Prevention of 5-Fluorouracil-Caused Growth Inhibition in *Sordaria fimicola*

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Growth (dry weight accumulation) of *Sordaria fimicola* in standing liquid culture (sucrose-nitrate-salts-vitamins) is inhibited by the presence of 5 μ M 5-fluorouracil in the medium. This inhibition is completely prevented by uracil, deoxyuridine, and 5-bromouracil, partly prevented (40 to 90% of growth observed without 5-fluorouracil) by uridine, thymidine, and 5-bromodeoxyuridine, and slightly prevented by trifluorothymine, cytosine, cytidine, deoxycytidine, and 5-methylcytosine (all at 0.5 to 1 mM). Thymidine and thymine riboside were without any apparent effect. Growth is also inhibited by 0.2 mM 6-azauracil, and this inhibition was completely prevented by uracil and uridine, partly prevented by deoxyuridine, 5-bromouracil, cytidine, and 5-methylcytosine, and slightly prevented by thymine, thymidine, 5-bromodeoxyuridine, cytosine, and deoxycytidine. The data suggest that the observed inhibition of growth by 5-fluorouracil is due to inhibition of both ribonucleic acid and deoxyribonucleic acid synthesis. The data also allow inferences concerning pyrimidine interconversions in *S. fimicola*; i.e., thymine can be anabolized to thymidylic acid without first being demethylated, although demethylation appears to occur also.

There is much practical importance in knowing the pathways of nucleic acid precursor interconversions for the design of selective anticancer and antimicrobial drugs. The invention of the anticancer drug 5-fluorouracil (FU) followed the recognition that cancer cells use uracil for nucleic acid biosynthesis to a greater extent than normal cells (5). The success of the antimycotic drug 5-fluorocytosine appears to depend on the ability of fungi to deaminate the drug to FU and the inability of mammalian cells to do so (10).

In our laboratory we have been attempting to elucidate the modes of action of FU in the inhibition of growth and perithecial formation in *Sordaria fimicola*. One of the procedures employed has been to investigate the ability of various pyrimidines and pyrimidine derivatives to prevent FU inhibition. On the basis of these data we also have been able to propose a scheme for the interconversions of some pyrimidine metabolites in this fungus.

(This paper is based on a portion of the Ph.D. thesis of H. F. Schoen [City University of New York, 1975].)

MATERIALS AND METHODS

Organism. A wild-type strain of *S. fimicola* (rob.) Ces. et de Not. was obtained from the Ameri-

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can Type Culture Collection (culture 14517) and was maintained on slants of basal medium solidified with 2% agar. Transfers were allowed to grow and develop at room temperature (20 to 28°C) until mature perithecia were present, about 6 days, and then stored at 4 to 6°C.

Medium. A sucrose-nitrate medium, with thiamine and biotin added, modified slightly from that of Bretzlöff (1), was the basal medium used in all experiments. It contained (in 1.00 liter of glass-distilled water): sucrose, 20.0 g; KNO₃, 1.0 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.12 g; FeCl₃, 0.1 mg; MnSO₄·4H₂O, 0.4 mg; CuSO₄·5H₂O, 0.4 mg; Zn(NO₃)₂·6H₂O, 0.9 mg; thiamine-hydrochloride, 0.5 mg; and biotin, 12.5 μ g. The pH of the medium is 4.6 before autoclaving and was not adjusted except for experiments in which additions to the medium altered the pH, in which cases it was readjusted to 4.6 with HCl.

The medium was autoclaved at 121°C for 15 min. Additions to the basal medium were incorporated into the medium before autoclaving, except for trifluorothymine solutions, which were filter sterilized separately and then added to the cooled, autoclaved medium.

Inoculum and incubation conditions. The inoculum was prepared by transferring scrapings from a stock slant to a 250-ml Erlenmeyer flask containing 50 ml of basal medium, allowing this to grow without shaking for 5 to 6 days, and then blending in a Virtis homogenizer.

Experimental cultures contained 40 ml of medium in a 250-ml Erlenmeyer flask and were inoculated with 0.4 ml of the blended inoculum. The flasks were

incubated without shaking at $24 \pm 2^\circ\text{C}$ in constant fluorescent white light (Sylvania Powertube, Cool White, FA8T12-CW-VHO).

Dry weight determination. The mycelium was collected on a tared filter paper circle in a Büchner funnel, washed twice with water, dried overnight in an oven at 90°C , and weighed.

RESULTS

Table 1 gives the results of a series of experiments on the prevention of FU inhibition of growth by various pyrimidines and derivatives. Uracil and deoxyuridine were the most effective compounds; at 0.50 mM they completely prevented the growth (dry weight accumulation) inhibition by 5.0 μM FU. Uridine was less effective. Thymine was also quite effective but, at 5.0 μM FU, the effect reached a plateau at 0.50 mM at less than complete restoration of growth. In the presence of 10 μM FU, thymine

was more effective at 1.0 mM than at 0.50 mM, but the response was still less than complete. The thymine nucleosides were apparently without any effect.

Some analogues of thymine also were tried as preventives. 5-Bromouracil (bromouracil) was even more effective than thymine, and 5-bromodeoxyuridine (bromodeoxyuridine) was quite effective even though thymidine was not.

Finally, cytosine, its nucleosides, and 5-methylcytosine showed a slight preventive effect.

Dry weights were determined also in older cultures (15 to 25 days old) (data not shown). The overall pattern of results was similar to that seen in the 5-day-old cultures. The percentage of inhibition by FU alone was less. Where the growth as a percentage of control for a preventive was high (as, for example, with 0.50 mM thymine), there was in general no

TABLE 1. Prevention of FU inhibition by pyrimidines and pyrimidine nucleosides

FU concn (μM)	Base series	Base series concn (mM)	Growth (%) ^a		
			Free base	Ribonucleoside	Deoxyribonucleo- side
5.0	None		10.6 $\pm$ 6.7		
	Uracil				
		0.0010	15.3 $\pm$ 1.8	— ^b	—
		0.0050	24.7 $\pm$ 1.0	13	19
		0.050	76.5	21	47
		0.50	113 $\pm$ 33.2	28.8 $\pm$ 8.0	90.6 $\pm$ 28.9
		1.0	87	51.3 $\pm$ 32.9	109
	Thymine	0.0050	0	—	—
		0.050	26.3 $\pm$ 0.4	—	—
		0.50	65.1 $\pm$ 14.4	—	—
		1.0	68.0 $\pm$ 5.3	5.5	19 $\pm$ 9.9
	Bromouracil	0.0050	20	—	5.0
		0.050	39	—	9.5
		0.50	95.0	—	51.0
		1.0	87.0 $\pm$ 16.3	—	82.5
	Azathymine	1.0	14.3 $\pm$ 0.4	—	—
	Trifluorothymine	0.75	28	—	—
		1.0	32.8 $\pm$ 3.2	—	—
	Cytosine	0.50	26	27	22
		1.0	38	—	—
	5-Methylcytosine	0.50	28	—	—
10	None		6.5 $\pm$ 3.1		
	Thymine	0.050	12	—	—
		0.10	17 $\pm$ 1.4	—	—
		0.50	37	—	—
		1.0	58.5 $\pm$ 16.3	—	—

^a Dry weight after 5 days, as percentage of control without FU, plus or minus the standard deviation of the mean of two replicates where experiment was repeated.

^b —, Data not available.

further increase, relative to the control, in older cultures. Where the degree of reversal was smaller (as with 0.0050 mM uracil), there was an increase in the percentage dry weight in older cultures. However, since the culture with FU alone also increased in percentage dry weight, there was no increase in the relative degree of prevention (in fact, there was usually a decrease).

There was, however, one exception to this pattern. The growth, as percentage of control, with deoxyuridine typically decreased in older cultures (for 0.50 mM deoxyuridine, the average in cultures aged 15 to 25 days was 72%, and for 1.0 mM it was 76%).

In general, the degree of perithecial development was correlated with the degree of restoration of growth. Even in cases where growth restoration was complete, however, there were usually noticeable differences in overall appearance between the control and the cultures with additions. The only preventive restoring the appearance of the culture to one identical to the control was uracil at 0.50 mM and above; 1.0 mM uridine gave almost normal perithecial development, but the overall appearance of the culture was still noticeably different from the control. Thymine and uridine together also gave an appearance like that of the control (see below).

Two additional types of experiments were performed to check on the specificity of the preventive effect. In one, the ability of purines and a purine ribonucleoside (inosine) at 100 times the molarity to prevent inhibition by 5 μ M FU was tested. They were totally without effect. In the other, several pyrimidine compounds at 0.50 mM were tested in the absence of FU for their effect on growth. Uracil, uridine, deoxyuridine, and bromouracil were slightly stimulatory (ca. 20% more growth after 5 days than with no additions), and thymine and bromodeoxyuridine were without effect. At 18 days no effects were seen on growth, but bromouracil and bromodeoxyuridine inhibited perithecial development somewhat. (Cytosine compounds were not tested in this manner.)

To account for the effects of uptake and usage peculiarities in *S. fimicola* of these compounds, the ability of the above compounds to prevent the inhibitory effects of the inhibitor of pyrimidine anabolism 6-azauracil (azauracil) was tested. Azauracil inhibits the de novo synthesis of pyrimidines (14). The ability of a compound to prevent the inhibition would indicate: (i) that the compound was taken up and anabolized to the extent that it could replace the needed components that were not being made

de novo because of the inhibitor, or (ii) that it interferes with the transport of the inhibitor itself and exerts its preventive effect that way. The procedure is not foolproof, as intermediate steps involved in the interconversions are not known for *S. fimicola*.

The results of the experiment with azauracil are given in Table 2. Surprisingly, all the pyrimidines tested gave some stimulation of growth compared to azauracil alone. Uracil and uridine completely prevented inhibition; deoxyuridine was less effective. Thymine gave relatively less growth here than in the FU experiments; bromouracil, as in the FU experiments, was more effective than thymine, but bromodeoxyuridine was much less effective than in the FU experiments.

One additional experiment was performed to try to determine whether the effects of thymine and deoxyuridine on FU inhibition are separable from the effects of uridine. That is, do thymine and deoxyuridine stimulate growth by a different mechanism (presumably by providing an increased endogenous supply of thymidylic acid) than uridine (presumably by increasing the endogenous supply of uridylic acid)? The experiment consisted of testing the effects of combinations of thymine, uridine, and deoxyuridine. The results are shown in Table 3. The effects of thymine plus deoxyuridine were intermediate between those of thymine alone and deoxyuridine alone. Thymine plus uridine and uridine plus deoxyuridine had a greater effect than either alone. Of the combinations, only thymine plus uridine was like the control in perithecial development and overall appearance. Although these results seem to indicate that thymine and uridine do act in different ways in preventing inhibition by FU, the differ-

TABLE 2. Prevention of azauracil inhibition by pyrimidines and pyrimidine nucleosides^a

Preventive ^b	Growth (%) ^c
None	1
Uracil	117
Uridine	112.5
Deoxyuridine	78.5
Thymine	21
Thymidine	14
Bromouracil	47.5
Bromodeoxyuridine	27.5
Cytosine	29.5
Cytidine	76.5
Deoxycytidine	28
5-Methylcytosine	47

^a Azauracil at 0.20 mM.

^b Preventives at 0.50 mM each.

^c Dry weight after 5 days, as percentage of control without azauracil. Average of two replicates.

TABLE 3. Prevention of FU inhibition by thymine, uridine, and deoxyuridine, separately and in combinations^a

Preventive	Concn (mM)	Growth (%) ^b	
		5 days	15 days
None		14	27
Thymine	0.50	57.5	67
Deoxyuridine	0.50	100.5	56.5
Uridine	0.50	23	81
Thymine + deoxyuridine	0.25	86	70
Thymine + uridine	0.25	73.5	94
Deoxyuridine + uridine	0.25	128	100.5

^a FU at 5.0 μ M.^b Dry weight as percentage of control without FU. Average of two replicates.

ences are not necessarily those presumed above (see Discussion).

DISCUSSION

The following mechanisms of the prevention of FU inhibition have been reported (6, 11, 12): (i) inhibition of uptake, (ii) inhibition of anabolism to the active derivative, (iii) inhibition of incorporation of the active derivative into ribonucleic acid (RNA), and (iv) bypass of the block in thymidylic acid synthesis by supplying the cell with an alternate source of thymidylic acid, usually thymine or thymidine (4, 5, 13, 15). Since FU is incorporated into RNA only as 5-fluorouridylic acid residues (5, 12), presumably only those compounds that are ultimately converted to uridine triphosphate or an analogue thereof can prevent FU inhibition by method (iii).

The greater preventive effects of uracil and uridine, compared with cytosine and cytidine, could be due to the action of the former two compounds by all three mechanisms, the action of the latter two being restricted to methods (ii) and/or (iii), presumably after being deaminated to uracil or a uracil derivative.

The situation with regard to deoxyuridine and deoxycytidine is less clear. They may act by somehow reversing the block in thymidylic acid synthesis, possibly by raising the endogenous levels of deoxyuridylic acid. However, the block in thymidylic acid synthesis would by itself be expected to cause the endogenous level of deoxyuridylic acid to rise, and it is questionable whether any exogenously added compound would raise this level further. We feel, therefore, that deoxyuridine and deoxycytidine act by being ultimately converted to uracil or uridylic acid, rather than deoxyuridylic acid, and act through method (ii) or (iii).

Thymine would, at first thought, be expected to act by method (iv). Similarly, bromouracil, which in some organisms is incorporated into deoxyribonucleic acid (DNA) in place of thymine without severe effects on DNA function (2), might also, along with bromodeoxyuridine, be expected to act this way. In *Neurospora crassa*, however, there are no known enzymes for thymine anabolism (3, 10). Thymine and thymidine are, however, converted in vivo to uracil (3, 9, 10). If this were the case in *S. fimicola*, then thymine could not act by method (iv) and would presumably act by method (ii) or (iii). Bromouracil and bromodeoxyuridine could presumably also act this way if they were debrominated (2). But if thymine, bromouracil, and bromodeoxyuridine can be converted to uracil, then one would expect them to prevent inhibition caused by azauracil also. As shown in Table 2, however, these compounds are much less effective in preventing azauracil inhibition than FU inhibition (Table 1). We conclude, therefore, that the enzymes for thymine anabolism are present in *S. fimicola* and that at least part of the preventive effects of these compounds with FU is due to the bypassing of the block in thymidylic acid synthesis (method iv). (If the enzymes for thymine anabolism are indeed present, it is surprising that the analogues trifluorothymine and 6-azathymine have been reported not to inhibit growth or perithecial production in *S. fimicola* [8; H. F. Schoen, Ph.D. thesis, City University of New York, 1975]. On the other hand, if 6-azathymine is converted to azauracil, it should also have an inhibitory effect. Trifluorothymine did have a slight preventive effect with FU [Table 1].) Additional possibilities for the preventive effect of thymine, bromouracil, and bromodeoxyuridine are: antagonism of the uptake or anabolism of FU (and more effective than that of azauracil) and inhibition of catabolism of FU to the tricarboxylic acid cycle inhibitor fluoroacetic acid (7)—inhibition by thymine of FU catabolism has been reported (2).

In the experiment shown in Table 3, where pairs of preventives were used, it was noted that some pairs gave better prevention than the individual preventives, viz., at 5 days, thymine plus uridine, and, at 15 days, thymine plus uridine and deoxyuridine plus uridine. This suggests that the inhibition of both RNA and DNA synthesis is involved in the inhibition of growth by FU. It may, however, indicate only that the conversion of the members of each pair to uridine triphosphate occurs via different pathways, and that the ultimate endogenous level of uridine triphosphate achieved is

greater when both pathways are utilized. (We have already mentioned that deoxyuridine and uridine likely act by the same method.)

In summary, we believe that the inhibition of growth by 5 μ M FU involves inhibition of DNA synthesis and possibly also normal RNA synthesis. Whether this is also the case at lower FU concentrations, where inhibition of branch density and perithecia formation has been reported (8), is not known.

We can now attempt a synthesis of what the data may tell us about pyrimidine interconversions in *S. fimicola*, in spite of the fact that conclusions from this type of data can be only tentative without supporting enzyme and genetic data and experiments with labeled precursors.

From the experiment on preventives of azauracil-caused growth inhibition (Table 2) it can be seen that all the preventives tried were successful to some degree in preventing growth inhibition. Since azauracil is believed to act by inhibiting de novo pyrimidine synthesis, this implies that either all these compounds are capable of ultimately being converted to a uracil ribonucleotide, at least to some degree, or that they interfere with the uptake or anabolism of azauracil itself. On the basis of our data there is no way of clearly distinguishing between these two possibilities.

Since azauracil inhibits by blocking the de novo synthesis of pyrimidines, those compounds that are converted to a uracil compound past the block (e.g., uridylic acid) to only a very slight degree may nevertheless have a marked effect on allowing growth to proceed. On the other hand, FU is converted to 5-fluorouridine triphosphate and is subsequently incorporated into RNA. Uridine triphosphate formed from potential preventives will thus have to compete with the 5-fluorouridine triphosphate for incorporation, and we therefore can expect that relatively high levels will be required for the competition to be successful. In addition, uridine ribonucleotides presumably continue to be made de novo in the presence of FU, and hence additions to this pool resulting from transformation of exogenous pyrimidines may not have a great effect. Thus, we assume that prevention of FU inhibition is indicative of a major conversion of the preventive to a uridine ribonucleotide. Complications in this interpretation are the possible relief of the thymidylic acid block by certain preventives and the inhibition of the uptake and anabolism of FU by the preventive. One should also bear in mind that differences in the effectiveness of the preventives may be due to differences in the rate of uptake of the preventives themselves.

FIG. 1. Proposed scheme of pyrimidine interconversions in *S. fimicola*, as determined by studies of prevention of FU and azauracil inhibition by various pyrimidines and pyrimidine derivatives. Dashed lines indicate that the proposed reaction occurs only slightly. Arrows connect only those intermediates that are implicated directly by the data; they do not necessarily show the pathways involved. Abbreviations: U, uracil; UR, uridine; UdR, deoxyuridine; UMP, uracil ribonucleotide, presumably uridylic acid when derived from U or UR; T, thymine; TdR, thymidine; dTMP, thymidylic acid; C, cytosine; CR, cytidine; CdR, deoxycytidine.

The hypothesized interconversions are summarized in Fig. 1.

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