Cytotoxic Effects of Inhibitors of *de Novo* Pyrimidine Biosynthesis upon *Plasmodium falciparum*†

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ABSTRACT: The malarial parasite *Plasmodium falciparum* can only synthesize pyrimidine nucleotides *via* the *de novo* pathway which is therefore a suitable target for development of antimalarial drugs. New assay procedures have been developed using high-pressure liquid chromatography (HPLC) which enable concurrent measurement of pyrimidine intermediates in malaria. Synchronized parasites growing in erythrocytes were pulse-labeled with [^{14}C]bicarbonate at 6-h intervals around the 48-h asexual life cycle. Analysis of malarial extracts by HPLC showed that incorporation of [^{14}C]bicarbonate into pyrimidine nucleotides was maximal during the transition from trophozoites to schizonts. The reaction, *N*-carbamyl-L-aspartate → L-dihydroorotate (CA-asp → DHO) catalyzed by malarial dihydroorotase is inhibited by L-6-thiodihydroorotate (TDHO) *in vitro* (K_i = 6.5 μM), and TDHO, as the free acid or methyl ester, induces a major accumulation of CA-asp in malaria. Atovaquone, a naphthoquinone, is a moderate inhibitor of dihydroorotate dehydrogenase *in vitro* (K_i = 27 μM) but induces major accumulations of CA-asp and DHO. Pyrazofurin induces accumulation of orotate and orotidine in malaria, consistent with inhibition of orotidine 5′-monophosphate (OMP) decarboxylase with subsequent dephosphorylation of the OMP accumulated. Although TDHO, atovaquone, and pyrazofurin arrest the growth of *P. falciparum*, only moderate decreases in UTP, CTP, and dTTP were observed. 5-Fluoroorotate also arrests the growth of *P. falciparum* with major accumulations of 5-fluorouridine mono-, di-, and triphosphates and the most significant inhibition of *de novo* biosynthesis of pyrimidine nucleotides.

The intraerythrocytic stages of the malarial parasite *Plasmodium falciparum* obtain adenosine, inosine, and hypoxanthine from the erythrocyte and are unable to synthesize purine nucleotides *de novo* (Scheibel & Sherman, 1988). Erythrocytes do not contain significant levels of pyrimidine nucleotides (Szabados & Christopherson, 1991), and the parasite is unable to salvage pyrimidine bases or nucleosides; pyrimidine nucleotides can only be synthesized *via* the *de novo* pathway (Scheibel & Sherman, 1988). By contrast, humans are able to synthesize pyrimidine nucleotides *via* the *de novo* or salvage pathways. Thus, inhibitors of malarial *de novo* pyrimidine biosynthesis could be effective antimalarial drugs, as the patient could still synthesize UTP and CTP from uridine.

A number of enzyme-catalyzed reactions of the *de novo* pyrimidine pathway[1] (eq 1) can be blocked by specific inhibitors (Christopherson & Lyons, 1990), but none of these

$$HCO_3^- \rightarrow CAP \rightarrow CA\text{-}asp \rightarrow DHO \rightarrow Oro \rightarrow OMP \rightarrow UMP \rightarrow UDP \rightarrow UTP \rightarrow CTP \quad (1)$$

inhibitors are now of major interest as anticancer drugs. Excessive patient toxicity, due perhaps to the roles of pyrimidine nucleotide precursors in RNA, polysaccharide, and lipid biosyntheses, and drug resistance, attributed to the salvage synthesis of pyrimidine nucleotides from uridine and cytidine, have precluded their use as anticancer drugs. However, for reasons described above, the pyrimidine antagonists have considerable potential as antimalarial drugs, with the naphthoquinone atovaquone having undergone successful clinical trials (Hudson, 1993). We have synthesized several potent inhibitors of the enzyme dihydroorotase (EC 3.5.2.3; CA-asp → DHO, eq 1; Christopherson *et al.*, 1989) which, as alkyl ester prodrug derivatives, induce blockade of *de novo* pyrimidine biosynthesis in human CCRF-CEM leukemia cells growing in culture (Brooke *et al.*, 1990). These dihydropyrimidine analogues, TDHO and HDDP, may induce blockade of the *de novo* pyrimidine pathway in malaria because malarial dihydroorotase has properties similar to those of the rodent enzyme (Krungkrai *et al.*, 1990). Atovaquone blocks the respiratory chain of malarial mitochondria at Complex III, leading to inhibition of dihydroorotate dehydrogenase (EC 1.3.3.1; DHO → Oro; Hammond *et al.*, 1985; Hudson, 1993). Using isolated malarial mitochondria, Fry and Pudney (1992) found that atovaquone forms a covalent adduct with a protein of 11.5 kDa, probably derived from Complex III. Pyrazofurin, as the C-nucleoside, inhibits malarial orotate phosphoribosyltransferase (Oro → OMP) while the 5′-monophosphate derivative inhibits OMP decarboxylase (OMP → UMP; Scott *et al.*, 1986). Pyrazofurin was an effective antimalarial, retarding the maturation of trophozoites to schizonts; toxicity was not affected by addition of uracil or uridine to the culture. Queen *et al.* (1990) found that 5-fluoroorotate (FOro, ID_{50} = 0.042 μM) was a potent inhibitor of the growth of *P. falciparum in vitro* while 5-fluorouracil (ID_{50} = 5.2 μM) was far less effective. 5-Fluoroorotate was proposed to induce inhibition of orotate phosphoribosyltransferase (Oro → OMP, eq 1) and thymidylate synthase (dUMP → dTMP). Krungkrai *et al.* (1992) tested a variety of 5-substituted orotate derivatives as inhibitors

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[1] Abbreviations: CA-asp, *N*-carbamyl-L-aspartate; CAP, carbamyl phosphate; CoQ_6, ubiquinone coenzyme with six isoprenoid units in the side chain; DHO, L-dihydroorotate; Oro, orotate; Ord, orotidine; TDHO, L-6-thiodihydroorotate; the prefix "F" before an abbreviation indicates the 5-fluoro derivative of the pyrimidine ring (for example, FOro = 5-fluoroorotate).

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of dihydroorotase and dihydroorotate dehydrogenase *in vitro* and against *P. falciparum* growing in erythrocytic culture and found 5-fluoroorotate to be the most potent in each case. They found that 5-fluoroorotate and 5-aminoorotate eliminated parasitemia from mice infected with *Plasmodium berghei*.

We have developed procedures for quantification of intermediates of the pathway for *de novo* biosynthesis of pyrimidine nucleotides in *P. falciparum*. Malaria has been grown in human erythrocytes in synchronized culture, and pulse-labeling with [^{14}C]bicarbonate has shown that the flux through the *de novo* pyrimidine pathway is maximal at the transition from trophozoites to schizonts. Parasites have been exposed to a variety of pyrimidine antagonists during this period, and analysis of malarial extracts by high-pressure liquid chromatography (HPLC) has provided direct evidence for their mechanisms of toxicity.

EXPERIMENTAL PROCEDURES

Materials. L-Dihydroorotate (DHO), orotidine (Ord), UTP, CTP, dTTP, and other nucleotides were obtained from the Sigma Chemical Co. (St. Louis, MO). The pyrimidine antagonists pyrazofurin, 5-fluoroorotate, 5-fluorouracil, and 3-deazauridine were also from Sigma. *N*-Carbamyl-L-aspartate (CA-asp) was synthesized by the procedure of Christopherson *et al.* (1978), and L-6-thiodihydroorotate (TDHO) was made as described by Christopherson *et al.* (1987). Atovaquone (BW566C80) was a kind gift from Dr. W. E. Gutteridge of the Wellcome Research Laboratories (Beckenham, U.K.). Sodium [^{14}C]bicarbonate (38.6 mM, 51.8 Ci/mol) was from Amersham International plc (Amersham, U.K.). Yeast orotate phosphoribosyltransferase/OMP decarboxylase was from Sigma. For growth of *P. falciparum*, human O$^+$ erythrocytes and serum were obtained from the Australian Red Cross Society Blood Transfusion Service (Sydney, Australia), RPMI 1640 medium (bicarbonate-free and containing 25 mM K.Hepes buffer) was from Flow Laboratories (Sydney, Australia), and the gas mixture 90% N_2/5% O_2/5% CO_2 was from Commonwealth Industrial Gases (Sydney, Australia). All other chemicals were of analytical reagent grade.

Growth of Malaria in Culture. Plasmodium falciparum K1, resistant to chloroquine, was obtained as a synchronized culture in human O$^+$ erythrocytes from the Army Malaria Research Unit (Ingelburn, Australia). The hematocrit of the erythrocytes was 4% with up to 5% parasitemia. The malaria was grown in RPMI 1640 medium containing 25 mM K.Hepes and supplemented with 0.37 mM hypoxanthine, 25 mM $NaHCO_3$, 40 μg/mL of gentamycin sulfate, an additional 2 g/L of D-glucose, and O$^+$ human serum at a final concentration of 10% (v/v). After inoculation with parasitized erythrocytes, the culture flask was flushed with a 90% N_2/5% O_2/5% CO_2 gas mixture, sealed, and incubated at 37 °C. The culture medium was changed every 12 h to maintain the pH in the range 7.3–7.5. To radiolabel intermediates of the *de novo* pyrimidine pathway, the medium was changed to bicarbonate-free which had been bubbled with N_2 and stirred for 16 h at 4 °C. Cultures of 100 mL were then flushed with a 5% O_2/95% N_2 (CO_2-free) gas mixture, [^{14}C]bicarbonate (51.8 Ci/mol) was added to a final concentration of 500 μM along with a drug, as required, and the flask was sealed and incubated for 6 h prior to harvesting.

Preparation of Malarial Extracts. All procedures for extraction of metabolites were at 0–4 °C. Parasitized erythrocytes (100 mL) were harvested by centrifugation (200 g, 10 min), and erythrocytes were lysed by vortexing for 1 min in 0.15% (w/v) saponin in Hanks' Balanced Salt Solution (9 mL, pH 7.4) and left on ice for 5 min. The suspension was then diluted to 50 mL with Hanks' Balanced Salt Solution, and the freed parasites were harvested by centrifugation (2100*g*, 15 min). The parasites were resuspended twice more, and the final weight of the pellet was used to calculate the volume. The parasites were frozen in liquid nitrogen, an equal volume of 0.8 M $HClO_4$ was added, and the suspension was thawed briefly at 37 °C. The suspension was frozen and thawed twice more and and then left on ice for 15 min. After centrifugation (2100*g*, 15 min), the acidic supernatant was neutralized by vortexing for 1 min with an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Sherman & Fyfe, 1989). The phases were separated by centrifugation (9000*g*, 5 min), and the upper aqueous phase was retained for analysis.

High-Pressure Liquid Chromatography. Acid-soluble metabolites were separated by gradient anion-exchange HPLC on a Partisil 10-SAX column (0.42 × 22 cm; Whatman, Clifton, NJ) and quantified using a Spectra-Physics UV2000 ultraviolet detector, an LKB 2140 Rapid Spectral Detector (Bromma, Sweden), and an LKB 1208 Betacord Radioactivity Monitor (Wallac Oy, Turku, Finland) connected in series as described previously (Sant *et al.*, 1989). Metabolite concentrations in *P. falciparum* were expressed as amol/parasitized erythrocyte.

Purification of Dihydroorotase and Dihydroorotate Dehydrogenase. These two enzymes were partially purified from the same culture of *P. falciparum* using modifications of the initial steps of Krungkrai *et al.* (1990, 1991). A culture of malaria (100 mL) was harvested at the transition from trophozoites to schizonts (27 h), and the parasites were isolated by saponin lysis and resuspended in 3 volumes of 20 mM K.Hepes, 1 mM DTT, 1 mM EDTA, and 10 mM benzamidine (pH 7.4). The parasites were lysed by three cycles of freeze–thawing and the soluble and particulate fractions separated by centrifugation (39 000*g*, 30 min, 4 °C). Nucleic acids were removed from the soluble fraction by addition of streptomycin sulfate (3% w/v), and protein from the 30% to 60% ammonium sulfate fraction was collected by centrifugation (39 000*g*, 30 min, 4 °C). The pellet was redissolved in 20 mM K.Hepes, 1 mM DTT, 1 mM EDTA, 30% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol (pH 7.4) and desalted by centrifugal ultrafiltration, and this partially purified preparation (V_{max} = 4.84 pmol *N*-carbamyl-L-aspartate produced/(min μL), 2.1 μg protein/μL, 99 μL) was stored at −20 °C for dihydroorotase assays. The particulate fraction isolated above was resuspended in the same buffer (200 μL) and Triton X-100 was added to 0.15% (w/v). After stirring (40 min, 4 °C), the detergent extract was centrifuged (39 000*g*, 30 min, 4 °C), and DHO was added to 100 μM to the supernatant (V_{max} = 13.5 pmol orotate produced/(min μL) 3.2 μg protein/μL, 133 μL), which was stored at −80 °C for the DHO dehydrogenase assays.

Enzyme Assays. For assay of dihydroorotase, incubation mixtures contained (in a total volume of 25 μL) 50 mM K.Hepes (pH 7.4), 5% (v/v) glycerol, and 20 μM [^{14}C]-dihydroorotate (55.3 Ci/mol), and the reaction (DHO → CA-asp) was initiated with partially purified dihydroorotase (4.2 μg of protein). Three samples (7 μL) from each assay mixture was spotted at appropriate times onto poly(ethyleneimine)–cellulose chromatograms. The [^{14}C]CA-asp produced from [^{14}C]DHO in the dihydroorotase assay was isolated by

ascending chromatography with 0.34 M LiCl (Christopherson *et al.*, 1989). Radioactive spots were located by autoradiography with X-ray film followed by scintillation counting or by a Phosphor Imager (Molecular Dynamics Inc., Sunnyvale, CA) with computerized quantification.

Assay mixtures for DHO dehydrogenase in the presence of TDHO or atovaquone contained (in a total volume of 25 μL) 50 mM K.Hepes (pH 7.4), 20% dimethyl sulfoxide, 0.15% (w/v) Triton X-100, CoQ_6 (100 or 20 μM, respectively), [^{14}C]-DHO (10 or 100 μM, respectively, 10 Ci/mol), 500 μM P-Rib-PP, 5 mM $MgCl_2$, and 3.8 μg of yeast orotate phosphoribosyltransferase/OMP decarboxylase, and the reaction (DHO → Oro → OMP → UMP) was initiated with partially purified malarial DHO dehydrogenase (2.1 or 3.2 μg of protein, respectively) isolated from the particulate fraction. Three timed samples for each assay were analyzed on poly(ethyleneimine)–cellulose chromatograms using 0.4 M formic acid as the developing solvent, and the [^{14}C]UMP produced was quantified as for dihydroorotase.

Determination of Inhibition Constants. Data for inhibition of dihydroorotase by TDHO were fitted by nonlinear regression analysis (Duggleby, 1984) to the velocity equation describing competitive inhibition. Krungkrai *et al.* (1991) reported that the DHO dehydrogenase from *P. berghei* displays a ping pong bi bi mechanism for DHO and CoQ_6. Consistent with the mechanism proposed by Krungkrai *et al.* (1991), we have assumed that TDHO is binding competitively as an analogue of DHO and atovaquone is binding competitively with CoQ_6. The inhibition constant (K_i) for TDHO as an inhibitor of DHO dehydrogenase was determined as a Dixon plot with a saturating concentration of CoQ_6 (100 μM) and a subsaturating concentration of DHO (10 μM). The K_i value for atovaquone was determined as a Dixon plot with a saturating concentration of DHO (100 μM) and a subsaturating concentration of CoQ_6 (20 μM).

RESULTS

Growth of *P. falciparum* in the presence of [^{14}C]bicarbonate results in ^{14}C-labeling of all intermediates of the *de novo* pyrimidine pathway. Analysis of a perchloric acid extract of *P. falciparum* by HPLC gave the elution profiles for ultraviolet absorbance and ^{14}C shown in Figure 1. Both purine and pyrimidine nucleotides have strong absorbance at 260 nm, but only the pyrimidine nucleotides incorporate [^{14}C]bicarbonate. While most of the peaks of the ultraviolet profile have been assigned, there are at least 11 peaks representing malarial metabolites which remain to be identified. Most of these unknown peaks are unique to malaria and are not apparent in extracts of human cells (cf. Brooke *et al.*, 1990).

Under the conditions of erythrocytic culture used, the asexual life cycle of *P. falciparum* was approximately 48 h. For a pyrimidine antagonist to have the maximum effect, the *de novo* pyrimidine pathway should be active at the time of drug addition. To determine the period of maximum flux through the pathway, synchronized parasites were pulse-labeled with [^{14}C]bicarbonate for 6-h intervals around the cycle from 0 to 48 h. At zero time, a culture was divided into eight equal volumes (100 mL) which were grown in parallel during the 48 h of the experiment. At 6-h intervals, [^{14}C]-bicarbonate was added to one of the cultures, and 6 h later, parasites of the culture (99 mL) were harvested. After extraction, the eight acid-soluble extracts obtained from the 6-h intervals around the 48-h cycle were analyzed by HPLC, yielding concurrent elution profiles for ultraviolet absorbance

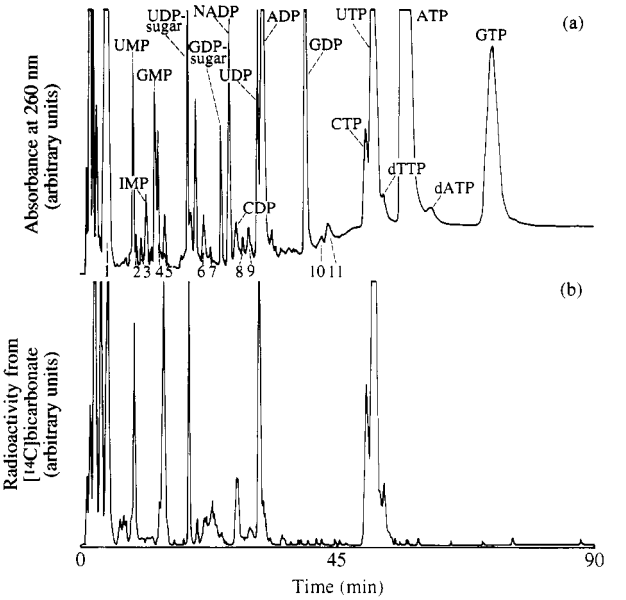

FIGURE 1: Analysis by HPLC of nucleotides extracted from *P. falciparum*: (a) total nucleotides quantified by ultraviolet absorbance; (b) incorporation of [^{14}C]bicarbonate during 6 h into pyrimidine nucleotides. Peaks numbered 1–11 are not found in extracts from human cells.

and ^{14}C (Figure 2). Absorbance peaks, representing total purine and pyrimidine nucleotides in the parasite, are largest for the 18–24-h sample (Figure 2g), and the incorporation of [^{14}C]bicarbonate into pyrimidine nucleotides (Figure 2h) is also maximal during this period. The rate of *de novo* biosynthesis of pyrimidine nucleotides is greatest at the transition from trophozoites to schizonts at 24 h (Figure 2g,h,q).

These procedures for measurement of intermediates of *de novo* pyrimidine biosynthesis have been used to determine the effects upon the pathway of a variety of pyrimidine antagonists. *P. falciparum* was exposed to the methyl ester of thiodihydroorotate (TDHO-Me, 250 μM, 6 h), and the ultraviolet elution profile (Figure 3c) indicates some decrease in the levels of UTP, CTP, and dTTP, consistent with inhibition of the pathway. The ^{14}C-profile (Figure 3d) confirms this depletion of UTP, CTP, and dTTP and shows a large accumulation of CA-asp, consistent with inhibition of dihydroorotase by TDHO and a smaller peak for DHO. In addition to accumulation of ^{14}C-labeled CA-asp and DHO, two other metabolites accumulate (A and B) which have not been identified. The free acid of TDHO gave similar results. *P. falciparum* treated with atovaquone (25 μM, 6 h) shows a similar response with decreases in UTP, CTP, and dTTP and major accumulations of ^{14}C-labeled CA-asp and DHO (Figure 3e,f). The depletions of UTP, CTP, and dTTP induced by these two specific inhibitors do not seem to be sufficient to result in cell death which could result from some other effect. For example, CA-asp or DHO at high concentrations in *P. falciparum* could be toxic or a metabolite of CA-asp or DHO, perhaps peak A or B (Figure 3d), could be toxic.

Pyrazofurin (250 μM, 6 h) induces moderate decreases in UTP, CTP, and dTTP, and accumulations of orotidine (Ord) and orotate (Oro) are apparent in both the ultraviolet profiles and ^{14}C-profiles (Figure 3g,h). Pyrazofurin has antimalarial activity, but again, major decreases in the end-products of the *de novo* pyrimidine pathway are not observed under these conditions. The accumulations of Ord and Oro are consistent with inhibition of OMP decarboxylase by pyrazofurin 5′-

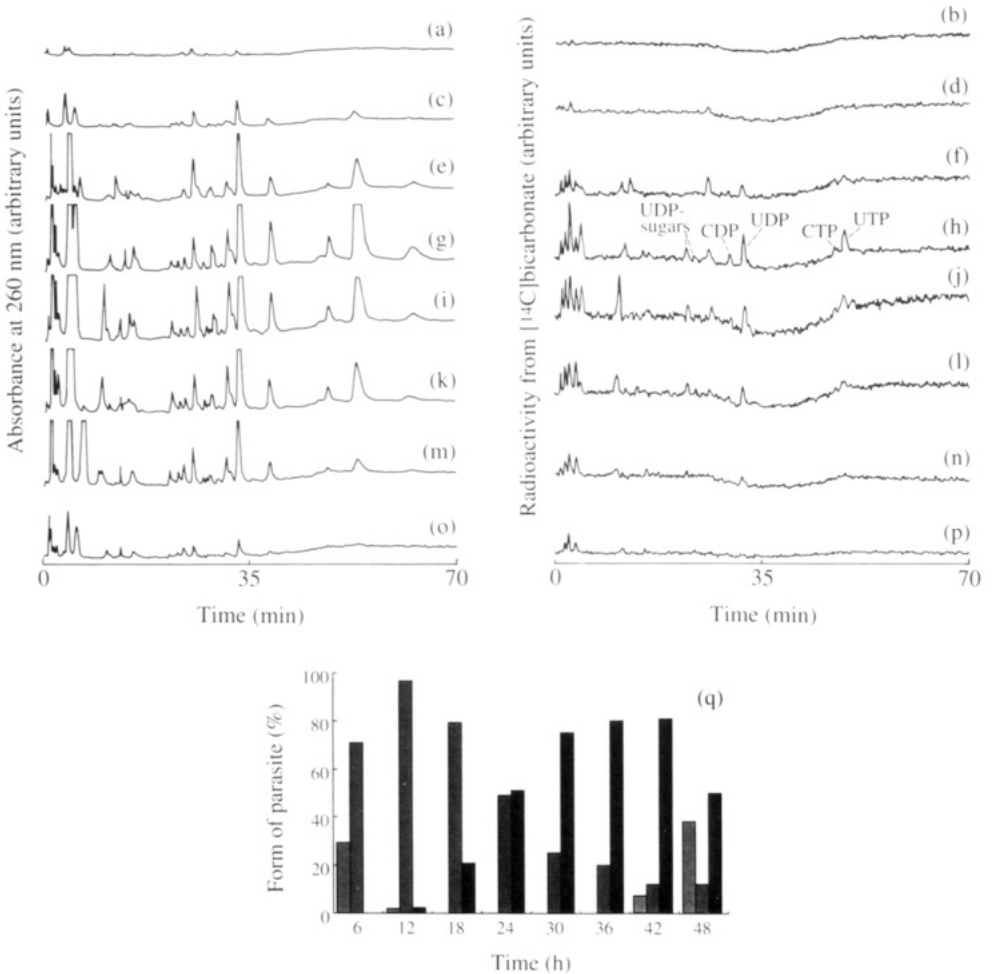

FIGURE 2: Determination of the period of maximum flux through the *de novo* pyrimidine pathway in *P. falciparum*. Parasites were pulse-labeled with [^{14}C]bicarbonate for 6 h, and extracts were analyzed by ultraviolet absorbance (left side) and for ^{14}C (right side). Periods for pulse-labeling were as follows: (a,b) 0–6 h; (c,d) 6–12 h; (e,f) 12–18 h; (g,h) 18–24 h; (i,j) 24–30 h; (k,l) 30–36 h; (m,n) 36–42 h; (o,p) 42–48 h. (q) Percentage of each form of the parasite during the 48-h asexual growth cycle. Parasite forms were counted after Giemsa staining of thin blood films from samples of the cultures analyzed above by HPLC. Forms of the parasite are designated as follows: (stippled bars) rings; (hatched bars) trophozoites; (solid bars) schizonts.

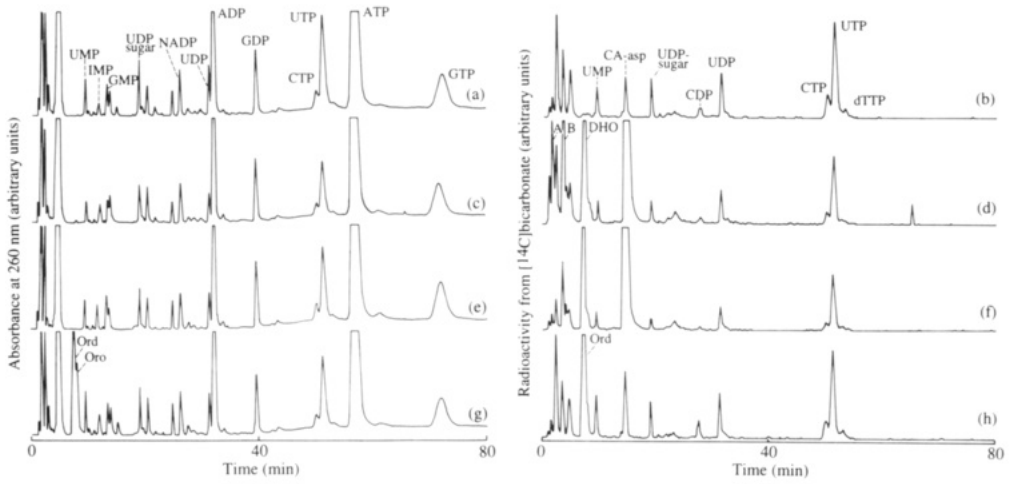

FIGURE 3: Effects of inhibitors upon the *de novo* pyrimidine pathway in *P. falciparum*. Drug and [^{14}C]bicarbonate were added to cultures at the transition from trophozoites to schizonts, and a sample of parasites was extracted 6 h later. The cultures analyzed concurrently by ultraviolet absorbance and ^{14}C were as follows: (a,b) control; (c,d) +250 μM TDHO methyl ester; (e,f) +25 μM atovaquone; (g,h) +25 μM pyrazofurin. A and B are unidentified material metabolites.

monophosphate; similar observations have been made with mouse L1210 leukemia cells (Sant *et al.*, 1989). Inhibition by pyrazofurin 5′-monophosphate would result in accumulation of OMP, which could be degraded *via* the sequence OMP → Ord → Oro. 3-Deazauridine induces potent inhibition of CTP synthetase in mouse L1210 leukemia cells (Sant *et al.*, 1989), but exposure of *P. falciparum* to this pyrimidine analogue (250 μM, 6 h) did not affect pyrimidine biosynthesis (data not shown). Metabolite peaks of interest from the elution profiles of Figure 3 have been integrated, and cellular levels

Table 1: Effects of Inhibitors upon Pyrimidine Intermediates, ATP, and GTP in *P. falciparum*[a]

metabolite	control		+250 μM TDHO-Me		+25 μM atovaquone		+250 μM pyrazofurin	
	amol/pe	aCi/pe	amol/pe	aCi/pe	amol/pe	aCi/pe	amol/pe	aCi/pe
DHO	*b*	*b*	12.5	88.1	15.6	85.9	*b*	*b*
Ord/Oro	*b*	*b*	*b*	*b*	*b*	*b*	12.1	99.7
UMP	1.24	12.3	0.712	7.74	0.741	5.49	1.45	14.2
CA-asp	1.91	19.9	115	807	231	1270	3.43	28.4
UDP-sugars	1.57	12.1	1.76	7.89	0.964	3.15	1.86	10.0
CDP	0.436	6.30	0.630	3.43	0.435	1.72	1.14	7.98
UDP	1.86	23.0	1.24	12.9	1.24	9.19	1.78	21.1
CTP	2.36	15.6	1.73	7.25	1.12	4.94	1.89	11.5
UTP	8.96	74.7	5.43	43.0	5.69	35.7	7.44	60.8
dTTP	0.458	7.21	0.312	4.49	0.410	2.94	0.535	6.57
ATP	20.9	*b*	21.3	*b*	19.0	*b*	20.6	*b*
GTP	6.10	*b*	5.97	*b*	5.75	*b*	5.60	*b*
pyrimidine nucleotides synthesized from [^{14}C]bicarbonate[c] (% of control)	100		56.5		41.5		84.6	

[a] Parasites were grown in synchronized erythrocytic cultures (100 mL) to the transition from trophozoites to schizonts, exposed to drug for 6 h, harvested, extracted, and analyzed by HPLC as described in Experimental Procedures. For each culture, the specific radioactivities (*r*) of CTP, CDP, UTP, and UDP were calculated as the ratio r = (aCi/parasitized erythrocyte (pe))/(amol/parasitized erythrocyte (pe)). Average values of *r* obtained for the 4 pyrimidine nucleotides in each culture were as follows: control, 10.43 ± 3.59 Ci/mol; methyl ester of TDHO (TDHO-Me), 7.03 ± 4.58 Ci/mol; atovaquone, 5.51 ± 1.62 Ci/mol; pyrazofurin, 8.27 ± 2.51 Ci/mol. These *r* values were used to calculate cellular levels of CA-asp, DHO and Ord/Oro (amol/pe) from the radioactivity (aCi/pe) as described in the text. [b] Not detected. [c] Calculated from the sum of UMP + UDP-sugars + CDP + UDP + CTP + UTP (aCi/pe).

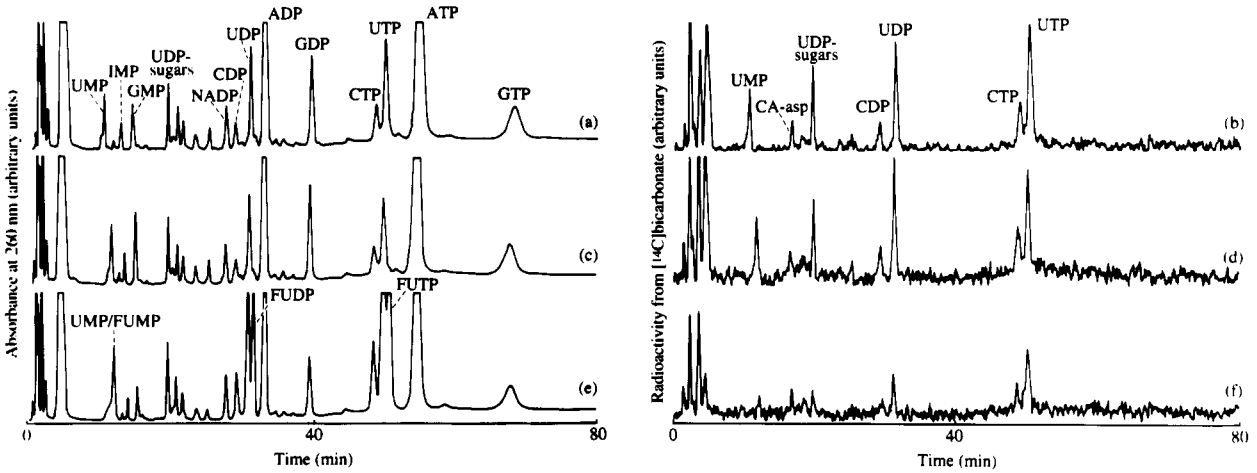

FIGURE 4: Effects of 5-fluorouracil and 5-fluoroorotate upon the *de novo* pyrimidine pathway in *P. falciparum*. Experimental procedures were as for Figure 3: (a,b) control; (c,d) +25 μM 5-fluorouracil; (e,f) +25 μM 5-fluoroorotate.

are shown in Table 1. For the ribonucleoside triphosphates (CTP, UTP, ATP, and GTP), peak areas for both the ultraviolet and ^{14}C-profiles were determined and cellular levels calculated as amol/parasitized erythrocyte and aCi/parasitized erythrocyte, respectively. The specific radioactivities of these nucleotides in cell extracts could then be calculated as the ratio to give Ci/mol. The cellular levels of CA-asp and DHO, which accumulated after drug exposure, were only measurable as radioactivity (aCi/parasitized erythrocyte). Molar levels of these metabolites were calculated by dividing the ^{14}C content of the metabolite (aCi/parasitized erythrocyte) by the specific radioactivity of pyrimidine nucleotides in that culture (aCi/mol). The methyl ester of TDHO (250 μM, 6 h) induced accumulation of CA-asp and DHO with a decrease in total pyrimidine nucleotides to 56.5%. Atovaquone (25 μM, 6 h) induced accumulation of CA-asp and DHO with a decrease in total pyrimidine nucleotides to 41.5%. The metabolic effects of pyrazofurin (250 μM, 6 h) were less severe with accumulations of Oro plus Ord and CA-asp and a decrease in total pyrimidine nucleotides to 84.6%.

Queen *et al.* (1990) screened 64 purine and pyrimidine analogues, known to inhibit steps of nucleotide biosynthesis, for inhibition of growth of *P. falciparum*. They found 5-fluoroorotate to be the most potent inhibitor with an IC_{50} value of 42 nM. To determine the mechanism of this toxicity, *P. falciparum* was exposed to 5-fluoroorotate (25 μM, 6 h) and 5-fluorouracil (25 μM, 6 h) was added to a parallel culture for comparison. 5-Fluorouracil induced only moderate decreases in pyrimidine nucleotides as indicated by the ultraviolet profiles or ^{14}C-profiles (Figure 4c,d, Table 2). However, large amounts of 5-fluoro-UDP (λ_{max} = 266 nm) and 5-fluoro-UTP (λ_{max} = 268 nm) accumulated in malaria treated with 5-fluoroorotate (Figure 4e, Table 2), and the incorporation of [^{14}C]bicarbonate into total pyrimidine nucleotides was decreased to 32.7% (Figure 4f, Table 2) of the control culture (Figure 4b) during the 6 h of drug exposure. Using [^{14}C]DHO (5.56–33.3 μM) and TDHO (0–25.0 μM), with partially purified DHOase (4.2 μg protein/25 μL), a full inhibition pattern showed TDHO to be a competitive inhibitor with a K_i value of 6.5 ± 0.6 μM. A Dixon plot for TDHO (0–250 μM) with partially purified DHO dehydrogenase (2.1 μg protein/25 μL), 10 μM [^{14}C]DHO, and 100 μM CoQ_6 gave a K_i value for TDHO at 37.0 ± 5.0 μM. A Dixon plot for atovaquone (0–80 μM) with partially purified DHO dehydrogenase (3.2 μg protein/25 μL), 100 μM [^{14}C]DHO, and 20 μM CoQ_6 gave a K_i value for atovaquone of 27 ± 1.6 μM.

Table 2: Effects of 5-Fluorouracil and 5-Fluoroorotate upon Pyrimidine Intermediates, ATP, and GTP in *P. falciparum*[a]

metabolite	control		+25 μM 5-fluorouracil		+25 μM 5-fluoroorotate	
	amol/pe	aCi/pe	amol/pe	aCi/pe	amol/pe	aCi/pe
UMP	1.54	50.6	1.63	27.8	2.40	5.14
FUMP[b]	c	c	c	c	2.35	c
CA-asp	1.02	8.00	0.510	4.86	2.54	5.43
UDP-sugars	1.76	23.1	1.87	19.5	2.17	5.68
CDP	2.08	11.8	1.99	14.0	3.69	c
UDP	3.81	39.6	3.47	42.6	6.09	13.5
FUDP	c	c	c	c	14.5	c
CTP	4.03	22.5	3.22	23.9	6.73	12.5
UTP	6.49	63.6	4.53	51.6	12.5	29.5
FUTP	c	c	c	c	23.2	c
ATP	31.0	c	27.5	c	21.6	c
GTP	8.09	c	7.36	c	5.63	c
pyrimidine nucleotides synthesized from [^{14}C]bicarbonate (% of control)	100		84.1		32.7	

[a] Parasites were grown in synchronized erythrocytic cultures (50 mL) to the transition from trophozoites to schizonts, exposed to drug for 6 h, harvested, extracted, and analyzed by HPLC as described in Experimental Procedures. [b] FUMP cochromatographed with UMP, but the cellular level was calculated from the additional area of the ultraviolet peak for UMP compared with the equivalent ^{14}C-peak. The cellular levels of CA-asp and FUMP were calculated using the following *r* values: control, 7.86 ± 2.58; 5-fluorouracil, 9.53 ± 2.70; 5-fluoroorotate, 2.14 ± 0.26. [c] Not detected.

DISCUSSION

Growth of *P. falciparum* in human erythrocytes in the presence of [^{14}C]bicarbonate enables concurrent quantification of the total levels of purine and pyrimidine nucleotides in the parasite and the rate of *de novo* pyrimidine biosynthesis. The gradient anion-exchange HPLC procedure used to analyze malarial extracts has revealed a number of metabolites, currently of unknown structure, which are not found in human cells (Figure 1). In synchronized *P. falciparum*, the total levels of purine and pyrimidine nucleotides and the rate of *de novo* pyrimidine biosynthesis are maximal at the transition from trophozoites to schizonts (Figure 2). Consistent with these observations, Newbold *et al.* (1982) found that 60% of DNA synthesis in *P. chabaudi* occurs at or just prior to schizogony, and Gero *et al.* (1984) found activities of enzymes of the pyrimidine pathway to be maximal at this stage of the asexual growth cycle of *P. falciparum*. Thus, specific inhibitors of the *de novo* pyrimidine pathway should arrest the growth and kill parasites at this stage of the 48-h growth cycle.

To determine the mechanisms of cytotoxicity of TDHO, atovaquone, pyrazofurin, 5-fluoroorotate, and 5-fluorouracil, parasites were exposed to these pyrimidine antagonists for 6 h during this transition from trophozoites to schizonts. Concurrent detection of metabolites separated by HPLC by ultraviolet absorption and radioactivity enabled quantification of molar amounts of CA-asp, DHO, and Ord + Oro. The approximate IC_{50} values in ascending order for the five pyrimidine antagonists are atovaquone, 0.7 nM (Fry & Pudney, 1991); 5-fluoroorotate, 42 nM (Queen *et al.*, 1990); 5-fluorouracil, 5.2 μM (Queen *et al.*, 1990); pyrazofurin, 6.3 μM (Scott *et. al.*, 1986); and TDHO, 35 μM (data not shown). Consistent with this order, atovaquone induced severe inhibition of the *de novo* pyrimidine pathway with accumulation of CA-asp to 231 amol/parasitized erythrocyte and of DHO to 15.6 amol/parasitized erythrocyte. However, the rate of *de novo* pyrimidine nucleotide biosynthesis after the blockade at DHO dehydrogenase (DHO → Oro; UMP, UDP-sugars, CDP, UDP, CTP plus UTP) was still 41.5% of that of the control culture (Figure 3b,f, Table 1), suggesting that death of the parasites was not due primarily to a deficiency of pyrimidine nucleotides. The moderate inhibition of DHO dehydrogenase by atovaquone *in vitro* (K_i = 27 μM) supports the proposal that inhibition of pyrimidine biosynthesis is indirect, resulting from inactivation of Complex III of the electron-transport chain (Fry & Pudney, 1992). Atovaquone could disrupt another unknown but essential function of the electron-transport chain in malaria. The inhibitions of the *de novo* pyrimidine pathway induced by TDHO and pyrazofurin (Table 1) also may not be sufficient to kill the parasite. CA-asp, DHO, Oro, or Ord, which accumulate, or metabolites of these intermediates, perhaps peaks A and B, which are induced by the methyl ester of TDHO (Figure 3d), may be toxic. Alternatively, the moderate decreases in UTP and CTP observed may be sufficient to cause an imbalance between the deoxynucleoside triphosphates which could result in genetic miscoding (Bebenek *et al.*, 1992). With the assumption that trophozoites occupy approximately 40% of the volume of an erythrocyte (Bosia *et al.*, 1993; 0.1 pL), the cellular concentration of CA-asp induced by the TDHO methyl ester (Table 1) would be approximately $115 \times 10^{-3}/0.1 \times 0.4 \approx 2.9$ mM. The free acid of TDHO is a potent inhibitor of malarial dihydroorotase (K_i = 6.5 μM), but accumulation of CA-asp in the trophozoite due to inhibition of dihydroorotase may result in development of "metabolic resistance" (Christopherson & Duggleby, 1983). The substrate of the inhibited reaction (CA-asp) may accumulate to a concentration sufficient to compete with TDHO for binding to dihydroorotase, resulting in restoration of the original flux through the *de novo* pyrimidine pathway. TDHO is also a weaker inhibitor of DHO dehydrogenase (K_i = 37 μM, DHO → Oro); this second inhibition "down-stream" results in accumulation of DHO (Figure 3d). TDHO was not detectable in the ultraviolet elution profile of Figure 3c and must be present at concentrations of less than 0.05 amol/parasitized erythrocyte or approximately 1 μM in the trophozoite.

5-Fluoroorotate induced the most severe decrease in *de novo* pyrimidine synthesis to 32.7% of the control (Figure 4b,f). Significant accumulations of 5-fluoro-UMP, 5-fluoro-UDP, and 5-fluoro-UTP were apparent in the ultraviolet profile (Figure 4e), but there was no accumulation of a pyrimidine intermediate (Figure 4f) as observed for the other pyrimidine antagonists. UTP is a potent inhibitor of mammalian carbamyl phosphate synthetase, which catalyzes the first reaction of the *de novo* pyrimidine pathway ($HCO_3^- \rightarrow$ CAP; eq 1; Lyons & Christopherson, 1985). UTP also inhibits malarial carbamyl phosphate synthetase (Gero *et al.*, 1984). The 5-fluoro-UTP which accumulates may be a potent inhibitor of carbamyl phosphate synthetase in malaria, blocking the pyrimidine

pathway at the first step, but this inhibition may not be sufficient to kill the parasite. The cytotoxicity could result from misincorporation of FUTP into RNA or of dFUTP into DNA or from inhibition of malarial thymidylate synthase by FdUMP. Rathod *et al.* (1992) have found that 5-fluoroorotate (5 μM) induced a 50% reduction in proliferation of *P. falciparum* and a 50% reduction in thymidylate synthase activity while high incorporation (9% of uridine residues) of 5-fluoroorotate into nucleic acids occurred at 1000-fold higher concentrations. While Rathod *et al.* (1992) proposed that thymidylate synthase is the primary target of 5-fluoroorotate in malaria, cellular levels of FdUMP, dTTP, and related metabolites should be measured to obtain direct proof for this inhibition in growing parasites.

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