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THE EFFECT OF 6-AZAURACIL UPON *TRYPANOSOMA EQUIPERDUM*

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Abstract—6-Azauracil, an analog of uracil which is an inhibitor of the growth of a number of experimental murine neoplasms and of several species of bacteria, prevents the reproduction of *T. equiperdum* in mice. This action results in growth suppression without trypanocidal sequelae. When treatment is stopped, the drug is rapidly cleared from the body, and the rate of reproduction of the trypanosomes rapidly returns to normal. The potency of a given dose of 6-azauracil is significantly enhanced when infected mice are fed a diet totally lacking in purines and pyrimidines. Conversely, the inhibitory activity of the analog is partially reversed when infected mice are fed a diet supplemented with uracil. The ribonucleoside of 6-azauracil, i.e. 6-azauridine, a much more potent inhibitor of the growth of certain transplanted neoplasms in mice than is 6-azauracil, does not inhibit the growth of *T. equiperdum* in the same species. Also, experiments with *T. equiperdum in vitro* have shown that 6-azauracil is at least 100 times more potent than 6-azauridine as an inhibitor of orotidylic acid decarboxylase, an enzyme selectively inhibited by the ribonucleotide of 6-azauracil (i.e. 6-azauridine-5′-phosphate).

6-AZAURACIL (AzU) was originally synthesized[1, 2] in the hope that, as a potential antagonist of uracil, it might be useful in cancer chemotherapy. It inhibited the growth of a number of experimental murine neoplasms[3–6] and several species of bacteria.[7, 8] Studies of the mechanism of its action revealed that the analog does not act directly as an antagonist of uracil. Rather, it interferes with *de novo*-biosynthesis of pyrimidine derivatives by being converted to 6-azauridine 5′-phosphate (AzUMP), which inhibits the enzymic decarboxylation of orotidylic acid to uridylic acid.[9, 10] Thus, only those cells will be adversely affected by the antimetabolite which possess the ability to synthesize uridylic acid from preformed uracil or uridine (since the same pathways are followed by the analog), while at the same time depending primarily upon *de novo*-biosynthesis of uridylic acid for their pyrimidine requirements.

Canellakis[11] has shown that there are two enzymic pathways of uracil anabolism. One, which seems to be characteristic of all mammalian cells studied so far, converts uracil to uridine by means of a nucleoside phosphorylase in the presence of ribose-l-phosphate. Uridine is then converted to uridine monophosphate (UMP)and to derivatives with additional phosphate groups by means of uridine kinases. The other pathway, disclosed in a number of bacterial species,[7, 11] converts uracil directly to UMP by means of uracil phosphoribosyl-pyrophosphorylase in the presence of pyrophosphoryl-ribosyl-5′-phosphate (PRPP).

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The data presented herein indicate that 6-azauracil, but not its ribonucleoside (6-azauridine (AzUR)), prevents the reproduction of *T. equiperdum* in mice, and, furthermore, that on a molar basis AzU is at least 100 times more potent than AzUR in its ability to inhibit the decarboxylation of orotidylic acid by *T. equiperdum* incubated *in vitro*.

MATERIALS AND METHODS

Ha/ICR Swiss female mice were inoculated intraperitoneally with 1×10^6 trypanosomes, obtained from the blood of infected mice and suspended in isotonic saline solution fortified with 1% glucose. In untreated mice death invariably occurred between 55 and 70 hr after inoculation. Drug treatment of each group of ten mice was begun within $\frac{1}{2}$ hr following the inoculation of the trypanosomes. The dosage schedule employed depended upon the drug under investigation and will be indicated later in the text. The duration of treatment was generally 6 days; any deviation from this time will also be indicated.

Studies of the effects of AzU and AzUR upon the ability of intact trypanosomes to convert orotidylic acid *in vitro* were designed as follows: $50–55 \times 10^6$ trypanosomes suspended in Krebs phosphate buffer (pH 7·3) were placed in each of a series of Warburg vessels, together with 0·1 ml of a given molar concentration of AzU or AzUR, 0·1 ml of 2% glucose, and sufficient Krebs phosphate buffer (pH 7·3) to make a final volume of 2·5 ml. Each center well contained 0·2 ml of 2 N NaOH to trap $^{14}CO_2$. One side-arm of each vessel contained 0·3 ml of 6 N $HClO_4$ to stop the reaction at the end of the incubation period. Thus prepared, the vessels containing the trypanosomes were pre-incubated for 10 min in a Dubnoff shaker at 37 °C. At the end of the pre-incubation period, orotic acid-7-^{14}C (0·1 ml of a solution having a specific activity of $1·12 \times 10^5$ counts/min per μmole per 0·1 ml) was added, and the mixture was allowed to incubate for an additional hour. At the end of the incubation period, the reaction was stopped by tipping in the perchloric acid solution. After allowing ten more minutes for stabilization, an aliquot of 0·1 ml of NaOH was removed from the center well of each vessel and placed in a glass vial into which was also introduced 10 ml of a counting mixture containing a phosphor*. Radioactivity, in the form of $^{14}CO_2$, was measured in a liquid scintillation counter (Technical Measurements Company).

RESULTS

Effects of various schedules of equivalent daily doses of AzU injected intraperitoneally

It was found previously that the antitumor activity of AzU was highest when the compound was given to mice for 6 days in the drinking water or when the comparable total daily dose (750 mg/kg) was injected parenterally in three equal portions at 8-hr intervals. It was decided at the outset, however, to treat mice infected with *T. equiperdum* by parental administration of AzU at a higher dose level (960 mg/kg per day), since this amount is roughly equivalent to that imbibed when mice are permitted to drink a solution of AzU at a concentration of 8 mg/ml, its maximum solubility at 20 °C. This was done in order that the activity of the most effective parenteral dosage regimen could later be compared with that of the highest oral dose available to the

* Toluene–absolute ethanol (2:1) counting mixture containing 0·27% DPO (2:5-diphenyl-oxazole) and 0·0033% POPOP (1:4-di-(2-(5-phenyloxazole))-benzene) (Pilot Chemicals, Waltham, Mass.)

mice *ad libitum*. Table 1 shows the relation between dosage schedule, duration of treatment, and the ability of a particular daily dose of AzU to prolong the lives of infected mice. It can be seen that the best results were obtained in every instance when AzU was injected every 8 hr. The optimal duration of treatment with this dose of AzU appeared to be 6 days, in keeping with an earlier finding that, at this concentration, the toxicity of AzU becomes too severe if treatment is prolonged beyond this point.

TABLE 1. EFFECT OF 6-AZAURACIL ON SURVIVAL TIME OF MICE INFECTED WITH *T. equiperdum*—I. VARIOUS SCHEDULES OF EQUIVALENT DAILY DOSAGE INJECTED INTRAPERITONEALLY

Dose (mg/kg)	Interval between injections (hr)	Duration of treatment (days)	Survival time (hr)
—	—	—	61 ± 1*
960	24	7	144 ± 3
480	12	7	168 ± 5
320	8	7	175 ± 5
960	24	6	132 ± 3
480	12	6	153 ± 4
320	8	6	194 ± 6
960	24	5	121 ± 3
480	12	5	150 ± 5
320	8	5	167 ± 4

* Standard error of the mean.

TABLE 2. EFFECT OF 6-AZAURACIL ON SURVIVAL TIME OF MICE INFECTED WITH *T. equiperdum*—II. COMPARISON OF EQUIVALENT DAILY DOSES ADMINISTERED PARENTERALLY AND ORALLY

Dose	Route of administration	Day of onset of treatment	Survival time (hr)
—	—	—	56 ± 0*
320 mg/kg every 8 hr; 8 mg/ml water *ad lib.* for 5 days	I.P.	0	164 ± 4
	oral	0	144 ± 8
	I.P.	1	146 ± 3
	oral	1	128 ± 6
	I.P.	2	121 ± 3
	oral	2	94 ± 5

* Standard error of the mean.

Comparison of equivalent daily doses of AzU administered parenterally and orally

Table 2 shows that the parenteral administration of AzU at 8-hr intervals is more effective than is treatment by permitting infected mice to drink a solution of AzU *ad libitum*, even though the total dose received daily was found to be roughly equivalent. AzU is excreted quite rapidly,[12] and these results indicate that in order for the antimetabolite to exert its full effect upon the trypanosomes, adequate blood levels must be maintained with more precision than is afforded by ingestion of the drug

ad libitum. In addition, it can be seen that maximal effectiveness was obtained when treatment was started shortly after the trypanosomes were injected into the mice. A delay of 24 or 48 hr before the onset of treatment was reflected in a subsequent 24- or 48-hr decrease in the expected survival time.

TABLE 3. EFFECT OF 6-AZAURACIL ON SURVIVAL TIME OF MICE INFECTED WITH *T. equiperdum*—III. EFFICACY OF INCREASING CONCENTRATIONS INJECTED INTRAPERITONEALLY

Dose (mg/kg per 8 hr) for 6 days	Survival time (hr)
—	70 ± 1*
80	96 ± 5
160	132 ± 8
320	178 ± 7

* Standard error of the mean

TABLE 4. EFFECT OF 6-AZAURACIL ON SURVIVAL TIME OF MICE INFECTED WITH *T. equiperdum*—IV. EFFICACY OF INCREASING DURATION OF TREATMENT WITH UNIFORM DOSE (250 MG/KG PER 8 HR) INJECTED INTRAPERITONEALLY

Days of treatment	Survival time (hr)
0	56 ± 0*
1	81 ± 1
2	94 ± 1
3	112 ± 1
4	133 ± 2
5	159 ± 3
6	184 ± 6

* Standard error of the mean.

Effect of increasing concentrations of AzU injected intraperitoneally

The cumulative 6-day dose of AzU employed in experiments described so far was known to be close to the minimal lethal dose determined for this period of treatment. Consequently, a study of the relationship between dose and therapeutic response was made in order to ascertain whether a lower and safer dose might be selected for future experiments. Table 3 indicates the positive correlation between increasing dose and therapeutic efficacy. Considering the parameters of therapeutic efficacy and host toxicity, it was judged that that dose of AzU previously found to have the highest antitumor activity with minimal host toxicity, namely, 250 mg/kg per 8 hr for six consecutive days, should also be satisfactory in experiments with mice infected with *T. equiperdum*.

Effect of increasing duration of treatment with a uniform dose (250 *mg AzU/kg per* 8 *hr*)

Table 4 shows that the survival time of infected mice was prolonged approximately 24 hr as a result of each day's treatment with AzU. These results indicate that AzU suppresses the growth of the trypanosomes, but fails to kill the parasites, since Chen *et al.*[13] have demonstrated that survival time is directly proportional to numbers of viable trypanosomes in the peripheral blood. Concomitant studies on changes in the numbers of trypanosomes in the blood of mice treated with AzU demonstrated that in the presence of therapeutic levels of the drug, reproduction of the organisms was almost completely suppressed. When treatment was terminated and the drug was essentially cleared from the body, reproduction recommenced without lag and proceeded at the normal rate of one doubling of the preexisting population approximately every 4–6 hr.[13]

TABLE 5. EFFECT OF DIET ON 6-AZAURACIL-INHIBITION OF *T. equiperdum* IN MICE*

Regimen	Survival time (hr)
Powdered Lab Chow (PLC)	58 ± 1†
Purine and pyrimidine-free diet (PFD)	59 ± 1
PLC and AzU (inj.)	172 ± 7
PLC and AzU (oral)	152 ± 6
PFD and AzU (inj.)	216 ± 9
PFD and AzU (oral)	214 ± 10
PLC and 1% U	58 ± 1
PLC and 1% U and AzU (inj).	102 ± 5

* 6-Azauracil was administered either intraperitoneally (250 mg/kg per 8 hr) or orally (0·5% solution vailaable *ad libitum*), starting ½ hr after the intraperitoneal injection of approximately 1×10^6 trypanosomes per mouse and continuing for six consecutive days.
† Standard error of the mean.

Effect of diet upon the activity of Azu

It has been mentioned that the action of AzU upon susceptible cells is attributable to its anabolism to azauridylic acid and its interference in this form with the biosynthesis *de novo* of pyrimidines, by way of inhibition of orotidylic acid decarboxylase. Since the available evidence indicates that the anabolism of azauracil and the normal counterpart, uracil, proceeds along the same pathway, the potency of the antimetabolite should be affected by the amount of uracil supplied exogeneously. Table 5 shows the results of such an experiment. Mice infected with trypanosomes either were treated with AzU or served as controls. In one series, the animals were given a diet complete in all respects (vitamins, minerals, fat, carbohydrates and protein), but totally lacking in purines and pyrimidines. In another series, the usual diet, Purina Lab Chow, was provided in powdered form to approximate the consistency of the test diets. In a third series, the animals were given powdered Purina Lab Chow to which uracil (1%) had been added. The findings indicate that the growth-suppressing activity of a given dose of AzU was considerably enhanced when the infected animals

concomitantly ate a diet lacking in purines and pyrimidines. Conversely, the activity of AzU was partially reversed when infected mice ate a diet rich in uracil. The test diets *per se* apparently had no influence upon the fate of the infected mice. It is also apparent that in the presence of a reduced amount of pre-formed uracil, the activity of AzU given orally *ad libitum* is roughly equivalent to that following parenteral administration.

TABLE 6. COMPARISON OF THE ABILITY OF AzU AND OTHER ANTIMETABOLITES TO PROLONG THE LIVES OF MICE INFECTED WITH *T. equiperdum*

Compound	I.P. dosage mg/kg per day for 6 days	Dose schedule	Per cent increase in survival time*
Pyrimidine derivatives			
6-Azauracil	750	t. i. d.	250
5-Fluorouracil	25	qd	25
6-Uracil methyl sulfone	150	t. i. d.	23
6-Azathymine	450	qd	21
5-Iodo-2′-deoxyuridine	150	qd	13
6-Methyluracil	960	t. i. d.	9
5-Nitrouracil	960	t. i. d.	0
Pyrimethamine	(0·3% in powdered Lab Chow)		0
6-Azauridine	120	t. i. d.	0
Purine derivatives			
6-Mercaptopurine	30	qd	37
Psicofuranine	800	single injection	30
6-Selenopurine	25	qd	20
Psicofuranine	100	b. i. d.	7
Folic acid antagonist			
Methotrexate	1·5	qd	18
Antibiotics			
Puromycin	200	single injection	curative
Azaserine	5	qd	50
Chloramphenicol	100	qd	0
Alkylating agent			
Uracil mustard	5	qd	0
Agents which interfere with glycolysis			
2-Deoxy-D-glucose	(1% in powdered Lab Chow)		0
N-Acetylglucosamine	400	qd	5

* The average survival time of mice receiving approximately 1×10^6 trypanosomes by intraperitoneal injection is 60 hr.

Effect of other antimetabolites on T. equiperdum in vivo

Of various antimetabolites tested in the past, only puromycin (Stylomycin) (6-dimethylamino-9- (3′-deoxy-3′- (*p*-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl)purine)—and the corresponding aminonucleoside—have been shown thus far to be effective against the growth of several species of trypanosomes both *in vitro* and *in vivo*.[14–17] Having now established that the pyrimidine analog, AzU, is active against *T. equiperdum in vivo*, it was of interest to determine whether other antimetabolites of different character and mechanisms of action might also be effective. Table 6 shows that the number of antimetabolites capable of prolonging the lives of

infected mice to a significant degree remains small. Puromycin stands out as the only curative, trypanocidal agent in this series. Of the remainder, AzU is distinguished by its potent growth-suppressing action, with azaserine exhibiting activity of only borderline therapeutic significance. Most of the antimetabolites studied were administered at maximal tolerated doses for the standard 6-day treatment period. It should be noted that one compound, 6-azauridine (AzUR), which was given at a level twice that previously found to have maximal antitumor activity in mice, was completely ineffective. Before this result could be interpreted in a definitive way, it remained to be determined whether the primary biochemical mechanism of action of AzU was the same in *T. equiperdum* as that found in cells of mammalian origin. [9, 10]

TABLE 7. THE EFFECT OF 6-AZAURACIL (AzU) AND 6-AZAURIDINE (AzUR) UPON THE CONVERSION OF OROTIC ACID-^{14}COOH* TO $^{14}CO_2$ BY *T. equiperdum in vitro*

	Conc. of analog (μmole/ml)	$^{14}CO_2$ recovered (counts/min)	Inhibition (%)
AzU	0	1232	—
	0·36	79	94
	0·18	98	92
	0·09	159	87
	0·045	223	82
AzUR	0·36	884	28
AzU	0	2377	—
	0·02	855	64
	0·01	1193	50
	0·005	1399	41
	0·002	1804	24
	0·001	1957	18
AzUR	0·18	1934	19

* The incubation medium in each flask contained 0·1 ml of orotic acid-^{14}COOH (0·1 μmole; 1·1 × 10^5 counts/min).

Effect of AzU and AzUR upon the ability of intact trypanosomes to convert orotic acid-^{14}COOH *to uridine nucleotides and* $^{14}CO_2$ in vitro

The methodology of these experiments has already been described. Under the conditions set forth, AzU on a molar basis was at least 100 times more active than AzUR in its ability to interfere with the decarboxylation of, presumably, orotidylic acid (Table 7). The possibility exists that the very small amount of activity exhibited by AzUR at the highest concentrations used could be due to contamination with trace amounts of the free base, since, as can be seen in Table 7, only 0·002 μmoles of AzU per ml of medium (0·226 μg/ml) would cause approximately 25 per cent inhibition of orotidylic acid decarboxylase activity. In these studies, the pertinent enzyme, its immediate substrate, and the non-gaseous products of the reaction were not isolated and characterized, but only inferred on the basis of earlier experience with other cells. In other experiments, the details of which will be published later, these substances have been identified, and the results have confirmed the present interpretation in every detail. In addition, there is clear evidence that the observed difference in activity of AzU and AzUR *in vitro* is not due to differences in permeability, since both compounds readily enter viable trypanosomes by passive diffusion.[18]

DISCUSSION

While nucleic acid antagonists have been designed primarily as agents with potential usefulness in cancer chemotherapy, studies of their mechanisms of action in neoplastic cells can lead to knowledge which may be applied to investigations concerning the nature of growth and development of other living systems. Thus, in this instance, the sensitivity of *T. equiperdum* to the action of the pyrimidine analog, 6-azauracil, elucidates a facet of the metabolism of this micro-organism which hitherto was unknown. It can now be suggested with a considerable degree of confidence that this species of trypanosome depends primarily upon *de novo*-biosynthetic reactions for its pyrimidine requirements, while possessing at the same time a limited ability to assimilate preformed uracil. The inability of AzU to cause the death of these protozoa, although it prevents their reproduction, may be due to the fact that sufficient uridylic acid is formed from the anabolism of the limited amount of preformed uracil available to provide pyrimidine-containing cofactors necessary for vital functions. Also, it may be inferred that *T. equiperdum* lacks or is deficient in the enzyme uridine kinase, since 6-azauridine (the ribonucleoside) is completely inactive as an inhibitor of the proliferation of this organism. This enzymic deficiency in *T. equiperdum* stands in contrast to the observation that uridine kinase is present in all cells of mammalian origin thus far studied.[11]

The sensitivity of *T. equiperdum* to the action of AzU does not indicate necessarily that this antimetabolite will be effective against other species of trypanosomes. For example, if, as has been reported, *T. cruzi* depends primarily upon preformed pyrimidines for its requirements,[19] it can be predicted that AzU will not be an effective inhibitor of the growth of this pathogen. Whether other pathogenic protozoa are susceptible to the action of AzU is a matter for future investigation.

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