J. King Saud Univ., Vol. 9, *Science* (1), pp. 11-23 (A.H. 1417/1997)

ZOOLOGY

In vitro Conversion of Proguanil to an active Metabolite Using Rat Liver Microsomes and NADPH

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(Received 7-5-1994 ; accepted for publication 15-1-1995)

Abstract. Attempts were made to improve drug screening procedures by the inclusion of a mechanism or a system for metabolising the biguanide proguanil to its active metabolite cycloguanil *in vitro*. In this study the *in vitro* growth of *Plasmodium falciparum* was assessed in the presence of the antimalarial proguanil incubated with the following compounds separately: a) Mouse peritoneal macrophages, b) Minced rat liver and minced rat kidney, both from two groups of rats, one group dosed with phenobarbitone, while the other was not, c) Human liver cell line, d) Rat liver microsomes and NADPH together. With all the above mentioned systems success was achieved only with rat liver microsomes and NADPH.

Introduction

A limitation of existing *in vitro* drug testing is that it fails to identify activity in compounds which *in vivo* are inactive, *per se*, but, which possess activity through one or more of their metabolites. Attempts have been made, therefore to develop an *in vitro* system for testing candidate antimalarial compounds which would also include the capacity to identify active metabolites. The test compound employed in this work was proguanil. This biguanide has almost no antimalarial activity *in vitro* [1], being converted, *in vivo*, to the highly active triazine cycloguanil [2,3], for which the parmacokinetics parameters were measured in a bioassay which assesses *in vitro* growth inhibition of a cycloguanil sensitive strains of Plasmodium falciparum, produced by dilutions of plasma collected after oral administration of the prodrug proguanil [4].

The aim of this work is to study the metabolism of proguanil *in vitro* using different preparations from rats and mice as well as human liver cell line.

Materials and Methods

The S2 isolate of *Plasmodium falciparum* used in this work was selected, at random, from Sudanese isolates, collected in the field. It was maintained in continuous culture, modified, from Jensen and Trager [5], using RPMI-1640 medium supplemented with HEPES buffer, Sodium bicarbonate and human serum blood group AB positive.

Drug assay

The method of regular drug assay was modified from that of Desjardins *et al.* [6] in which, the *in vitro* culture of *Plasmodium falciparum* was incubated with the different concentrations of the drug under test, together with ^{3}H radioactive hypoxanthine the uptake of which by the parasite in each concentration of the drug was measured and expressed as percentage of the uptake by the undosed control parasite culture. 10^{2} Molar proguanil was prepared in 70% ethanol and then further dilutions of the drug were made in complete RPMI medium.

The culture of *Plasmodium falciparum* in a ring stage, synchronized when necessary with Sorbitol [7], was diluted in medium and fresh red blood cells to constitute a suspension of 5% haematocrit and 0.5-1.5% parasitaemia. The culture was dispensed into the assigned wells and incubated for 24 hours after which ^{3}H hypoxanthine was added to each well and reincubated for another 24 hours, after the completion of the 48 hours incubation, the cells were harvested with a cell harvester (Titertek) onto a glass fiber mat through a vacuum generated suction, after complete dryness at room temperature, the individual discs (each coming from a single well) were cut off the fiber mat and introduced into the scintillation counter for determination of the radioactivity, the scintillation counter (Packard 3385) was linked to an Apple lie computer, programmed to calculate the disintegration per minute (DPM) from the counts per minute (CPM) of the radioactive material in each tube and then to find the mean for the first six control tubes, and that for each set of triplicate, separately. The mean DPM of the background, uninfected red blood cells, should be subtracted from the control mean DPM as well as from that of each triplicate of the drug concentrations. The mean DPM of each triplicate which represents the radioactivity uptake by the parasite at that particular concentration of the drug was expressed as a percentage of the uptake by the control parasite i.e. the drug-free parasite. A graph of these percentages was plotted against the drug concentrations in a log graph manner, the IC 50 and IC 90 (the inhibitory concentrations) could be read from the graph.

Mouse peritoneal macrophages

Mouse peritoneal macrophages were obtained from NMRI mice by aseptic dissection under a sterile laminar flow hood (Microflow, Hepaire) after killing by decapitation. The

collected material from different mice was pooled into a sterile container which was placed on ice, and a sample was estimated for the number of the macrophages, using a haemocytometer, then the required number of cells i.e. 1 x 10^6 cells/ml was allowed to adhere into the wells of a micro titre plate which was incubated at 37°C for four hours after which the medium was aspirated, without disturbing the adhered cells. The decanted medium was replaced with 100 µL of fresh medium containing the required concentrations of proguanil to be used for the test of *Plasmodium falciparum* sensitivity as described in the method of regular drug assay. In another series of experiments proguanil hydrochloride and mouse peritoneal macrophages were incubated together in 10 ml complete RPMI medium at a final concentration of 10^{-4} molar and 10^6 cells/ml respectively at 37°C for four hours after which they were spun in a Chilspin (4°C) at 4000 r.p.m. for half an a hour and the required concentrations of proguanil used to test for *Plasmodium falciparum* sensitivity as described before, were made by diluting the supernatant in RPMI medium. This system is used to abolish the effect of macrophages present in the culture, during cell harvesting.

Sliced rat liver and kidney tissues

The rat tissue was prepared as follows: Female outbred Sprague-Dawley rats weighing about 300 grams were killed, one rat was killed by a rapid intravenous injection of pentobarbitone sodium 150 mg/Kg body wt. Two further rats were killed by decapitation, one of which was killed immediately after recovery from anathesia produced by i.v. injecton of 30 mg pentobarbitone sodium. The third rat received no drug before decapitation. The rats were dissected aseptically under the sterile hood, the liver and kidneys were removed and transferred to separate sterile Petri dishes and finely sliced with sterile pairs of scissors and forceps. 6 ml of the finely sliced, semi liquid tissues, were added to 10 ml of complete RPMI medium in 75 ml tissue culture flask (Nunc). From each barbitone dosed rat, the sliced organs were incubated either free or with proguanil hydrochloride at a final concentration of 10^{-4} M at 37°C for four hours. From the undosed rat each liver and kidney preparation was divided into four portions, each portion was incubated for four hours at 37°C in the following manner: 1) with proguanil hydrochloride at a final concentration of 10^{-4} M and 10^{-4} M phenobarbitone sodium, 2) with 10^{-4} M proguanil hydrochloride alone, 3) with 10^{-4} M phenobarbitone sodium alone and 4) free of any drug to act as a control. After incubation, using sterile centrifuge tubes, all the different preparations were spun in a Chilspin (4°C) at 4000 r.p.m. for half an hour and the supernatants were diluted in medium to the required concentrations of proguanil which were inoculated into the respective wells of the microtitre plates, used for testing *Plasmodium falciparum* sensitivity as described in the method of regular drug assay.

Human liver cell line

The human liver cells preparation used in this work was the commercially available Chang liver cell line obtained from Flow laboratories, it was maintained in a monolayer form, in Minimum Essential Medium (MEM) supplemented with 10% calf serum, both obtained also from Flow laboratories. Two tissue culture flasks of the monolayer were set up, one was incubated with 10^{-4} M proguanil hydrochloride while the other was left drug-free to act as a control. A separate flask containing only MEM and fetal calf serum was inoculated with progunail hydrochloride at a final concentration of 10^{-4} M proguanil. All the tissue culture flasks were incubated at 37°C for 16 hours and that is because it is a monolayer and consequently the number of the hepatocytes is relatively fewer than that in the crude rat liver preparation, after incubation the medium was transferred into sterile centrifuge tubes and spun at 40000 r.p.m. in a Chilspin at 4°C for half an hour. The supernatants were used to prepare a set of three microtitre plates, one containing the required concentrations of proguanil was incubated with the liver preparation and another containing the required concentrations in the liver-free medium, while the third contained the drug-free medium which was incubated with the liver preparation. All the three plates were used for testing *Plasmodium falciparum* sensitivity as decribed before.

Rat liver microsomes plus NADPH

Rat liver microsomes were prepared by Braithwaite (Liverpool, School of Tropical Medicine, Department of Pharmacology) by homogenizing the liver in ice cold phosphate buffer and centrifugation of the homogenate at 13,000 g at 4°C followed by centrifugation of the supernatant at 105,000 g at 4°C. Microsomal protein content was determined by the method of Lowry *et al* . [8]. Cytochrome P450 protein content was determined by the method of Omura and Sato [9].

The stock solution of microsomes containing 6.4345 mg protein/ml and 0.335 n mole P450 /mg protein was diluted to 3 mg microsomal protein / ml of Phosphate buffer, to the diluted solution, proguanil hydrochloride was added to give a concentration of 10^{-6} molar proguanil before the final solution was divided into 2 ml aliquot into six glass centrifuge tubes. To the first (blank) tube, 2 ml of ethanol was added and then blown off with nitrogen gas to restore the original 2 ml volume. NADPH was added to the other five tubes to give a final concentration of 4×10^{-3} molar, the tubes were incubated at 37°C and the reaction in each tube was terminated by the addition of 2 ml ethanol at the following times: 0, 5, 10, 15 and 30 minutes.

After the termination of the reaction ethanol was blown off with nitrogen gas to restore the original 2 ml volume, then each solution was filtered through an acrodisc filter 0.2 m (Gilman) and from the sterile solutions, the required dilutions were made in complete RPMI medium and utilized for *Plasmodium falciparum* sensitivity as described previously.

Results

The biguanide proguanil was used in all tests to investigate the conversion of the drug to its metabolites *in vitro*. There was no growth inhibitory effect *in vitro* on the S2 isolate of *P. falciparum* at concentrations below 10^{-6} molar, its IC 50 value was 8.6 x 10^{-6} M. in the presence of the mouse peritoneal macrophages experiments to determine proguanil sensitivity gave disappointing results.

Uninfected red blood cells incubated with macrophages in the presence of ^{3}H hypoxanthine gave very high radioactivity i.e. a high uptake of ^{3}H hypoxanthine compared to uninfected red blood cells incubated without macrophages (Table 1). Addition of proguanil in this culture system with infected blood cells provided an IC 50 value which was similar to that obtained in macrophage free culture i.e. proguanil had not effect on the parasite culture (Fig. 1). Preincubation of proguanil with the mouse

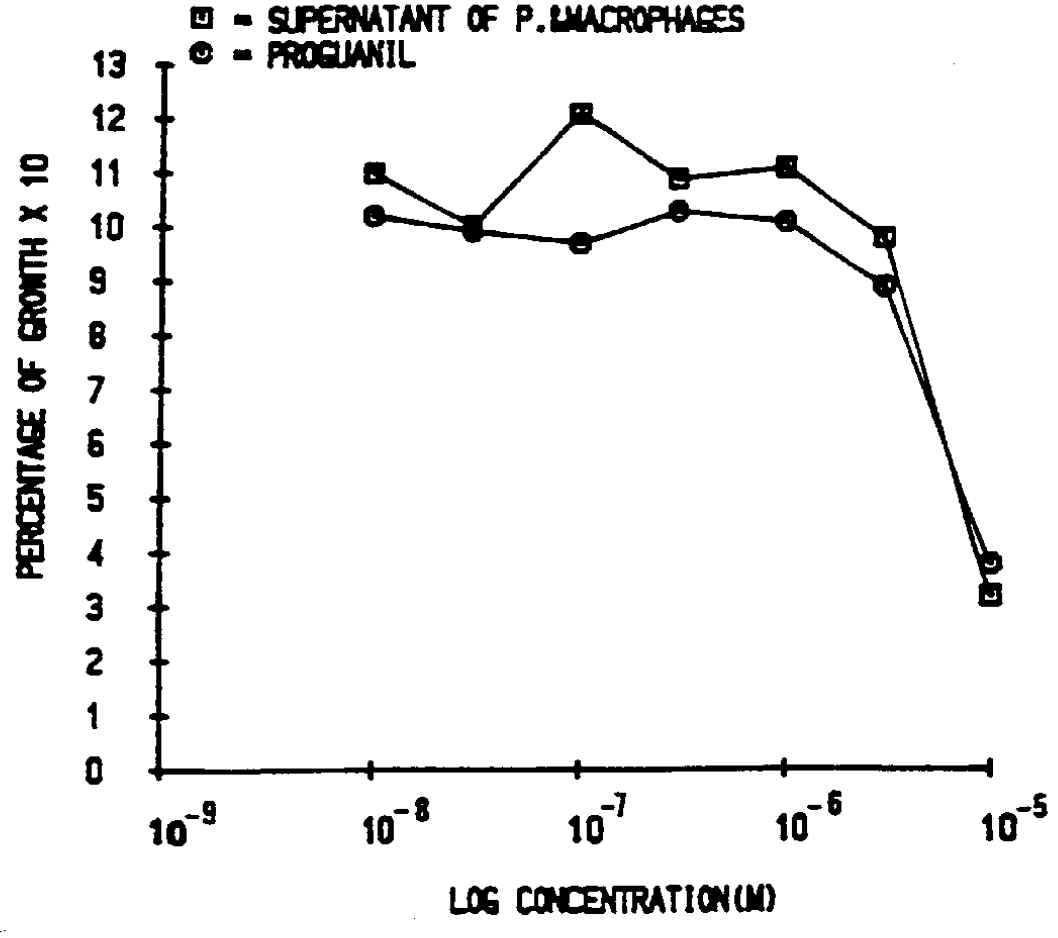

Fig. 1. The sensitivity of S2 isolate of *P. falciparum* to proguanil hydrochloride after being incubated with mouse peritoneal macrophages in RPMI medium □ as compared to sensitivity to proguanil hydrochloride incubated with RPMI medium only ①.
The growth indicator was H^{3} hypoxanthine incorporation.

peritoneal macrophages, minced rat liver and minced rat kidney was also ineffective (Figs. 2 and 3 respectively).

The IC 50 value of the drug by the use of a Chang human liver cell line was unaltered from that of proguanil alone (Fig. 4).

Table 1. The marked difference of the uptake of ^{3}H hypoxanthine by red blood cells and macrophages as compared to the uptake by the red blood cells alone

Background tube number	Details	CPM	DPM
1-3	Uninfected red blood cell + Macrophages	1985 ± 462.5	8632 ± 2045
4-6	Uninfected red blood cells only	185 ± 59.5	746.6 ± 225

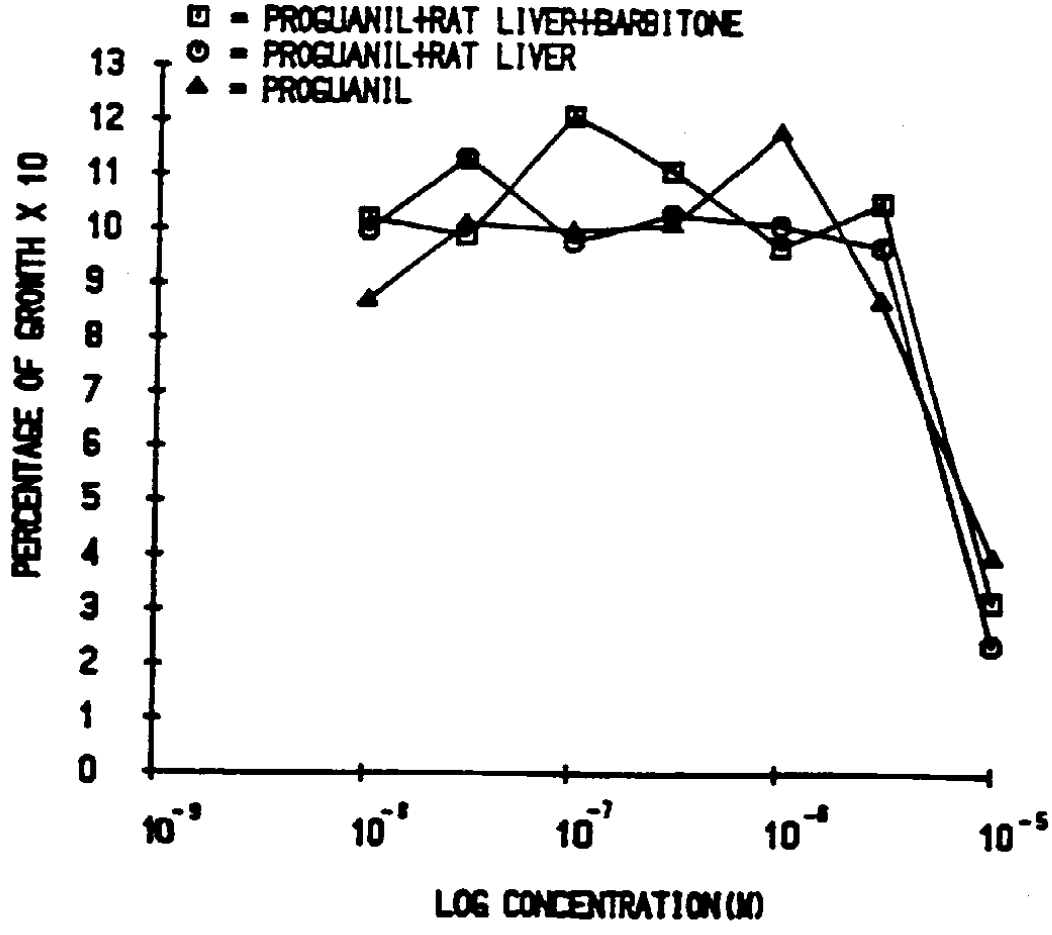

Fig. 2. The sensitivity of S2 isolate of P. falciparum to
△ Proguanil hydrochloride incubated with RPMI medium
○ Proguanil incubated with minced rat liver in RPMI medium
□ Proguanil incubated with minced rat liver and 10^{-4} molar phenobarbitone in RPMI medium
The growth indicator was H^{3} hypoxanthine incorporation
N.B.: Results with livers from rats dosed *in vivo* with phenobarbitone were similar.

Using microsomal preparations of rat liver to which NADPH had been added, a marked activation of proguanil was obtained. Five minutes incubation of the drug with the microsomal preparation gave a reaction product with an IC 50 level of 4×10^{-9} M compared to 8.6×10^{-6} M for proguanil alone. Similar lower value also being obtained after incubation for upto 30 minutes (Fig. 5).

Discussion

Mouse peritoneal macrophages were used as a feeder layer to increase the growth rate in *Plasmodium falciparum* culture [10] in this series of experiments they were used to eliminate their interfering role in drug screening in the future if they are goining to be used routinely for the above mentioned purpose. The experiment of Hawking and Perry

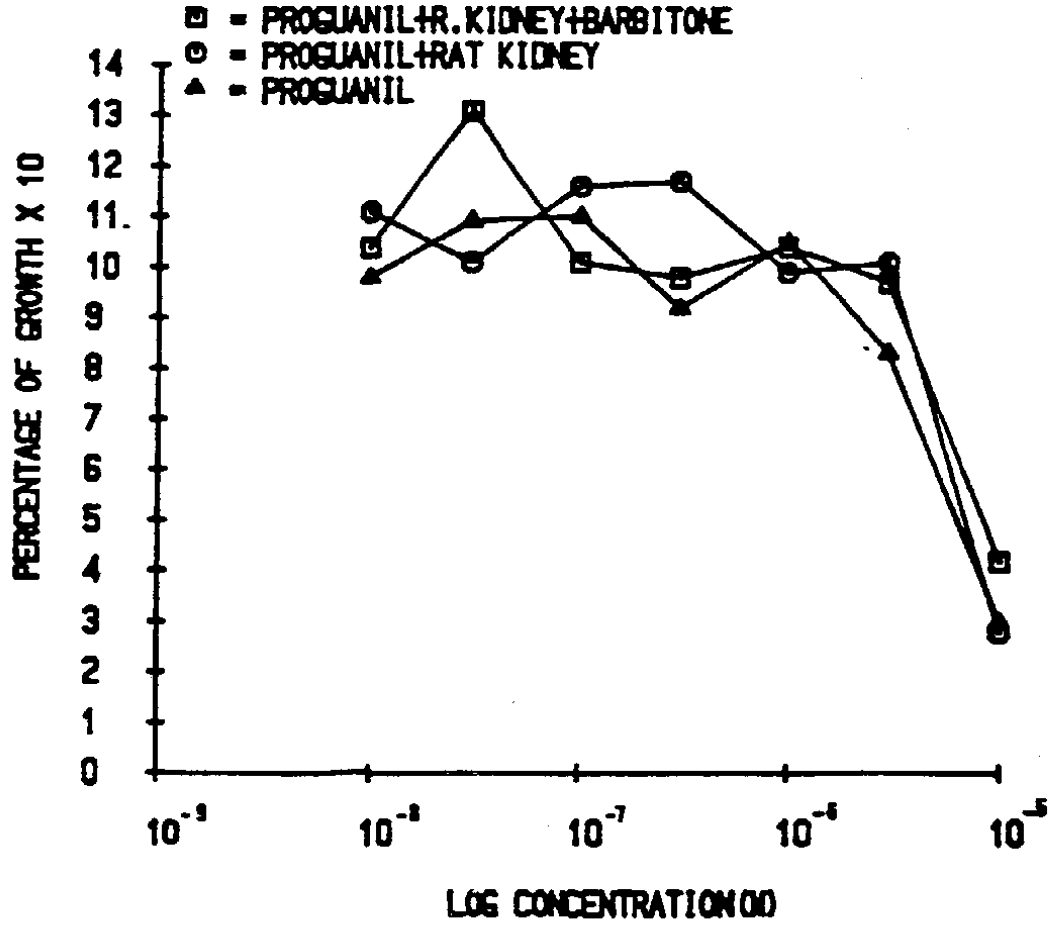

Fig. 3. The sensitivity of S2 isolate of *P. falciparum* to
△ Proguanil hydrochloride incubated with RPMI medium
⊖ Proguanil incubated with minced rat liver in RPMI medium
⊟ Proguanil incubated with minced rat kidney and 10^{-4} molar phenobarbitone in RPMI medium
The growth indicator was H^3 hypoxanthine incorporation.
N.B.: Results with kidneys from rats dosed *in vivo* with phenobarbitone were similar.

[11], in which proguanil was converted to an active metabolite *in vitro* by incubation with minced rat liver, was repeated in this work using the sexual blood stages of *P. falciparum* instead of the exoerythroctic stages of the avian malaria parasite *P. gallinaceum*, however in contrast to their result, the IC 50 value obtained after the incubation of progunail with the sliced rat liver was similar to that of progunail alone and this could be due strain variation as it has been shown that proguanil was effective *in vitro* against the exoerythrocytic stages of *Plasmodium cynomolgi* and *P. knowlesi* infecting cultured simian liver [12], though it was ineffective *in vitro* against the same stages of *Plasmodium yoelii* infecting cultured BALB/c hepatocytes [13].

Addition of phenobarbitone to the rat liver preparation, pentobarbitone pretreatment of the rats did not influence the results, however enzyme induction with phenobarbital is a time dependant process and requires a daily injection of phenobarbital for at least 72 hours prior to the start of the *in vitro* experiment, and in this series of experiments lack of induction *in vitro* cannot be concluded at a large scale, but the immediate lack of

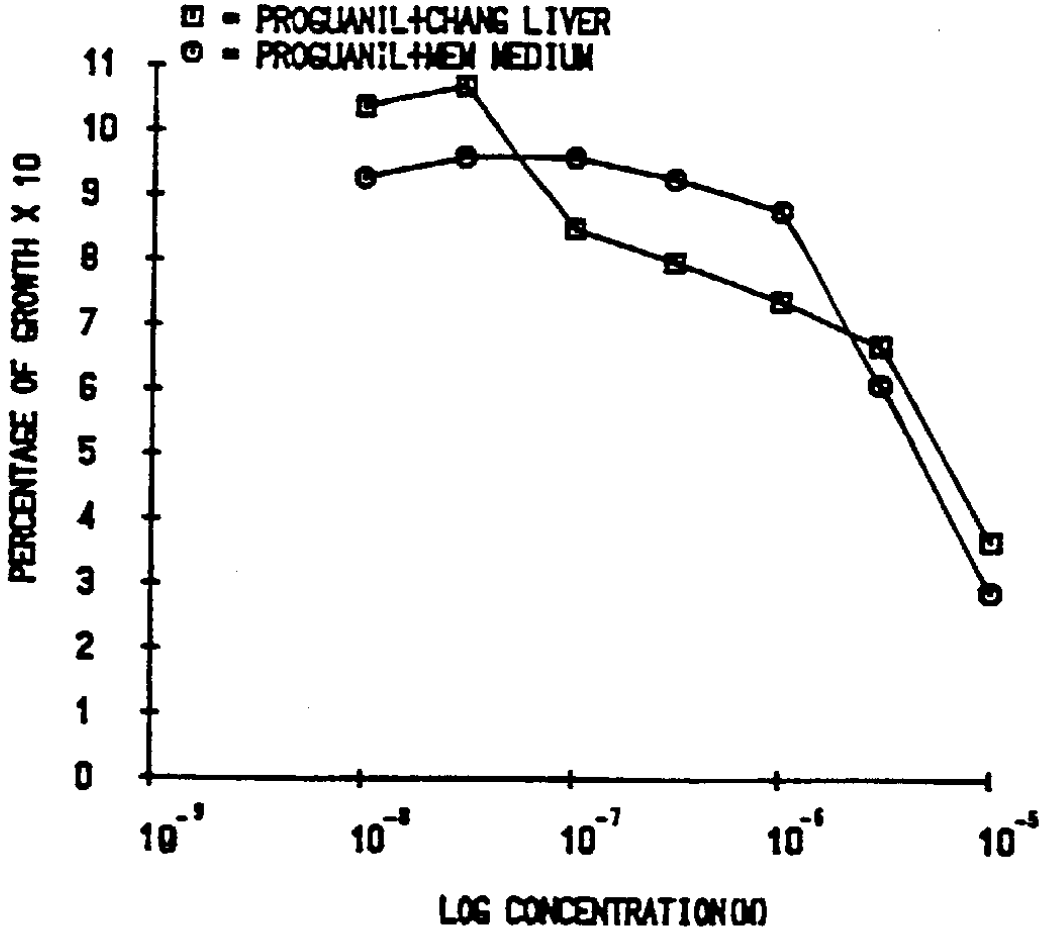

Fig. 4. The sensitivity of S2 isolate of *P. falciparum* to proguanil incubated with Chang human liver cells in MEM medium ◻ as compared to proguanil incubated in MEM medium only ○.
The growth indicator was H^3 hypoxanthine incorporation.

induction can be confirmed. Similar lack of activation was obtained with minced rat kidney which was used here to eliminate any non specific inherent rat tissue inhibitory effect, if the rat liver preparation had any positive effect.

Two metabolites of proguanil were isolated from monkey urine [14], the first was identified as N-P-chlorophenyl biguanide (PBG) and the second which had most antimalarial activity was a triazine. The triazine was identified by Carrington *et al.* [2] as 1-P-chlorophenyl-2-4-diamino-6, 6-dihydro-1,3, 5-triazine (CGT or cycloguanil). The pharmacokinetics of both compounds were studied in man using a highly sensitive and specific high-performance liquid chromatographic assay [15]. Cycloguanil was found to be less active than proguanil against *Plasmodium cynomolgi* in a similar host [16] and against *Plasmodium falciparum* in man [17]. Armstrong and Smith [18] were able to demonstrate species variation in proguanil metabolism among animals. Recently, Ward *et al.* [19] showed marked inter-subject variation in the metabolism of the drug in man in

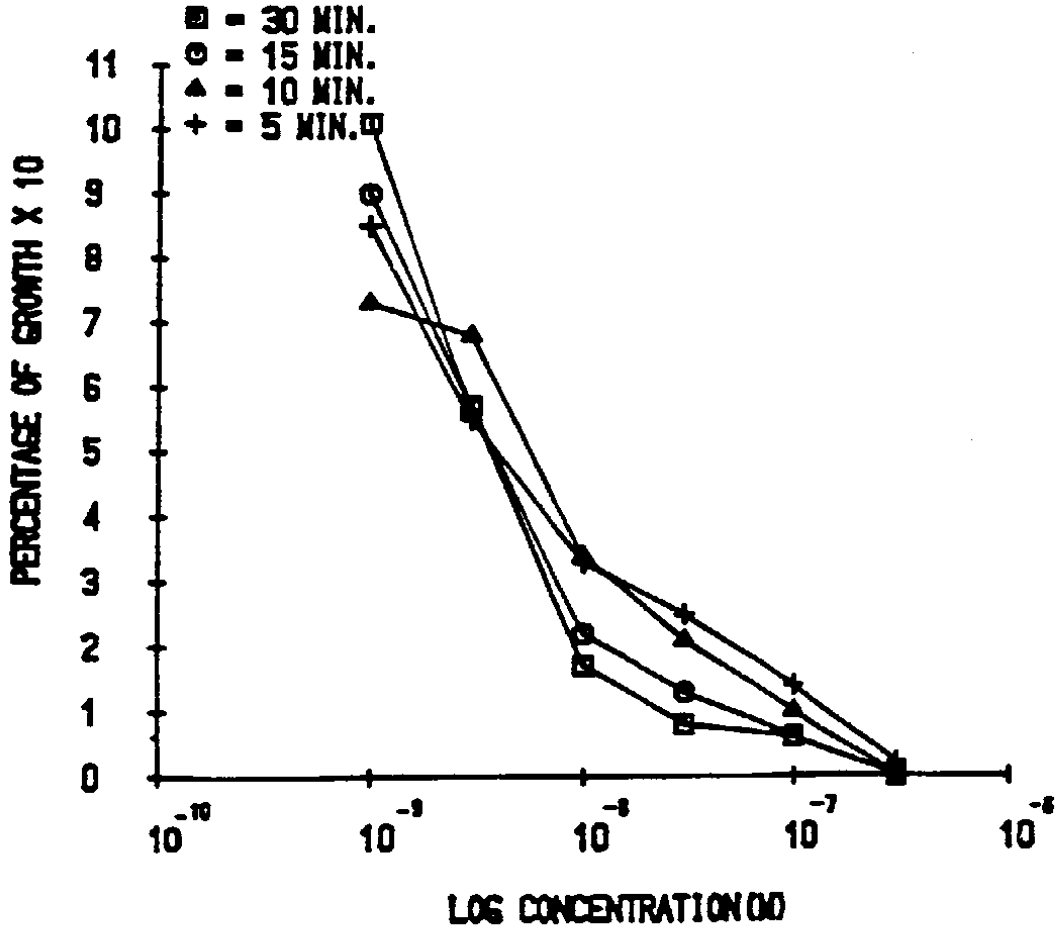

Fig. 5. The sensitivity of S2 isolate of *P. falciparum* to proguanil hydrochloride after being incubated with rat liver microsomes + NADPH.

N.B.: The control *P. falciparum* + proguanil without rat liver microsomes and NADPH was similar to that in Fig. 1.

two different ethnic groups, namely, British Troops and Kenyan school children. Armstrong and Smith [18] suggested that proguanil metabolism is accomplished by hepatic microsomal mixed function oxidase system and it is enhanced by phenobarbitone and 3-methyl cholanthrene (3-MC), and inhibited by microsomal mixed function oxidase inhibitors. They also explained that proguanil is N-dealkylated by cytochrome P-450 to p-chlorophenyl biguanide and cyclised by cytochrome P-448 to cycloguanil.

This series of experiments has demonstrated that effective metabolism of proguanil *in vitro* may be readily achieved by a preparation of rat liver microsomes as previously shown in a biochemical assay by Armstrong and Smith [18], but was not obtained with mouse peritoneal macrophages, the Chang human liver cell line or the crude sliced liver preparation [11]. The preparation of rat liver microsomes plus NADPH is a multistep procedure and costly in time and materials. This is, thus, unlikely to be appropriate as a routine component of a primary drug screening program. Nevertheless, the experiments can be performed utilizing the 13,000 g supernatant fraction which contains the membrane-bound enzymes including mixed function oxidases, soluble enzymes and NADPH-generating systems. The reasons for performing the experiments utilizing the 105,000 g microsomal fraction which contains the membrane-bound enzymes of the endoplasmic reticulum, is to rule out the metabolic effect of the soluble enzymes.
The slight increase in activity of proguanil metabolite cycloguanil *in vitro* (IC 50.4 $x10^{-9}$ M) as compared to that of cycloguanil *pèr se*, which had an IC 50 to 2.1 x 10^{-8} M for the same parasite strain (unpublished work) is consistent with a similar observation *in vivo* which was attributed in part to antimalarial activity inherent in the parent drug i.e. the unmetabolised proguanil [20]. This slight increase in activity could not be attributed to synergism between the unmetabolised proguanil and the metabolite cycloguanil as Watkins *et al.* [21] had demonstrated an antagonistic rather than a synergistic action between proguanil and cycloguanil in his *in vitro* experiments with *P. falciparum* after the initial finding of a retained weak activity of proguanil against cycloguanil-resistant parasites. Change of proguanil activity *in vitro* due to parasite variation has already been referred to [12] and [13]. The other metabolite of proguanil, namely the *p-chlorophenylbiguanide* (PBG) is a minor metabolite with insignificant antimalarial activity [21 and 22].

It was concluded that to incorporate into the *in vitro* test for drug action, a device for generating drug metabolites, crude liver preparations, similar to those employed by Hawking and Perry [11], were inadequate, as were test systems incorporating feeder layers of mouse macrophages or Chang human liver cells as they all showed a negative activation response. The most effective device was the incubation of the test compound, proguanil, with rat liver microsomal preparations in the presence of NADPH. This technique is inappropriate for routine large scale screening systems but may have value in the examination of antimalarial activities in restricted series of compounds, an example

are several natural plant products which are employed as traditional remedies, and where *in vivo* test using rodent malaria is not established or difficult to establish and maintain.

Acknowledgment. I wish to thank Professor G.S. Nelson for the use of facilities in the Department of Parasitology, Liverpool School of Tropical medicine where this work was done, and Professor R.E. Howells for his guidance and encouragement throughout the study.

I thank the following for their assistance: Mr. I. Braithwaite for the preparation and supply of the rat liver microsomes, Mr. P. Bray and Miss G. Smith for their technical assistance in the laboratory.

Finally, I would like to thank the Sudanese National Council for Research for the financial support of this work.

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تحوّل دواء البروجوانيل إلى مستقلبه الفعال خارج الجسم الحي بإضافة مستحضر الأجسام الدقيقة من كبد الجرذان ومستحضر إن آ دي بي إتش NADPH

أبو بكر مكي العجيل

مستشفى الأمل بالرياض، ص. ب ٢٩٩٣٢ ،

الرياض ١١٤٦٧ ، المملكة العربية السعودية

(استلم في ٢٦ ذي القعدة ١٤١٤هـ؛ قبل للنشر في ١٤ شعبان ١٤١٥هـ)

ملخص البحث. تمت عدة محاولات لتحسين طرق تقصّي العقار بإدخال آلية، أو نظام لاستقلاب عقار البروجوانيل ثنائي الجوانايد (the biguanide proguanil) إلى مستقلبه الفعّال السايكلوجوانيل خارج الجسم الحي *in vitro*، وذلك باستعمال تحضيرات مختلفة من الفئران والجرذان.

استخدمت لهذه الدراسة سلالة طُفيل الملاريا *Plasmodium falciparum* لقياس درجة نموه خارج الجسم الحي بعد إضافة البروجوانيل فقط أو بالإضافة إلى استعمال خلايا بلعية من التجويف البريتوني للفئران أو إضافة نسيج مقطع من الكبد أو الكلى، وجرذان محقونة أو غير محقونة بالفينوباربيتون، أو استعمال خط خلايا كبد آدمية.

ولقد وجد أن كل هذه التحضيرات لم تنجح في تحويل دواء البروجوانيل إلى مستقلبه الفعّال. وعند استخدام مستحضر الجسيمات الصغيرة لكبد الجرذان، وإضافة مستحضر إن آ دي بي إتش NADPH مع دواء البروجوانيل لطفيل الملاريا، لوحظ عدم نمو الطفيل. ويدل ذلك على أن مستحضر الجسيمات الصغيرة لكبد الجرذان وإضافة NADPH يساعدان على تحويل دواء البروجوانيل إلى مستقلبه الفعّال السايكلوجوانيل خارج الجسم الحي.