Mitt. Österr. Ges.
Tropenmed. Parasitol. 17 (1995)
229 - 234

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In vitro Sensitivity to Atovaquone of *Plasmodium falciparum* in Northeastern Tanzania 1993

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Introduction

The antimalarial activity of naphthoquinones has been known since the mid-1940s when a series of 2-hydroxy-1,4-napthoquinones was investigated in avian malaria models (3). Later, several series of naphthoquinones were studied by the Walter Reed Army Institute of Research, Washington D.C., but even the then most advanced compound, 3-(8-cyclohexyloctyl)-2-hydroxy-1,4-naphthoquinone (menoctone or WR 49 808) proved to have only a very low therapeutic acitvity against *Plasmodium falciparum* in man. It was concluded that "poor absorption from the gastrointestinal tract is characteristic of compounds of this group and may explain the lack of demonstrable activity by WR 49 808" (13).

There was renewed interest in the antimalarial activity of the naphthoquinones since the 1980s when 2-(4-t-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone (compound BW 58C) proved to be highly active against *P. falciparum* in vitro and against rodent and avian plasmodia in vivo (7). The compound was also active against *Theileria parva, Th. annulata* and *Eimeria tenella*. In the *P. yoelii nigeriensis* mouse model it showed blood schizontocidal and causal prophylactic activity. Although well tolerated the development of this candidate drug was discontinued for cosmetic rather than technical reasons, searching now for an improved analogue. This was found in the form of atovaquone, i. e. 4-chlorophenyl-2-cyclohexyl-3-hydroxy-1,4-naphthoquinone (Fig. 1).

While initially developed as an antimalarial candidate drug, atovaquone was found to possess strong activity against various infective agents, especially *Pneumocystis carinii* (6, 8) and it has been registered for the treatment of such infections. Nevertheless, atovaquone continues to hold strong interest in malaria due to its blood schizontocidal, causal prophylactic and sporontocidal activity (11). It is assumed to act as an antagonist of the ubiquinones and thereby to interfere with mitochondrial electron transport (5). It does not seem to have activity correlations with the standard antimalarials such as 4-aminoquinolines and 4-quinolinemethanols (11). Clinical trials in falciparum malaria showed good initial clinical and parasitological response, but a relatively high recrudescence rate (9). However, combination therapy with atovaquone and proguanil produced acceptable cure rates.

As atovaquone is a novel antimalarial compound it was of interest to investigate the in vitro response of fresh natural isolates of *P. falciparum* in an area with intensive malaria transmission and marked resistance to chloroquine.

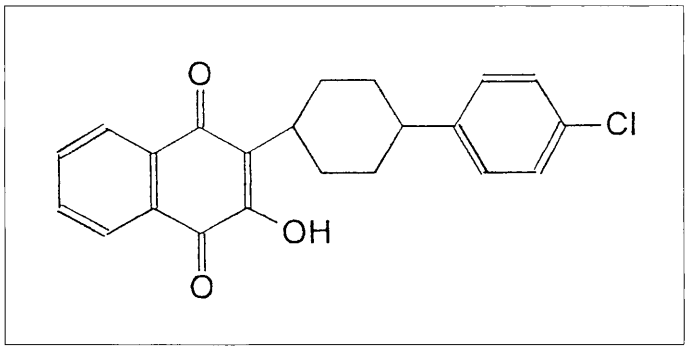

Figure 1:
Atovaquone

Material and methods

The study was carried out in May - July 1993 in the District of Muheza, Tanga Region, Tanzania, with the laboratory base at the Ubware Field Research Station of the Amani Research Centre, National Institute of Medical Research of Tanzania. The investigation formed part of an assessment of the response of *P. falciparum* to operationally used antimalarials such as chloroquine, quinine, mefloquine and sulfadoxine/pyrimethamine. In the District of Muheza malaria transmission is intensive. Malaria is highly stable and holo-endemic. *P. falciparum* dominates the plasmodial species formula (approximately 95% of all positive blood specimens), followed by *P. malariae* (approximately 10% of all positives), and *P. ovale* (approximately 3% of all positives). Most of the *P. malariae* and *P. ovale* infections are mixed with *P. falciparum*. There is very little seasonal variation in the positivity rates for malaria and in the parasite species formula.

Study population

The parasite isolates came from persons with *P. falciparum* mono-infections who were either suffering from clinically manifest malaria and reporting for treatment, or found to be chronic, oligosymptomatic carriers of the parasite. The persons had no recent antimalarial drug intake and an asexual parasitaemia of 1,000 - 50,000/µl blood. In total 36 *P. falciparum* isolates were tested. They came from persons between 4 and 17 years of age (mean 9.9 ± 2.4 years). The relatively young age of the patients can be explained by the high degree of malaria endemicity in the study area where asexual *P. falciparum* parasitaemia ≧ 1,000/µl is rare among persons over 15 years of age.

Drug sensitivity testing

The drug sensitivity tests were carried out with blood samples obtained by fingerprick, following the WHO Standard Microtest Method for the assessment of schizont maturation inhibition (12, 14). In consideration of the atovaquone plasma concentrations measured in pharmaco-kinetic studies and of the results of preceding validation studies, the following concentration range was tested:

Well	Concentration
A	Control (no drug)
B	10 nmol / l blood-medium mixture
C	30 nmol / l blood-medium mixture
D	100 nmol / l blood-medium mixture
E	300 nmol / l blood-medium mixture
F	1000 nmol / l blood-medium mixture
G	3000 nmol / l blood-medium mixture
H	10000 nmol / l blood-medium mixture

The tests were performed using the material of the WHO standard test kits, supplied through the WHO Regional Office for the Western Pacific, Manila, except for the microplates dosed with atovaquone which were prepared at the Institute of Specific Prophylaxis and Tropical Medicine, University of Vienna, Austria. RPMI 1640 LPLF (i. e. RPMI 1640 with reduced contents of para-aminobenzoic acid and folic acid) that is routinely supplied with the WHO test kits was used for the tests as it could be anticipated that ordinary RPMI 1640 would produce erroneous readings as is the case with the antagonists of the folate pathway.

Atovaquone was obtained from Wellcome Reasearch Laboratories, Beckenham, U.K.

Counting methods

The procedure for assessing pre-incubation parasitaemia followed the WHO standard methodology (15). In addition, 100 thick film fields of the Giemsa-stained slides were read in order to exclude mixed infections.

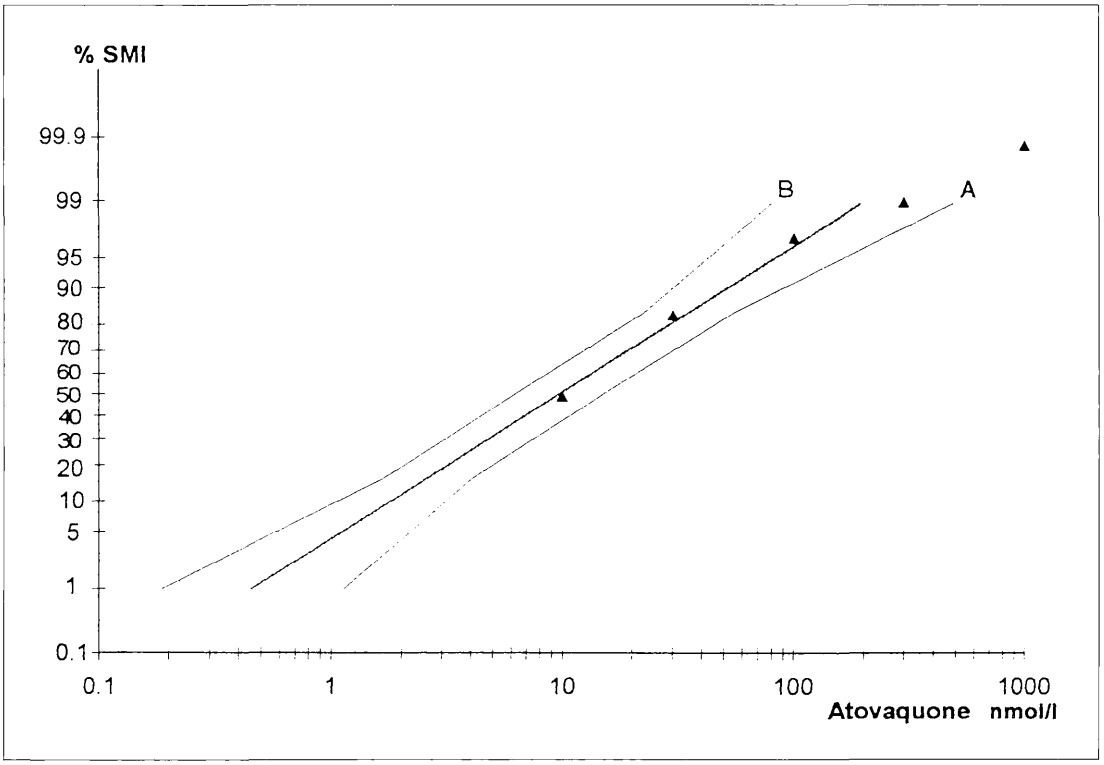

Figure 2:

Sensitivity of *Plasmodium falciparum* to atovaquone in Northeastern Tanzania, 1993. % SMI = % inhibition of schizont maturation · 95% confidence intervals of regression line indicated (A = higher, B = lower confidence interval).

The schizont counts were read by counting the total number of schizonts per 200 asexual *P. falciparum* parasites in accordance to the WHO method (14). Tests were considered successful when at least 10% of the parasites in the control wells (A) had reached the schizont stage.

Statistical analysis

Following the most widely practised method for the evaluation of dose response experiments, the results were analysed by the log concentration / response probit procedure described by LITCHFIELD and WILCOXON (10). This method is based on the "least squares" procedure. An adaptation to EDP (4) facilitated the analysis.

Results

The success rate of the tests was 92% (33 out of 36). The geometric mean parasite count of the successfully tested isolates was 2,153/µl blood. The mean schizont count of the control wells was 107.4 / 200 asexual parasites (53.7%).

None of the isolates was fully inhibited at the lowest concentration tested (10 nmol / l blood-medium-mixture [BMM]). The large majority of isolates had a cut-off point (i. e. the concentration at which no schizont maturation had occurred) of 300 nmol / l BMM. The last of the isolates had a cut-off point of 3,000 nmol / l BMM (Tab. 1).

The relative inhibition of schizont maturation is quite marked already at the lowest concentration (49.4% at 10 nmol / l BMM), rising consistently to 99.9% at 1,000 nmol / l BMM. The log concentration / response probit regression line shows a good fit to the observed data points of inhibition (Fig. 2), with a Chi^2 value for heterogeneity of 0.7045 (maximum acceptable 9.49). This results in relatively narrow confidence intervals of the regression line.

The regression parameters are shown in Table 2. The comparison of EC_{50} (9.52 nmol / l BMM), EC_{90} (50.76 nmol / l BMM) and EC_{99} (198.65 nmol / l BMM) denotes a relatively flat regression line.

Discussion and conclusions

The high success rate of the tests (92%) reflects an efficient sampling process and especially the exclusion of donors with residual drug. The same is implied by the rather high mean counts of schizonts in the controls (53.7%).

Table 1:

Sensitivity of *Plasmodium falciparum* to atovaquone in Northeastern Tanzania, 1993 (number of isolates tested: 33).

Drug concentration in nmol / l BMM	No. (%) fully inhibited	% Inhibition of schizont maturation
10	0 (0)	49.37
30	5 (15)	83.28
100	16 (48)	97.10
300	30 (91)	98.99
1000	32 (97)	99.87
3000	33 (100)	100.00

Table 2:

Sensitivity parameters of atovaquone in *Plasmodium falciparum* from Northeastern Tanzania, 1993

Regression	EC in nmol / l BMM	
Probit $y = a + b \times \ln_x - 5$	EC_{50}	9.5225
A = 3.27337	EC_{90}	50.7596
b = 0.76596	EC_{95}	81.5563
Chi^2 = 0.70455 (Max. acc. 9.49)	EC_{99}	198.6515

Slope Function S = 3.6633
95% Confidence Intervals of S 2.6245 to 5.1133
Factor EC_{50} = 1.5569 · Factor $_S$ = 1.3958

The selected drug concentration range of the microtitre plates was correct. Ideally, one would prefer to start with an inhibition of approximately 20% at the lowest concentration and locate the cut-off point of schizont maturation at well F. This will not be possible in the case of atovaquone unless a geometric progression of 4 is adopted which would carry the risk of loosing important detail, especially data points between EC_{16} and EC_{84} which are crucial determinants of the confidence intervals.

The good fit of the data points to the regression line, confirmed by the low Chi^2 value for heterogeneity, indicates very little, if any, specific selection pressure of atovaquone or related drugs in the study area. This is quite understandable for atovaquone since this compound has not yet been used in Tanzania. It also seems to sustain the assumption that the mechanism of action of atovaquone is quite different from that of other blood schizontocidal drugs (5, 11).

The rather flat concentration-reponse line would explain the substantial parasitological recrudescence rate after therapeutic challenge. This problem has obviously been overcome by combining atovaquone with proguanil (1, 9). In view of individual differences in cytochrome P-450 activity responsible for the metabolic conversion of proguanil to the active metabolite, cycloguanil (2), the use of tetracyclines as potential partners of atovaquone seems to be worth pursuing.

Given the precarious state of drug response of *P. falciparum* in many parts of the world and the paucity of suitable alternative candidate drugs, atovaquone is definitely a compound that merits further development as an antimalarial. An additional asset is its causal prophylactic and sporontocidal potential.

Summary Atovaquone, a naphthoquinone, is an anti-infective agent with a relatively wide spectrum of activity which also includes plasmodia. Its specific acitvity seems to be independent of that of the standard antimalarial drugs currently in use. Clinical studies in multi-drug resistant falciparum malaria yielded encouraging results. The in vitro sensitivity of *Plasmodium falciparum* to atovaquone has been studied in Muheza District, Tanzania, in 1993, using a schizont maturation inhibition test newly developed for this purpose. The results from 33 fresh isolates showed an EC_{50} of 9.52 nmol / l BMM and an EC_{90} of 50.76 nmol / l BMM. The log concentration / response probit regression line was rather flat but showed a good fit to the data points. In some isolates schizont maturation was completely inhibited only at a concentration which might be clinically not reached with the usual dose regimen. This would explain the need for combining atovaquone with a suitable synergistic partner drug in order to obtain radical cure in falciparum malaria.

Key words *Plasmodium falciparum,* drug response, atovaquone, naphthoquinones, Tanzania.

Zusammenfassung

Atovaquone-Empfindlichkeit in vitro von Plasmodium falciparum in Nordost-Tansanien, 1993

Atovaquone, eine Naphthochinonverbindung, besitzt ein relativ breites antiinfektiöses Wirkungsspektrum, welches auch Plasmodien einschließt. Seine Wirkungsweise scheint sich von jener der gegenwärtig gebrauchten Malariamittel zu unterscheiden. Klinische Studien bei multiresistenter Falciparum-Malaria haben ermutigende Ergebnisse gezeigt. Die in vitro-Sensibilität von *Plasmodium falciparum* gegenüber Atovaquone wurde 1993 im Distrikt von Muheza, Tansanien, untersucht. Die Ergebnisse der Schizontenreifungstests von 33 frischen Isolaten ergaben eine EC_{50} von 9,52 nmol / l Blut-Medium-Mischung (BMM) und eine EC_{90} von 50,76 nmol / l BMM. Die Logkonzentrations-Hemmprobitregression war relativ flach, zeigte jedoch eine gute Übereinstimmung mit den beobachteten Meßpunkten. Einige Isolate zeigten erst bei Atovaquone-Konzentrationen eine vollständige Hemmung der Schizontenreifung, welche unter normaler Dosierung schwerlich erreicht werden dürften. Daher ist es notwendig, Atovaquone mit einem geeigneten synergistischen Partner zu kombinieren, um eine komplette Beseitigung der Falciparum-Malaria sicherzustellen.

Schlüsselwörter *Plasmodium falciparum,* Arzneimittelempfindlichkeit, Atovaquone, Naphthochinone, Tansanien.

Acknowledgements The writers wish to express their thanks to Mrs. Zeina Ally, Mr. Ezechiel Malecela and Mr. John Hiza from the Amani Research Centre, National Institute of Medical Research of Tanzania, for their most valuable assistance in the field and laboratory work. Thanks are also due to Wellcome Research Laboratories, Beckenham, U.K., for providing the test compound.

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