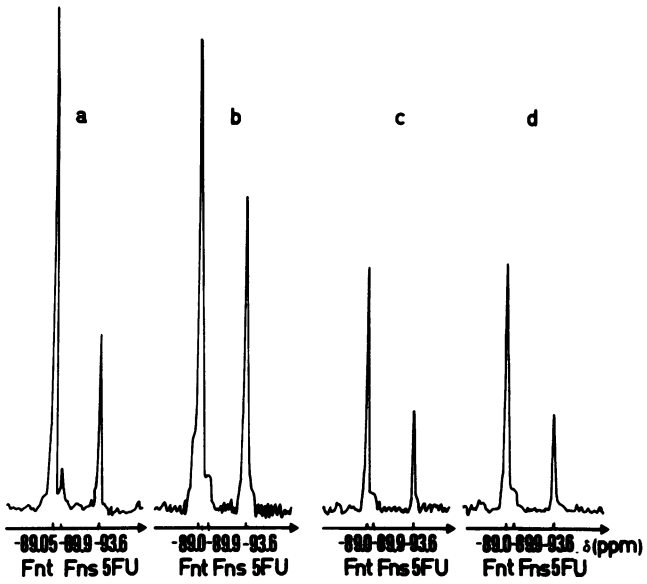

FIG. 2. ^{19}F NMR spectra of a highly susceptible *C. albicans* strain treated with FC (10 μg ml^{-1}) for 16 h and recorded at 37°C without (a and b) or with (c and d) perfusion. Time of NMR recording: 0 to 1.5 h (a and c), 1.5 to 3 h (b), and 3 to 4.5 h (d). The time of each NMR recording block was 1.5 h, and the percentages of the various metabolites are considered to be representative of the state of the metabolism 45 min after the beginning of each NMR recording block. The resonance positions are referenced to an external reference, CF_3COOH (5-g $liter^{-1}$ aqueous solution), resonance peak.

The results of the quantitative study at 4°C are presented in Table 1. The total concentration of the intracellular fluorinated metabolites was high (≈4,600 pmol/10^6 cells). The proportion of Fnt (≈78%) was superior to that of 5FU (≈17%) and Fns (≈5%). The absolute amounts of drug uptake showed a reasonable variation among the experiments. The average standard error was ±15%.

Highly susceptible *C. albicans* strain: perfused studies. During the NMR recording, the *C. albicans* cells that had previously been incubated with FC for 16 h were perfused with culture medium at 37°C for 4.5 h. Two major signals were observed on the ^{19}F NMR spectra (Fig. 2c and d), one attributed to 5FU (δ = −93.6 ppm), the other to Fnt (δ = −89.0 ppm). There was also a small shoulder at the base of the Fnt signal which may be attributable to Fns (δ = −89.9 ppm). During the NMR recording of the perfused cells, no significant variation in the proportions of the intacellular fluorinated metabolites was noticed; 5FU represented ≈25% of all the metabolites between 0 and 1.5 h and ≈20% between 3 and 4.5 h (Fig. 2c and d).

Highly susceptible *C. albicans* strain: PCA extracts. In the ^{19}F NMR spectrum of the PCA-soluble fraction (Fig. 3b), three signals were observed for Fnt (δ = −89.0, −89.1, −89.4 ppm); the most shielded did not correspond to FdUMP because a signal at a δ of −89.3 ppm appeared when the acid extract was spiked with FdUMP (Fig. 3a). A minor

TABLE 1. Intracellular concentrations of FC metabolites in highly susceptible, partially resistant, and highly resistant strains of *Candida* after 16 h of exposure to FC

Strain	Highly sensitive *C. albicans* MIC=0.25 μg ml^{-1}	Partially resistant *C. tropicalis* MIC=32 μg ml^{-1}		Highly resistant *C. albicans* MIC=128 μg ml^{-1}
5FC concentrations	10 μg ml^{-1}	20 μg ml^{-1}	200 μg ml^{-1}	200 μg ml^{-1}
Total intracellular concentrations of fluorinated metabolites (picomoles per 10^6 cells)[a,b]	4,600	800	700	25
Percentages of the different intracellular fluorinated metabolites[a,c]	16.8 / 5.0 / 78.2	13.7 / 5.0 / 81.3	25.7 / 8.5 / 65.8	100

[a] ^{19}F NMR spectra were recorded at 4°C for 6 h. Each result is the mean of two or three experiments.

[b] The intracellular concentration estimates are a lower limit of the real intracellular concentration since the fluorinated metabolites bound to macromolecules were not detected under our experimental conditions.

[c] Symbols: ▩, 5FU; ■, Fns; □, Fnt.

signal corresponding to 5FU was also detected, but the resonance of Fns was not observed (Fig. 3).

The ^{19}F NMR spectrum of the PCA-insoluble fraction showed no large signal characteristic of fluorinated metabolites bound to macromolecules, even after 12.5 h of NMR recording (25,000 scans).

Partially resistant *C. tropicalis* strain. *C. tropicalis* isolates were treated with two doses of FC (20 and 200 μg ml^{-1}) for 16 h. The ^{19}F NMR spectrum recorded at 4°C showed the same signals as those described above for the highly susceptible *C. albicans* strain, i.e., 5FU, Fns and Fnt; the Fnt signal was also predominant. The concentrations of the various fluorinated metabolites were very similar (700 and 800 pmol/10^6 cells) when the *C. tropicalis* strain was treated with 200 or 20 μg of FC ml^{-1} (Table 1). The proportions of the various intracellular fluorinated metabolites in the *C. tropicalis* strains were similar to those established for the highly susceptible *C. albicans* strain.

Highly resistant *C. albicans* strain. When *C. albicans* was treated with FC (200 μg ml^{-1}) for 16 h, only the 5FU signal was detected in the ^{19}F NMR spectrum recorded at 4°C. The intracellular concentration of this metabolite was very low (25 pmol/10^6 cells) (Table 1).

^{19}F NMR study of the culture media after incubation with FC. The ^{19}F NMR spectrum of the growing media of the various *Candida* strains after the 16-h incubation period with FC showed only the signals corresponding to FC and 5FU. The concentration of 5FU released into the culture medium was higher for the partially resistant strain (≈7,700 pmol/10^6 cells) than for the highly susceptible (≈4,700 pmol/10^6 cells) and the highly resistant (≈2,700 pmol/10^6 cells) strains. However, the concentrations of the total metabolized FC were close in the highly susceptible and partially resistant strains (≈9,300 and ≈8,500 pmol/10^6 cells, respectively) and superior to that estimated for the highly resistant strain (≈2,700 pmol/10^6 cells, with the intracellular fluorinated metabolite concentration [25 pmol/10^6 cells] being negligible).

DISCUSSION

The biochemical mechanism of action of FC has been the subject of several studies (5, 15, 19, 20). The intrafungal pathway of this drug is presented in Fig. 4. It has been suggested that the activity of FC after uptake by the fungi and intracellular deamination is a consequence of the intrafungal formation of two metabolites, 5-fluorouridine triphosphate (FUTP), which is incorporated into RNA in place of UTP, and FdUMP, which is a potent inhibitor of thymidylate synthetase (TS), a key enzyme of the DNA synthesis.

The ^{19}F NMR spectra of highly susceptible *C. albicans* or partially resistant *C. tropicalis* strains treated with FC generally show three signals corresponding to free intracellular fluorinated metabolites, 5FU, Fns, and Fnt. As can be concluded from the spectra of the PCA extracts of highly susceptible *Candida* yeasts (Fig. 3), the signal of Fnt in intact cells contains contributions from the different ribo- or deoxyribonucleotides or both. The intracellular Fns, FUR, and FUdR give identical resonances (11; Malet-Martino et al., in press). This difficulty in resolving the Fnt and Fns signals in intact cells is one of the drawbacks of the ^{19}F NMR methodology applied to this kind of study.

It has been reported that in *Saccharomyces cerevisiae* no

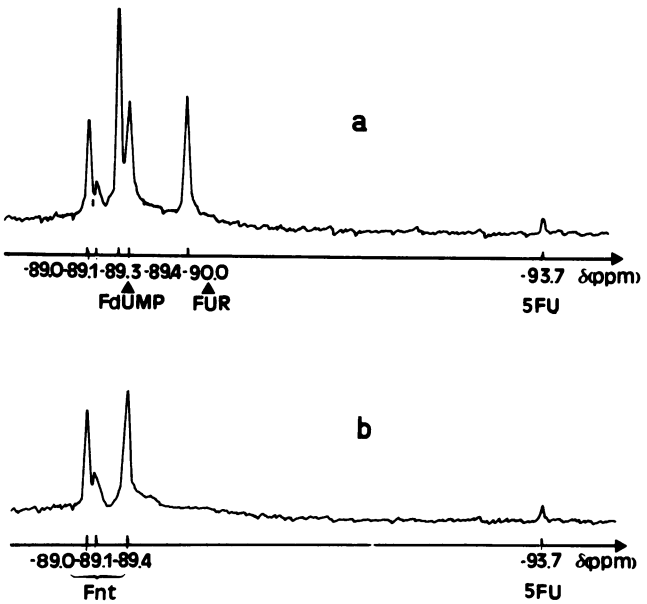

FIG. 3. ^{19}F NMR spectra of PCA extracts of a highly susceptible *C. albicans* strain after 16 h of FC treatment (10 μg ml^{-1}) (b) and spiked with FUR and FdUMP standards (a) (probe temperature, 25°C). The resonance positions are referenced to an external reference, CF_3COOH (5-g liter^{-1} aqueous solution), resonance peak.

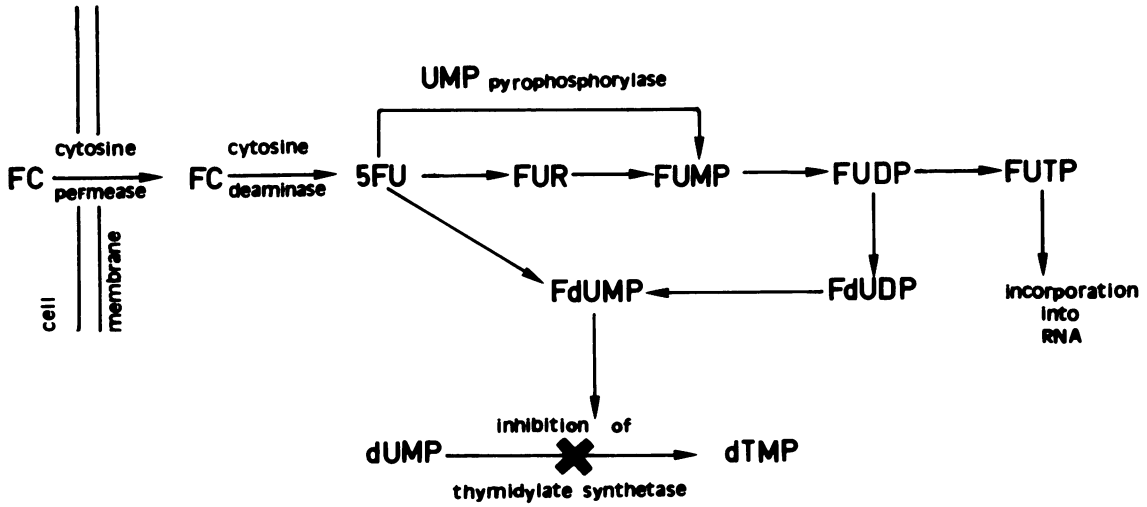

FIG. 4. Intrafungal pathway and mode of action of FC (from Scholer [16]). FUR is an Fns; FUMP, FUDP, FUTP, FdUMP, and FdUDP are Fnt.

FUR is formed during the FC pathway but 5FU is anabolized directly to 5-fluorouridine monophosphate (FUMP) by means of UMP pyrophosphorylase (10). The results of this study show that in highly susceptible and partially resistant *Candida* strains treated with FC, FUR (or FUdR, as ^{19}F NMR does not allow their differentiation) is present. However, because of the absence of the Fns signal in the PCA extracts of the highly susceptible *C. albicans* strain (Fig. 3), the Fns detected in intact cells probably come from an Fnt conversion between the end of the culture and the beginning of the ^{19}F NMR recording. The same reason can be evoked for a part of 5FU, as the 5FU/Fnt ratio is smaller in the ^{19}F NMR spectra of PCA extracts than in the spectra of intact cells recorded at 4°C.

In some experiments with the highly susceptible *C. albicans* strain, a low percentage of FC (≈1% of the intracellular fluorinated metabolites) was detected even after 16 h of incubation with this drug. This may seem surprising, as it has been reported that both transport through the fungus cell membrane and intracellular deamination take place rapidly (16); a large amount of radioactivity was found in the internal pool of *C. albicans* cells during the uptake phase, i.e., up to about 1 to 2 h after exposure to FC (14). We therefore studied the culture medium after 16 h of incubation with FC; a high percentage of residual FC (74%) was found compared with the percentage of 5FU excreted from the cells (26%). The results with the highly susceptible *C. albicans* strain may mean that the FC uptake or the FC deamination rates (or both) are lower than reported in the literature or that FC is constantly recycled across the cell membrane, as has been described for cytosine in *S. cerevisiae* (4).

A progressive conversion of Fnt into 5FU was observed during the NMR recording only when the *C. albicans* cells were studied at 37°C without perfusion (Fig. 2). This transformation stresses the necessity of a rapid ^{19}F NMR analysis in correct conditions of perfusion. We wished to demonstrate the efficiency of the perfusion by comparing the viability of *C. albicans* cells under ^{19}F NMR analysis conditions (6 h at 37°C) with and without perfusion and with and without FC treatment. The viability was complete whether or not the yeasts (treated or not) were perfused. This indicates that excellent viability is not a sufficient criterion to judge the physiological state of the cells, as a conversion of Fnt into 5FU occurs in the nonperfused yeasts which, however, have excellent viability. Nevertheless, the absence of Fnt conversion in perfusion studies emphasizes the interest of perfusion. Only an evaluation of the energetic metabolism of the yeasts by ^{31}P NMR could really demonstrate the normal functioning of the cells and, therefore, the efficiency of the perfusion system.

In *Escherichia coli* cells, the RNA-incorporated FUTP gives a very broad ^{19}F NMR signal which is located between 1 and 10 ppm downfield from 5FU (7, 9); this resonance region also corresponds to that of Fns and Fnt. In tumor cells, FdUMP forms a ternary covalent complex with the enzyme TS and with the cofactor 5,10-methylenetetrahydrofolate (8); the native complex prepared in vitro gives a broad ^{19}F NMR resonance at $\delta = -102.1$ ppm from external CF_3COOH (3). Under the experimental conditions of this study, we were unable to detect these signals even when the ^{19}F NMR analysis was performed with the insoluble fraction of PCA extracts of highly susceptible *C. albicans* cells treated with FC. This emphasizes a second drawback of the ^{19}F NMR method: its low intrinsic sensitivity. With the spectrometer used in our study and with *Candida* cells, the limit of sensitivity was about 10 pmol/10^6 cells for a compound with a narrow ^{19}F NMR signal and much higher for a compound bound to a macromolecule. Polak and Scholer (15) reported that incorporation of [2-^{14}C]FC into RNA after 24 h of incubation was about 60 ng/10^6 cells in *C. albicans* strains with normal FC susceptibility (MIC, <1.0 μg ml^{-1}), which corresponds to approximately 450 pmol of FUTP incorporated per 10^6 cells. Moreover, our ^{19}F NMR study was conducted after 16 h of incubation; Waldorf and Polak (20) recently demonstrated that the average incorporation of 5FU into RNA of highly susceptible *C. albicans* strains increased during a 48-h incubation period. The concentration of FUTP incorporated into RNA may therefore not have been maximal in our study. The weak concentration of FUTP incorporated into RNA and the width of its NMR signal suggest that we may not have detected it under our experimental conditions. It is possible that a recycle time similar to that used by Gochin et al. (7) for *E. coli* cells (≈0.1 s) and Keniry et al. (11) for tumor cells (0.15 s), i.e. much shorter than that used in our study (2 s), would have enabled the broad signal of FUTP incorporated into RNA to become

detectable over the 6-h period of NMR recording. However, it would have saturated partially the narrow signals of the free intracellular fluorinated metabolites, and their quantification would therefore have been more difficult. For FdUMP bound to TS, its concentration in yeasts has not, to our knowledge, been reported in the literature; studies made on 5FU-sensitive tumor cells indicate that the concentration of the ternary complex is too low to be detectable by ^{19}F NMR ($<<$ 1 pmol/10^6 cells) (17).

If the absolute quantification of the various fluorinated metabolites by ^{19}F NMR in biological fluids is relatively simple, provided that a few precautions have been taken (2), it is more difficult in cell cultures. It is necessary to make sure that the medium under study does not change during the NMR measurement, in other words, that there is no decantation in the NMR tube. To this end, we verified that the total intracellular concentration of fluorinated compounds measured every 1.5 h throughout the NMR experiment was constant; this demonstrates that there was no change in the density of the medium during the NMR experiment. We therefore assumed that the count of the cell pellet obtained after centrifugation is representative of the cellular density of the medium analyzed by NMR. This allowed us to convert the fluorinated metabolite concentration determined by NMR (millimoles per gram of cellular suspension) to the intracellular concentration (picomoles per 10^6 cells) by considering the number of cells per gram of cell pellet. Because the fluorinated metabolites bound to macromolecules were undetected under our experimental conditions, the intracellular concentrations thus determined represent only those of the free fluorinated metabolites. The total intracellular concentration reported is therefore a lower limit of the real total intracellular concentration.

^{19}F NMR methodology applied to the qualitative study of the metabolism of FC in *Candida* yeasts (or other microorgaisms) may become a tool for testing the susceptibility of various strains to FC. From the results presented in Table 1, it can be concluded that the more susceptible the *Candida* strain, the greater the intracellular concentration of fluorinated metabolites. With our spectrometer we were not able to measure the active anabolites, i.e., FUTP incorporated into RNA and FdUMP bound to TS; however, the measurement of unbound Fnt levels can be a criterion for an easy estimation of the susceptibility of a strain.

Resistance of *Candida* yeasts to FC is attributable to the following mechanisms: (i) deficiency in cytosine permease, (ii) deficiency in cytosine deaminase, (iii) deficiency in UMP pyrophosphorylase, and (iv) defect in regulation of de novo pyrimidine synthesis which would result in overproduction of endogeneous pyrimidine nucleotides and competition with the fluorinated pyrimidine nucleotides (10, 15, 21). ^{19}F NMR methodology may also be useful in defining the mechanism of resistance of a strain to FC. For the highly resistant *C. albicans* strain studied, only 5FU was observed and Fnt were undetected (in the limit of sensitivity of our spectrometer). This indicates that the resistance of this strain is primarily due to a defect in UMP pyrophosphorylase. Because an important feature of resistant *Candida* strains is the release of 5FU into the growth medium (15), we determined the amount of released 5FU; the total amount of metabolized FC therefore could be estimated ($\simeq$2,700 pmol/10^6 cells). It was much lower than the amounts obtained for the two other strains (although the strains were treated with different doses of FC, this comparison could be done because the amount of unmetabolized FC in the culture medium after the 16 h of incubation was always high). A deficiency in cytosine permease, cytosine deaminase, or both (if FC is constantly recycled across the cell membrane) might therefore also explain the resistance of this strain. The total amounts of metabolized FC are similar for the partially resistant *C. tropicalis* ($\simeq$8,500 pmol/10^6 cells) and the highly susceptible *C. albicans* ($\simeq$9,300 pmol/10^6 cells) strains. However, Fnt for these two strains represent $\simeq$8 and $\simeq$38%, respectively, of the total fluorinated metabolites. These results show that decreased activity of UMP pyrophosphorylase rather than defective FC uptake accounts for the mild resistance of the *C. tropicalis* strain.

In conclusion, despite some drawbacks (low sensitivity, unresolution of the signals of the different Fnt or Fns in intact cells), the noninvasive and quantitative ^{19}F NMR methodology has provided useful information concerning the metabolism of FC in susceptible and resistant *Candida* strains.

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