The use of 5-fluorocytosine and ketoconazole in the culture of the erythrocytic stages of *Plasmodium falciparum* and some tumor cell lines

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Summary. In vitro culture systems are often contaminated by bacteria and fungi. It is therefore often necessary to supplement culture media with agents such as penicillin/streptomycin, gentamycin or amphotericin B. The latter cannot be used in the in vitro culture of erythrocytic stages of *P. falciparum,* and thus anti-fungal agents have not been regularly used in this system. We describe the prophylactic use of 5-fluorocytosine (5-FC) and ketoconazole (KTZ) in tissue cultures at concentrations up to 300 and 10 μg/ml respectively which have no effect on the growth of *P. falciparum* (FCR-3 strain). A melanoma cell line (C32) and a line of uterine carcinoma (C41) were also unaffected by similar concentrations of 5-FC and KTZ. When dissolved in complete culture medium (RPMI 1640) with 10% human plasma, the minimum inhibitory concentration of 5-FC for a susceptible strain of *Candida* remained below 2 μg/ml. These experiments suggest that 5-FC (at 50 μg/ml) alone or in combination with KTZ (at 1 μg/ml) is a useful addition to the armamentarium of antimicrobials available to the tissue culture biologist for a variety of cell culture systems.
Key words. 5-Fluorocytosine; ketoconazole; malaria; tumor cell; cell culture.

Contamination of tissue culture materials remains a major problem for all classes of cell biologists who require a sterile environment. Most commonly, the culture medium which is a rich source of nutrients serves as a ready food supply for all kinds of ambient bacteria, fungi and even errant tumor cells which can inadvertently enter a culture and replace less aggressive living cells. Latent mycoplasma and viruses may also be present.

The malariologist working in vitro with erythrocytic stages of *Plasmodium falciparum* is relatively fortunate in that viruses and mycoplasma are not likely to be present, but fungi and bacteria are still capable of ruining experiments. In answer to the problem of contamination, it has been found that gentamycin (at 40 μg/ml) is useful in preventing bacterial growth without inhibiting protozoan proliferation. The temporary use of 5-fluorocytosine (5-FC) in cultures already contaminated with fungi has been recommended[1], but the continuous use of a suitable prophylactic anti-fungal agent in malaria cultures has not been documented. The polyene antibiotics such as amphotericin which are widely used in cell culture work are not suitable for red cells which cannot tolerate the disruption of membrane integrity which constitutes the mechanism of action of these agents[2]. We have tested in detail the use of 5-FC alone and together with ketoconazole (KTZ) in the malaria culture system and in two continuous tumor cell lines.

Materials and methods. Normal human blood was drawn by the Montefiore Medical Center Blood Bank in standard citrate-phosphate-dextrose-adenine (CPD-A) anticoagulant and used prior to its expiration date. The FCR-3 strain of *P. falciparum* (A2 clone) was obtained from Dr Seymour Schulman and Dr Jerome Vanderberg of the NYU School of Medicine, New York City and maintained in continuous culture by the candle jar method[3] with the following minor modifications which we have previously described[4]. Human plasma (from blood collected in CPD-A) was heat-inactivated at 56 °C for 30 min and used in place of human serum. Gentamycin, 40 μg/ml was added to the RPMI 1640 medium which was supplemented with extra glucose, sodium bicarbonate and TES buffer (N-tris-{hydroxymethyl} methyl-2-aminoethane sulfonic acid; 2-{2-hydroxy-1,1 bis {hydroxymethyl}ethyl} aminoethane sulfonic acid), pH 7.4 (Sigma Corp. St. Louis, Mo., USA). Cultures were initiated in triplicate in 24-well Falcon Multiwell Plates (Becton & Dickinson, Oxnard, CA, USA) at a packed red cell volume of 5% in 1.5-ml volumes, and fresh medium was supplied daily. 5-FC was kindly supplied as Flucytosine by Hoffman-LaRoche Nutley, NJ, USA, in the chemically pure form. It was readily dissolved in RPMI 1640 at a maximum concentration of 300 μg/ml. KTZ was kindly provided by Janssen Pharmaceutica, Inc., Piscataway, NJ, USA. The drug was dissolved in N-N′-dimethylacetamide (DMA) (Eastman Kodak, Rochester, N.Y., USA) at a concentration of 10 mg/ml. This stock solution was added to culture medium to obtain drug concentrations from 0.1 to 10 μg/ml with a maximum concentration of DMA of 0.1%. In separate experiments, DMA was shown not to be toxic to cells at this concentration. The medium with one or both drugs was sterilized by filtration through 0.22-μm filters (Nalgene) and diluted to final concentrations as indicated. The starting inoculum was derived from on-going cultures in normal A+ red cells. The parasitemia (percent infected red cells) was determined each day by counting Giemsa stained smears under the oil immersion lens of a light microscope.

The melanoma cell line (C32) was obtained from Dr Russell Howard, Parasitology Branch of the National

Institutes of Health (Bethesda, Md, USA), and the uterine carcinoma cell line (C41) was obtained as described previously[5]. In order to make a direct comparison, the C32 melanoma cells were grown in the same culture medium as the malaria parasites. However, flat 75 cm^2 Corning tissue culture flasks (Corning Laboratories, Corning, NY, USA) were used. The uterine carcinoma cell line was grown in MEM with 10% bovine fetal calf serum to which the antifungal agents had been added. The adherent cells were removed in 0.5% trypsin, diluted in isotonic saline (Isoton – Coulter Electronics, Hialeah, Fla, USA) and counted in a model ZBI Coulter counter. The data were expressed as the mean ± SD of the triplicate cultures. A Student t-test with correction for small sample size was used to evaluate the results[6]. The minimum inhibitory concentration (MIC) of 5-FC for the yeast *Saccharomyces cerevisiae* (ATCC 36375) was determined for 5-FC in both the complete RPMI 1640 culture medium and a standard broth medium as recommended[7,8]. The results were expressed as the minimum concentration of 5-FC in μg/ml needed for complete inhibition of fungal growth.

Results. Proliferation of *P. falciparum* in complete culture medium containing up to 300 μg/ml of 5-FC was completely unaffected by this anti-fungal agent (table 1). Since concentrations higher than 300 μg/ml were not assayed, no inhibitory concentration of 5-FC was defined for *P. falciparum*. In addition to normal growth rates, morphology of the ring, trophozoite and schizont stages also appeared normal (data not shown). A direct assay of anti-fungal activity of 5-FC in complete malaria culture medium against *Saccharomyces cerevisiae* revealed an MIC of 1.56 μg/ml. This MIC for 5-FC in complete culture medium was 15-fold greater than the MIC for the same agent in the standard beef broth medium in which the MIC was found to be 0.1 μg/ml.

The addition of KTZ to medium containing a fixed concentration of 5-FC at 50 μg/ml (table 2) had no effect on parasite growth. Proliferation proceeded normally for all 5 days of the culture period. A KTZ concentration of 15 μg/ml produced 80–90% inhibition of growth by days 4 and 5 (data not shown). Therefore, a single combination of drugs (50 μg/ml 5-FC and 1 μg/ml KTZ) was used for all other studies. The growth of both the C32 melanoma cells and the C41 uterine carcinoma cells was unaffected by this combination of anti-fungal agents (data not shown).

Table 1. The effect of 5-fluorocytosine (5-FC) on the growth of *P. falciparum* in normal human red cells. Data show the percent parasitemia (mean ± SD) of triplicate cultures.

Day	Control	5-FC 100 μg/ml	5-FC 200 μg/ml	5-FC 300 μg/ml
0	1.8 ± 0.10%	2.6 ± 0.02%	2.2 ± 0.22%	2.3 ± 0.15%
1	4.2 ± 0.30	5.1 ± 0.12	4.9 ± 0.48	4.3 ± 0.15
2	7.1 ± 0.19	6.9 ± 0.60	7.3 ± 0.36	6.1 ± 0.45
3	11.2 ± 0.28	10.5 ± 0.56	10.5 ± 0.37	10.6 ± 0.35
4	13.8 ± 0.96	14.5 ± 0.57	13.8 ± 0.85	14.0 ± 1.70

Table 2. The effect of combined 5-fluorocytosine (50 μg/ml) and ketoconazole on the growth of *P. falciparum* in normal human red cells. Data show the percent parasitemia (mean ± SD) of triplicate cultures.

Day	None	Ketoconazole (μg/ml) 0.1	1.0	10.0
0.	0.8 ± 0.05	0.7 ± 0.06	0.8 ± 0.03	0.8 ± 0.09
1.	2.0 ± 0.08	2.1 ± 0.12	1.8 ± 0.18	1.8 ± 0.02
2.	3.7 ± 0.18	3.4 ± 0.15	3.6 ± 0.32	3.5 ± 0.12
3.	5.0 ± 0.14	5.3 ± 0.08	4.9 ± 0.10	5.1 ± 0.16
4.	8.0 ± 0.12	8.2 ± 0.41	8.1 ± 0.26	7.6 ± 0.27
5.	9.7 ± 0.61	9.2 ± 0.23	9.1 ± 0.31	9.3 ± 0.22

Discussion. Mammalian malaria parasites cannot salvage pyrimidine nucleotides and thus must synthesize these nucleic acid precursors de novo[9]. Thus, it is not surprising that an agent such as 5-FC which primarily interferes with pyrimidine salvage has no toxic effects on the growth of plasmodia. For this reason, 5-FC is probably an ideal anti-fungal agent in this setting. 5-FC is transformed intra-cellularly into 5-fluorouracil and then into fluorouridine. Substitution of the latter compound in RNA disrupts protein synthesis[10]. 5-FC is primarily active against *Candida, Cryptococcus, Torulopsis* and *Aspergillus*. Resistant strains have been observed[10].

It has been previously observed that the actual fungicidal efficacy of 5-FC varies greatly according to the culture medium[11]. It is believed that media containing hydrolysates of biological materials may be rich in native pyrimidine nucleotides which could compete with 5-FC. In addition, there is also the problem of protein binding which affects the concentration of the free drug. For these reasons, it is not surprising to find that 5-FC must be increased about 15-fold in order to obtain an MIC for fungi in the complete culture medium used for malaria parasites. It should be noted however, that this level, which remains below 2 μg/ml is still readily attainable in the medium without toxic effects on *Plasmodia* or the two tumor cell lines tested here.

Because of the possibility of resistant fungi to 5-FC, a second agent, ketoconazole, was added. This agent is poorly soluble in aqueous solutions, and thus, a convenient relatively non-toxic organic solvent was found to serve as a vehicle for the stock solution. KTZ appears to act on a wide spectrum of pathogenic and other fungi by inhibiting ergosterol synthesis[12]. It is active against the mycelial stages of some *Candida* species in concentrations as low as 20 ng/ml[11]. Thus, the 0.5 μg/ml proposed in this study represents a large excess which should be inhibitory even to the less susceptible yeast forms of the same fungi[10].

KTZ has been described as having anti-plasmodial activity against *P. berghei* in vivo[13] and *P. falciparum* in vitro[14]. With respect to the *P. berghei* study in rodents, the effectiveness of KTZ appears to be slight. However, details of the methods and the parasite counts are not provided in this report[13]. The differences between our study and that of Pfaller and Krogstad[14] on the in vitro effect of KTZ on *P. falciparum* may reflect methodologic

or strain differences. Indeed, susceptibility to KTZ may vary in the same manner as susceptibility to chloroquine. The KTZ susceptible strains were derived from Honduras and Indochina [14], whereas the FCR-3 strain (used in this study) is of West African origin. However, because minimal anti-plasmodial effects may still occur with some strains at 1 μg/ml KTZ [15], we recommend that 5-FC be used alone initially. If anti-fungal contamination persists, KTZ can then be added by beginning at 0.5 μg/ml. No anti-plasmodial effect has been reported at this concentration.

Our data also demonstrate that the combination of 5-FC and KTZ is innocuous to two tumor cell lines and thereby suggest that these agents may have a wider applicability in tissue culture work. However, it would be safer to test each cell line before broadening the recommendations for use.

Finally, expense in media preparation is another consideration which unfortunately cannot be ignored. Using the 1988 price list of a popular U.S. chemical supply house as a basis for calculation [16], and assuming the use of 5-FC at a concentration of 50 μg/ml, addition of this reagent would increase the cost of each liter of culture medium by US$ 1.53. Obtaining KTZ from the same supplier would add only US$ 0.01 for each liter (if used at 1 μg/ml). These costs should be balanced against the loss incurred by discarding contaminated medium and plasticware and the labor required to repeat interrupted experiments.

As with all antimicrobials, these agents cannot replace meticulous sterile techniques which remain the mainstay of cell biology laboratory work.

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Decreased number of asialoglycoprotein receptors in diabetic BB Wistar rat

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Summary. The binding of asialoglycoproteins by hepatic binding protein was studied in freshly isolated hepatocytes from genetically diabetic BB Wistar rats. The number of cell surface asialoglycoprotein receptors was dramatically decreased (58,000 ± 38,000 for diabetic rats compared to 267,000 ± 70,000 for normal rats), while the association equilibrium constant was not changed. These results parallel those obtained with streptozotocin-diabetic rats and support the hypothesis that insulin deprivation is responsible for the decrease in the receptor number.
Key words. Diabetes; asialoglycoprotein-receptor; hepatocytes.

A transmembrane receptor, the hepatic binding protein (HBP), is responsible for the selective uptake and lysosomal degradation of asialoglycoproteins (ASGP) [1]. Previous reports have shown that in streptozotocin-diabetic rats, less ligand is bound, internalized and degraded due to a decreased amount of cell surface HBP [2, 3]. The toxicity of streptozotocin raises the question of whether this alteration is really a consequence of insulin deprivation.

The purpose of this study was to explore the binding affinity and the quantity of HBP at the cell surface of hepatocytes of genetically diabetic rats. We used livers from BB Wistar rats, in which diabetes is insulin-dependent [4, 5] and shares many features with the type I pathology in human diabetes.

In this report, we show that hepatocytes of BB rats, like those of streptozotocin-diabetic ones, present a de-