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IN VITRO TESTING OF ANTIMALARIAL EXO-ERYTHROCYTIC SCHIZONTOCIDES IN PRIMARY CULTURES OF HEPATOCYTES

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An in vitro *model using primary cultures of laboratory-bred* Thamnomys gazellae *hepatocytes and* Plasmodium yoelii yoelii *sporozoites was established for chemotherapeutic studies. The culture surface was reduced (0,5 cm) to allow more rapid performance and analysis of tissue schizontocidal tests, with less biological material (rodents, Anopheles, sporozoites), 20 compounds were tested. The results are discussed in comparison with those of other* in vivo *and* in vitro *systems.*

Four species of murine malaria parasites are known at present: *Plasmodium berghei* Vincke & Lips, 1948, *Plasmodium vinckei* Rodhain, 1952, *Plasmodium chabaudi* Landau, 1965, and *Plasmodium yoelii* Landau & Killick-Kendrick, 1966. Their life cycle can be maintained in the laboratory by cyclical transmission through *Anopheles stephensi* to common laboratory hosts (mice, rats and hamsters).

Work by different authors and personal observations have shown that the hepatic schizogony of these four species can be reproduced *in vitro*, either in cell lines or in primary cultures of rodent hepatocytes.

The first cultivation of hepatic stages of a mammalian *Plasmodium* was reported in 1976 by Doby and Barker who described intra-cytoplasmic bodies in cultures of embryonic human liver cells infected with sporozoites of *Plasmodium vivax*. In 1979, Strome, Santis & Beaudoin described schizonts of *P. berghei* developing in cultures of brain and liver from rats embryos, and of brain from turkey embryos. Hollingdale et al. (1981) succeeded in growing *P. berghei* in embryonic liver cultures from the rat, mouse, hamster and man, as well as in brain from the turkey. In 1983, Hollingdale, Leland & Schwarts, reported the complete development of *P. berghei* in a human hepatoma line and later (Hollingdale, 1986), the results of some chemotherapeutic tests. The first successful cultivation of exo-erythrocytic schizonts in adult hepatocytes was achieved by Lambiotte et al. (1981) who reported the development of *P. yoelii* in primary cultures of rat liver. This work was followed by that of Mazier et al. (1982) with *P. yoelii* in hepatocytes of the tree rat, *Thamnomys gazellae*.

The techniques established with the rodent Plasmodia have led to the cultivation of other species:

a) Two human parasites: *P. vivax* in human hepatocytes (Mazier et al., 1983, 1984a) and in hepatoma cells (Hollingdale, 1986); *P. falciparum* in human hepatocytes (Smith et al., 1984; Mazier et al., 1984b, 1985).

b) a simian *Plasmodium*, *P. cynomolgi bastianellii* in *Macaca rhesus* hepatocytes (Millet et al., in press).

We describe here an experimental model for *in vitro* screening and study of the mode of action of schizontocides potentially active against the tissue stages of *Plasmodium*.

Our system included:

– Parasite: *P. yoelii*; blood stages in white mice.
– Sporogony in *Anopheles stephensi*.
– Pre-erythrocytic schizogony in primary cultures of hepatocytes of the African murid *Thamnomys gazellae*.

P. yoelii was selected, having been used in our laboratory for many years for the *in vivo* study of hepatic schizogony. Its behaviour and ultrastructure are well known in normal conditions

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(Seureau et al., 1980) as well as under a restrictive host diet (Landau et al., 1982) or treatment by drugs (Boulard et al., 1983, 1985; Peters et al., 1984).

Two main reasons led us to select primary cultures of *Thamnomys* hepatocytes for a chemotherapy model: – (i) the adult hepatocyte is the normal host cell of the parasite. – (ii) the tropical rodent *Thamnomys gazellae* is closely related to *P. yoelii*'s natural hosts, *T. rutilans*. It can, moreover, be bred in the laboratory and its hepatocytes are easy to cultivate and quite resistant *in vitro*.

DESCRIPTION OF THE EXPERIMENTAL MODEL

Parasite strain: *P. y. yoelii* 265BY isolated from *Thamnomys rutilans* captured in the Central African Republic in 1969.

Vector: *Anopheles stephensi* from a colony established by Shute & Maryon (1966) in England.

Gametocyte donors: Aliquots ($7x10^{-4}$ ml) of parasitized blood cryopreserved at –195°C are injected intraperitoneally to Swiss white mice. Mosquitoes are usually fed on mice at day 6 after inoculation. The best day for feeding the mosquitoes is checked before using a new stock of frozen blood.

Rate of infection of mosquitoes: The number of oocysts in the midgut is first checked at day 7 after the infective meal. We have learned from previous experience that very large numbers of oocysts produce a low rate of infective sporozoites. The optimum number developing into schizonts is obtained from between 20 and 100 oocysts. Cages of mosquitoes used to infect the cultures are selected accordingly.

Cultivation on hepatocytes: Seglen's method (1973) was used for isolating hepatic cells by enzymatic dissociation.

The techniques for perfusing and seeding rodent liver cells and the supplemented culture medium (MEM) used for growing exoerythrocytic schizonts, have been described by Lambiotte et al. (1981) and Mazier et al. (1982).

In our previous work, schizonts were cultivated in continuous monolayers of hepatocytes spreading over the whole surface of a Petri dish. For the screening of drugs when all schizonts of a culture must be counted, this type of culture has many disadvantages, e.g., schizonts accumulate at the periphery of the cultures where they are difficult to detect; conting of all schizonts disseminated over a large surface is difficult and time consuming.

In order to obtain small surfaces of hepatocytes where schizonts are concentrated, the techniques for seeding cells were modified (Millet et al., 1985a, b); drops of 25μl of the hepatocyte suspension (200 to 350 cells per μl) obtained by enzymatic perfusion are deposited on the bottom of a Petri dish or a Falcon flask. They do not spread on the plastic and, 20 hours later, the cells form a continuous monolayer with approximately 4,000 hepatocytes in the shape of a 5mm diameter disc.

Infection of cultures with sporozoites: Infected mosquitoes are dissected aseptically. A suspension of ground-up salivary glands in culture medium (supplemented M.E.M) is made and an estimate of the number of sporozoites is carried out with a Malassez chamber.

The supernatant on each disc of hepatocytes (25μl) is replaced, using a micropipette, by an equal quantity of a suspension containing 10,000 to 20,000 sporozoites. Two hours later, 1ml of culture medium is added.

Drug tests: Tests are performed in Petri dishes each containing two discs of hepatocytes. The compound, dissolved in 1ml of culture medium, is added to the cultures two hours after the sporozoites; this solution is renewed after 24 hours. Each concentration of drug is tested on three Petri dishes (six discs); three infected Petri dishes are kept as untreated controls and three others are treated with primaquine at a concentration of 1mg/l or 0,1mg/l. Dead cells are counted after vival staining with trypan blue.

Cultures are fixed with methanol of Bouin's fluid. At 48 hours when the size of the schizonts varies between 20 and 40μm, they are counted with the x 20 objective. LD 50 and LD 90 values are calculated by the graphis method of Trevan.

This experimental model presents two main advantages; (i) it allows a considerable saving of biological material and (ii) rapid reading of the results.

RESULTS

The results of *in vitro* activity and cytotoxicity testing of 20 compounds are given in Table I. The zones of activity and citotoxicity are summarized in Fig. 1.

TABLE I

In vitro drug testing of 20 compounds.

Compound	Maximum Concentration Tested mg/1	Schizontocidal Activity mg/1		Cell Toxicity mg/1			
		LD 50	LD 90	1	10	50	100
Primaquine di P(PQ)	30	0,03	0,1	+	+++		
PQ Analogues:							
WR 249420	1	0,02	0,08	++	NT		
WR 242511	1	0,04	0,09	+	NT		
WR 238605	1	0,06	0,4	V	NT		
WR 225448	1	0,06	0,5	O	NT		
PQ Putative metabolites							
5-OH-PQ	50	15	40	O	O	+	
6-OH-PQ	50	6	25	O	O	O	
5,6-OH-PQ	50	6	30	O	O	+	
NH-COOH-PQ	50	25	45	O	O	V	
6-OCH_3-8AQ	50	8	45	O	O	+	
5-OH,6-OCH_3-8AQ	50	NA	NA	O	O	+++	
5,6-OH-8AQ	10	NA	NA	+	+++	NT	
6-OH-8AQ	10	NA	NA	O	++	NT	
Pyrimethamine	60	$1,5x10^{-4}$	$7,5x10^{-4}$	O	O	O	
Colchicine	100	NA	NA	++ (x)	NT	NT	++ (x)
Colchicine derivatives							
Thiocolchicine	100	NA	NA	+	NT	NT	++
3 demethylcolchicine	100	NA	NA	+ (x)	NT	NT	+ (x)
3 demethylcolchicine	100	NA	NA	+ (x)	NT	NT	+ (x)
Chloroquine base	10	NA	NA	V	+++		
Mefloquine	50	NA	NA	O	+++		

NT = not tested; NA = no schizontocidal activity; tested until cells are killed.
O = same number of dead cells as in controls; + = few dead cells at the periphery; ++ = areas of dead cells at the periphery and inside the discs of hepatocytes; +++ = all cells dead; V = no dead cells, but numerous intracytoplasmic vacuoles; (x) = nuclear alterations of cells.

a) Drugs known to be active against the tissue stages *in vivo* :

– Primaquine was active *in vitro* but the cytotoxic concentration approached the active one.

– Pyrimethamine was active and non-toxic at low concentrations.

b) Drugs used in the treatment of blood stages:

– chloroquine and mefloquine were inactive up to the cytotoxic concentration.

c) Primaquine analogues and derivatives:

Primaquine analogues: these were active at concentrations of 10^{-3} to 1mg/l which is comparable to primaquine. WR 225 448 was the least toxic.

Putative primaquine metabolities: hydroxy-PQ, carboxy-PQ and 6-methyl-8-AQ were active between 1 and 50mg/l; other derivatives were non-active up to the toxic concentration.

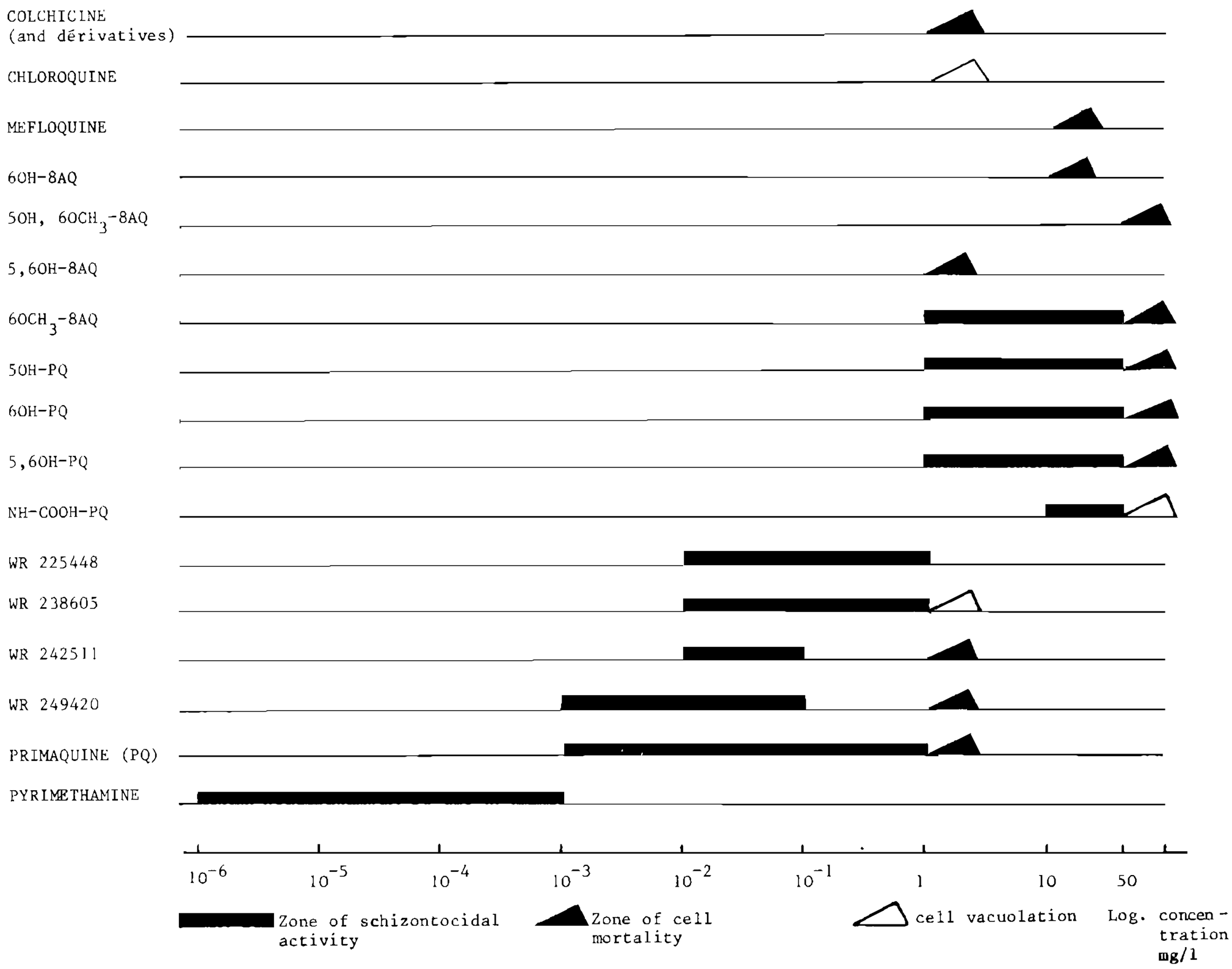
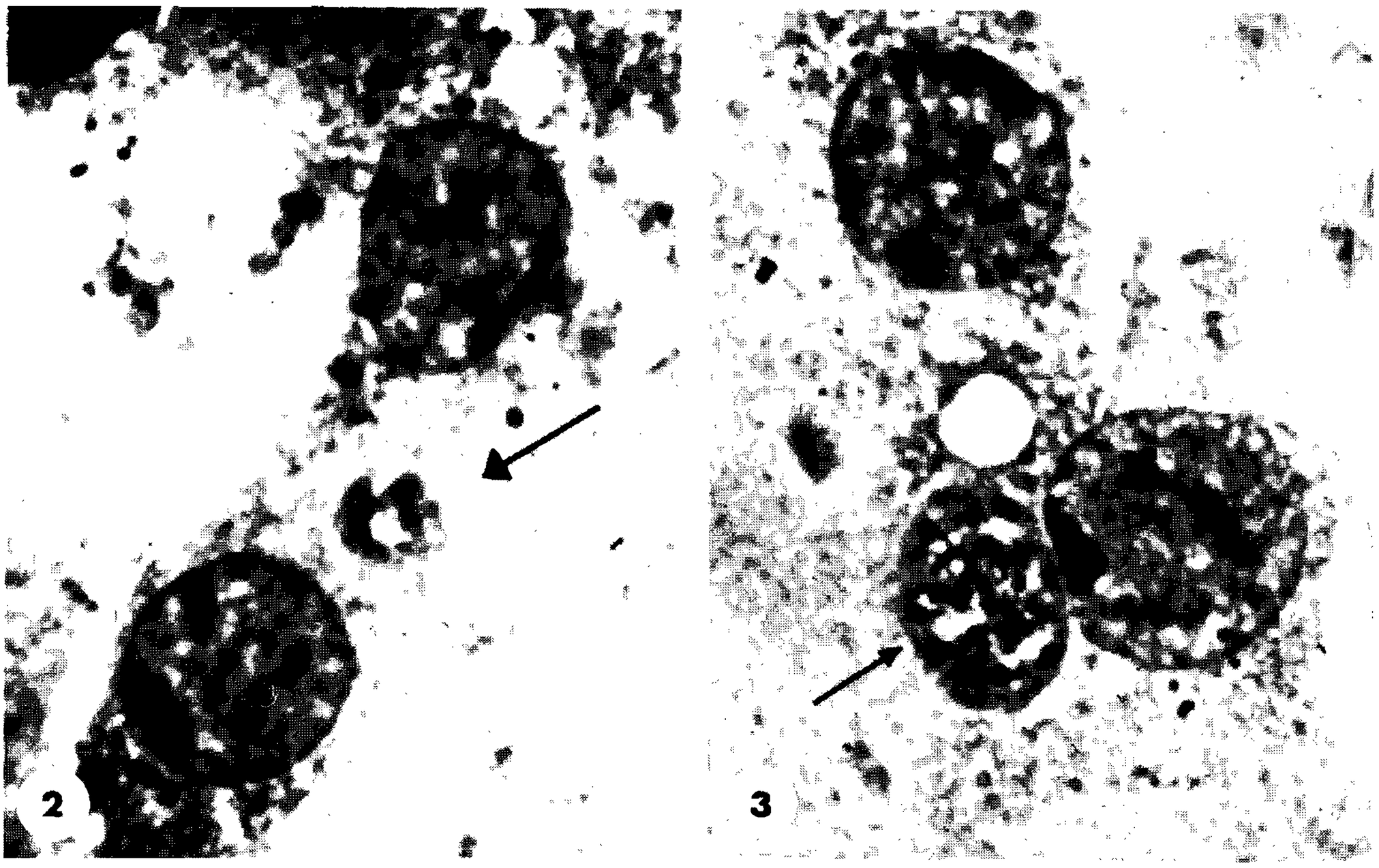


Fig. 1: Zones of schizontocidal activity and citotoxicity of 17 compounds.



Figs. 2-3: Schizonts of *P. y. yoelii* in hepatocytes; at 18 hours (Fig. 2); at 24 hours (Fig. 3).

d) Colchicine and derivatives (tested at two concentrations, 1 and 100mg/l).

Colchicine was non active and very cytotoxic.

Colchicine derivatives were non-active at 1mg/l; at 100mg/l schizonts were normal with 2 and 3-demethylcolchicine and altered with thiocolchicine; the cytotoxicity was similar to that of colchicine.

Fig. 4: Schizonts of *P. y. yoelii* in hepatocytes at 48 hours.

DISCUSSION

For compounds used as therapeutic agents, such as chloroquine, mefloquine, pyrimetamine and primaquine, the results of *in vitro* tests are in agreement with those obtained *in vivo* in animals and man.

The primaquine analogues studied have an activity and a toxicity comparable to those of primaquine, WR 225 448 being the least toxic. The lower toxicity of WR 225 448 was also found in *in vivo* studies (Peters et al., 1984) in the white rat.

The results obtained with primaquine putative metabolites are more difficult to interpret although they have been studied in very different systems. Data on the compounds which were tested in three different models are summarized in Table II:

TABLE II

Results of 8 PQ derivatives testing in 3 different experimental models

Compounds	*in vitro* model hepatocytes - *P. y. yoelii*	*in vivo* model Mouse - *P. y. nigeriensis*	*in vitro* model hepatoma - *P. berghei**
6 OH 8 AQ	inactive	active (10 - 30 mg/kg)	some activity
5 OH 6 OCH_3 8 AQ	inactive	NT	inactive
5,6 OH 8 AQ	inactive	active (10 - 30 mg/kg)	active
6 OCH_3 8 AQ	active (50 mg/1)	active (10 - 30 mg/kg)	some activity
5 OH PQ	active (50 mg/1)	active (30 - 60 mg/kg)	some activity
6 OH PQ	active (50 mg/1)	inactive (30 mg/kg)	NT
5.6 OH PQ	active (50 mg/1)	inactive (30 mg/kg)	NT
N-CAR BOXY PQ	active (50 mg/1)	active (30 mg/kg)	active

(NT = not tested; *one concentration tested: 5 mg/1).

1) *in vivo*: white mouse infected with *Plasmodium yoelii nigeriensis* (Peters & Robinson, 1986 in press).

2) *in vitro*: culture in an hepatoma line of human origin (Hollingdale, 1986 in press) infected with *Plasmodium berghei.*

3) *in vitro*: primary culture of *Thamnomys gazellae* hepatocytes infected with *Plasmodium yoelii yoelii.*

In Hollingdale's work, six drugs were tested: – four were found slightly active or inactive at the single concentration of 5mg/l: as higher concentrations were not tested, we cannot compare these results with those obtained with the other models; – two drugs were found active: N-Carboxy-PQ and 5,6 OH-8AQ. The first one was found active in both *in vitro* systems while the second one was found inactive in our hepatocytes model.

These discrepancies are likely to be explained by differences between the two models and particularly between hepatoma and hepatocytes metabolisms.

Our results are in agreement with those of Peters & Robinson *in vivo* for three compounds: 6 OCH^3 -8 AQ, 5 OH-PQ and N-Carboxy-PQ. They differ for two compounds: 6 OH-8 AQ and 5,6 OH-8 AQ which were found active *in vivo* and inactive *in vitro.* Finally, 6 OH-PQ and 5,6 OH-PQ were found inactive *in vivo* and 30mg/kg but were not tested with higher dosages.

Since primaquine was evidenced to be rapidly metabolized in different organs of the host in man and animal, its antimalarial activity could be due to still non identified metabolite(s) (WHO, 1984).

Divergent results obtained in these three models are likely to help understanding the metabolism of primaquine. According to these, one can assume that a compound can be active by itself, or through a metabolite issued either from the hepatocyte, or from another organ.

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