In the Name of God

the Merciful, the Compassionate

CHEMOTHERAPY OF MALARIA:

HALOFANTRINE RESISTANCE IN LABORATORY STRAINS OF *PLASMODIUM FALCIPARUM*

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CHEMOTHERAPY OF MALARIA : HALOFANTRINE RESISTANCE IN LABORATORY STRAINS OF *PLASMODIUM FALCIPARUM*

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The main aims of this study were focussed on the development of halofantrine resistance in *Plasmodium falciparum in vitro*, determination of the pattern of cross-resistance in halofantrine-resistant strains, and evaluating the ability of non-antimalarial drugs to reverse this resistance.

The asexual stages of eight strains of *P. falciparum* (K_1, FCR_3, $T_{9.96}$, CH150-R4, W2-mef, K_1HF, $T_{9.96}HF$ and $T_{9.96}HF4$) were used for cultivation and drug sensitivity testing.

[3H] hypoxanthine incorporation by chloroquine-resistant K_1 and sensitive $T_{9.96}$ parasites were compared. The results indicated that uptake of radiolabelled hypoxanthine by the K_1 parasites is significantly greater than in $T_{9.96}$ parasites.

The sensitivity of several strains of *P. falciparum* to both antimalarial and non-antimalarial agents was evaluated. The results demonstrated that chloroquine-resistant K_1 strain was sensitive to mefloquine and halofantrine, but not to quinine compared to chloroquine-sensitive $T_{9.96}$ parasites. Additionally, halofantrine was the most active antimalarial tested against the K_1 and $T_{9.96}$ parasites with IC_{50}s 2.2 and 6.6nmol/l respectively. Among the non-antimalarial drugs tested only penfluridol had a slight antimalarial activity against the K_1 and $T_{9.96}$ strains.

Antimalarial drugs in combination with the calcium channel blocker verapamil, calmodulin antagonists chlorpromazine and penfluridol or serotonin

inhibitors fluoxetine and praziquantel were performed. The IC_{50} values for chloroquine considerably decreased when it was employed in combination with verapamil, chlorpromazine and fluoxetine against the chloroquine resistant K_1 strain. In contrast, a relatively small increase in mefloquine sensitivity was seen in combination with verapamil or fluoxetine. There was no synergy between chloroquine and the three non-antimalarial drugs against the $T_{9.96}$ parasites. Additionally, verapamil potentiated the activity of chloroquine against chloroquine-resistant FCR_3 parasites. The effect of mefloquine in combination with verapamil was additive when tested against K_1 parasites but antagonistic against mefloquine-resistant W2-mef parasites. All three non-antimalarial agents antagonised the effect of halofantrine against the K_1 and $T_{9.96}$ parasites.

Intermittent exposure of both chloroquine-sensitive ($T_{9.96}$) and chloroquine-resistant (K_1) strains of *P. falciparum* resulted in a relatively rapid reduction in sensitivity to halofantrine. After six months the IC_{50} value for halofantrine increased nine-fold for chloroquine-resistant (K_1) parasites and three-fold for cloned chloroquine-sensitive ($T_{9.96}$) parasites. The halofantrine-resistant K_1HF became more sensitive to the actions of chloroquine and exhibited a reduced sensitivity to quinine, mefloquine and qinghaosu. The other halofantrine-resistant strain $T_{9.96}$HF remained sensitive to chloroquine and amodiaquine but exhibited a decreased sensitivity to mefloquine, quinine , qinghaosu and pyrimethamine. Resistance was stable in both strains either in the absence of drug pressure or when kept frozen in liquid nitrogen.

Combinations of penfluridol with halofantrine, mefloquine, quinine and chloroquine were assayed for synergy against halofantrine, mefloquine or

chloroquine resistant parasites. Synergy was observed between penfluridol and either halofantrine or mefloquine against $T_{9.96}$HF parasites, but not against K_1HF parasites. In contrast penfluridol antagonised the activity of quinine against both strains. An additive effect was observed with the combination penfluridol and mefloquine against mefloquine-resistant W2-mef parasites. The combinations chloroquine + verapamil and chloroquine + fluoxetine resulted in reduced potentiation against the K_1HF parasites.

[14C] halofantrine uptake by K_1HF, $T_{9.96}$HF, K_1 and $T_{9.96}$-infected erythrocytes was investigated. Halofantrine accumulation was greater in the halofantrine-sensitive parasites compared to resistant parasites. Penfluridol which is capable of reversing halofantrine resistance *in vitro* also enhanced halofantrine steady state concentration in halofantrine-resistant parasites. The mechanism of halofantrine resistance in these parasites appears to be related in part to drug transport either into or out of the parasites.

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CHAPTER 1 : INTRODUCTION AND REVIEW

1.1 Historical aspect

The chronicle of human malaria starts from time immemorial and continues for thousands of years through a path of sickness and death. The full antiquity of the malaria parasite's association with man remains unclear, but its clinical symptoms were fully described by Hippocrates 400 years before the birth of Christ (Bruce-Chwatt *et al*, 1981). The ancient Greeks had found some connection between swamps and malaria (Ackerknecht, 1965). Malaria was known in China long before the beginning of the Christian epoch. Emperor Huang Ti, in the Chinese medical classic Nei Ching, which has been edited by him, mentions the enlargement of the spleen connected with different types of fevers (Wernsdorfer & McGregor, 1988). Additionally, qinghaosu (a traditional Chinese herb) has been used for malaria therapy in China for over 1000 years (WHO, 1984). However, the aetiology of malaria remained a mystery until the end of the 19th Century, and no major progress in control and treatment was made for a long time except for the discovery at an unknown period of the curative virtue of cinchona bark by American-Indians (Garnham, 1966). According to Bruce-Chwatt *et al* (1981) although the precise date of the introduction of the new remedy into Europe is obscure, it was probably brought to Rome in 1632 by Spanish priests.

In 1716, Lancisi noted the presence of some black pigment in human spleens and brains, but did not associate these findings with malaria. Also, in 1847, Meckel described the black pigment in the blood and spleen of a patient who was suffering from acute disease. Although Meckel saw the parasites, he did not recognise them as such. The situation remained unchanged until 1880

when the organisms were recognised by Laveran, who named them *Oscillaria malariae*. Later on, these organisms became more famous as *Plasmodium falciparum* (Garnham, 1966). Laveran also demonstrated the phenomenon of exflagellation but had some problem in persuading other scientists of his discoveries owing to recent acceptance of the Klebs Tomassi-Crudeli bacillus as the etiological agent of malaria. In 1884, Danilewsky, a Russian researcher, started to investigate malaria in wild birds. His studies included the description of the morphology of parasites and the process of exflagellation in fresh blood (Garnham, 1966). The discovery of the differential stain, polychrome methylene blue, which stained the nucleus of the parasite a deep red in contrast to the cytoplasm which stained blue, by Romanowsky in 1891 made the study of malaria parasites much easier. The role of the exflagellating parasite was made clear by MacCallum in 1897 in avian malaria and later in *P. falciparum* in man.

Although in 1897 Ross made his famous discovery of the transmission of malaria by mosquitoes, Lancisi in 1717 had suggested a relationship between marsh insects and the occurrence of malaria. Also, in 1848 Nott proposed that mosquito involvement was the best explanation for the occurrence and distribution of both yellow and remittent fevers. Manson in 1896 reported the observation of exflagellation by *P. falciparum* gametocytes in the stomach of a mosquito (Coatney *et al*, 1971), and according to Russell (1955), Theobald in 1896 concluded that mosquitoes are the vectors of malaria. Grassi in 1898 identified the whole cycle of development of *P. falciparum* and *P. vivax* in the anopheline mosquitoes and, in 1899, sporogony of *P. malariae* was observed. At last, in 1900, Grassi's classic monograph was presented on the

subject (Garnham, 1966).

The tissue stages of primate malaria parasites remained obscure until Garnham (1947) discovered the exoerythrocytic stages of *Hepatocystis kochi* developing in the liver parenchyma cells of infected monkeys. The investigations were quickly followed by the identification of the full life cycle of *P. cynomolgi*, *P. vivax* and *P. falciparum* in monkey and man (Shortt & Garnham, 1948; Shortt *et al*, 1951).

1.2 The life cycle of malaria parasites

Malaria parasites are the organisms responsible for the disease of malaria in man. Although the disease usually is caused by one of four species of *Plasmodium*, namely *P. falciparum, P. vivax, P. malariae* and *P. ovale*, documented experiments demonstrated that man can easily be infected with certain species of simian malaria parasites such as *P. cynomolgi, P. knowlesi* and *P. simium* (Wernsdorfer & McGregor, 1988). Malaria parasites are intracellular protozoans that have adapted to a life cycle, which includes schizogony, involving a vertebrate host, and sporogony, occurring in the invertebrate host. Although there are variations in the individual life cycles of different malaria species, all of them follow a basic course of development through the vertebrate and invertebrate hosts. The life cycle of malaria parasites commences with the inoculation of sporozoites by an infected female mosquito into the blood of the vertebrate host. Development begins within less than an hour after the injection of the sporozoites by the vector. According to Fairley (1947), the sporozoites of *Plasmodium vivax* remain in the circulating blood up to one hour. The sporozoites then invade the

parenchymal cells of liver (or the reticuloendothelial system), where they undergo exoerythrocytic schizogony. The exoerythrocytic schizogony is completed towards the end of the incubation period of the infection, when large numbers of tissue merozoites from ruptured tissue schizonts are released into the blood circulation. In *P. falciparum*, there is a massive single generation of merozoites, about 40,000, in contrast to the liver schizonts of the other human malaria parasites which produce about 2,000-15,000 merozoites (Bruce-Chwatt *et al*, 1981). The incubation period which covers the time between the day of infection and the appearance of clinical symptoms, is usually short in *P. falciparum* (5.5-7 days), somewhat longer in *P. vivax* (6-8 days), and *P. ovale* (9 days), and longest (13-16 days) in *P. malariae* (Bruce-Chwatt *et al*, 1981). The phase of erythrocytic schizogony follows the exoerythrocytic schizogony. Liberated merozoites invade erythrocytes or reticulocytes and undergo a phase of growth and multiplication. The minimum time from inoculation of sporozoites by the mosquito until the first appearance of the merozoites in the peripheral blood is termed the 'prepatent period'. The period is constant and characteristic for each species of parasite. After erythrocytic invasion a vacuole is formed and the parasites assume the ring form, the cytoplasm becomes amoeboid and the uninucleate body is termed a trophozoite, feeding on the haemoglobin of the red cell by phagotrophy. The trophozoite continues to grow and assumes the schizont form, a body with a number of nuclei, varying from four to seventy-two depending on the species. The fully developed schizont ruptures releasing merozoites which invade fresh erythrocytes, initiating a new cycle of schizogony. These processes are followed by clinical symptoms and usually associated with patent parasitaemia. The

malaria paroxysm usually comprises three successive phases. The cold phase commences with shivering (rigor) and a feeling of intense cold. The pulse is rapid but weak and the skin is dry but pale. This phase sometimes is accompanied by vomiting. The cold stage lasts between fifteen minutes and one hour. The hot phase (second stage) starts with intense fever, the face is flushed, the skin dry and burning. Intense headache, nausea and vomiting are common. The temperature may rise to 41°C or more. The second stage lasts about two to six hours. In the last stage (the sweating phase) the patient sweats profusely and the temperature falls rapidly. The patient usually gets a deep sleep and on waking, feels weak but otherwise normal. The sweating phase lasts between two and four hours (Bruce-Chwatt, 1985).

Following erythrocyte penetration some merozoites develop into male and female gametocytes. The gametocytes give rise to the male and female gametes, and to sporogonic development after infected blood is taken up by a susceptible female anopheline mosquito. The fertilisation of microgamete and macrogamete occurs in the stomach of the mosquito and an ookinete develops which penetrates the epithelial lining of the stomach and develops as an oocyst. Sporozoites are formed in the mature oocyst, and are released when this ruptures. The sporozoites travel throughout the haemocoel of the insect, subsequently invading the mosquito salivary glands from where they can be introduced into a new vertebrate host when the mosquito takes a blood meal, completing the life cycle (Fig. 1.2.1). In *P. vivax* and *P. ovale* infections, a latent tissue stage (hypnozoites) has also been identified. It has long been thought that the relapses of *P. vivax* and *P. ovale* are due to the insistence of latent forms of the parasite

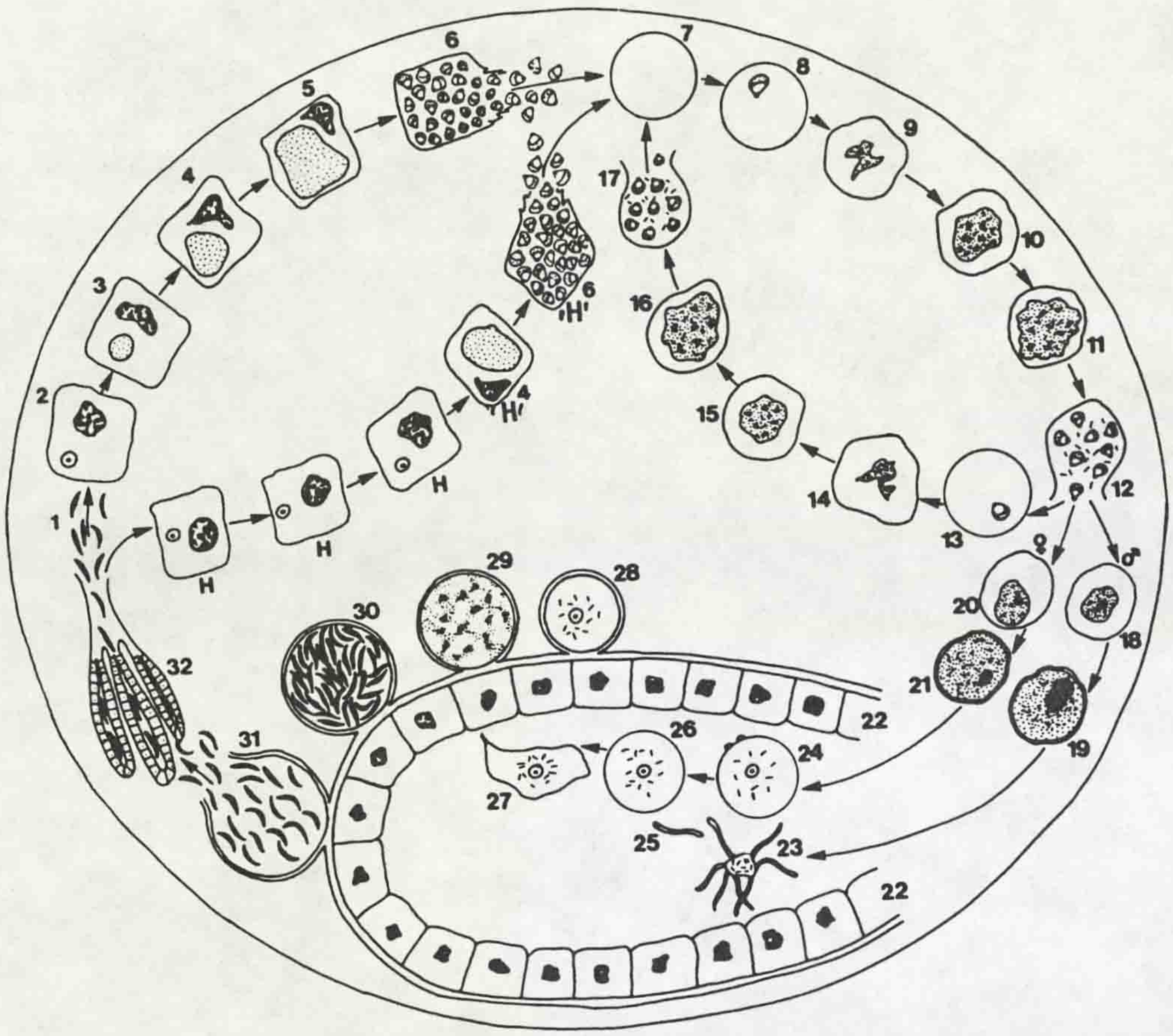


Fig. 1.2.1 **The life-cycle of a primate malaria parasite - 'vivax type' with dormant EE forms (modified from Bray & Garnham, 1982).**

1: sporozoites injected into skin by the mosquito; 2: 2-day-old exoerythrocytic form in a hepatocyte; 3, 4, 5: growing exoerythrocytic schizonts; H: hypnozoites in hepatocytes; 6: mature exoerythrocytic schizonts bursting, releasing merozoites into the blood; 7: erythrocyte; 8, 9: growing tropozoites; 10, 11: growing schizonts; 12: mature schizont releasing merozites; 13, 14, 15, 16, 17: erythrocytic cycle repeated; 18, 19: growth of the microgametocye; 20, 21: growth of the macrogametocyte; 22: mosquito has takne gametocytes up into its mid-gut; 23: exflagellation of the microgametocyte; 24: macrogametocyte escapes from erythrocyte to become a macrogemete; 25: microgamete; 26: macrogamete about to be fertilised; 27: zygote or ookinete; 28, 29, 30: oocyst growth on the mid-gut surface; 31: oocyst bursting, releasing sporozoites; 32: sporozoites in the mosquito salivery glands (two types: immediate growth 2-6; H, hypnozoites, eventually becoming activated 'H' 4, 'H' 6). (Wernsdorfer & McGregor, 1988)

in the liver. Attempts were made by several investigators to make clear the reason for relapses in *P. vivax* and *P. ovale* infections. Shortt & Garnham (1948) observed the presence of large multinucleated bodies in the liver of a monkey 102 days after it had received sporozoites. These organisms were later named the 'hypnozoites' on the suggestion of Dr C A Hoare FRS (Wernsdorfer and McGregor, 1988). At last, Krotoski and colleagues (1980), using immunofluorescent techniques, demonstrated the presence of hypnozoites, in the liver of monkeys which had received sporozoites of *P. cynomolgi bastianellii* seven days previously. These findings were latterly corroborated in *P. vivax* by a team of American-British investigators (Wernsdorfer and McGregor, 1988). Although the trigger which stimulates the hypnozoites for growth and multiplication is still unclear, some young activated parasites and also a number of larger schizonts have been found in late biopsies (Wernsdorfer McGregor, 1988).

1.3 Drugs used in the treatment and control of malaria

1.3.1 The development of malaria chemotherapy

Although attempts at treatment of malaria using the flowers, leaves and roots of many plants proved relatively unsuccessful, the powdered roots of ch'ang shan (*Dichroa febrifuga*) was used in China for at least two thousand years (Bruce-Chwatt *et al*, 1981). The bark of a South American tree having potent activity against fevers was used by Amerindians of Peru and Spanish conquistadors as a remedy for malaria infection. After the treatment of the Countess of Chinchon in 1638 the 'fever-tree' was named the cinchona tree (Russell, 1955; Guerra, 1977). Attempts to cultivate cinchona in other parts of

the world were initiated as early as 1743 by the French. In 1854, the Dutch started the first plantation of cinchona in Java. Clements Markham, a British geographer, also established the successful cultivation of cinchona in Ceylon and the Nilgiri Hills in India in 1872 (Bruce-Chwatt *et al*, 1986).

In 1820 quinine and cinchonine, the basic alkaloids of cinchona, were isolated by the French chemists Pelletier and Caventou. The first successful synthesis of quinine was achieved in 1944 (Woodward & Doering, 1944; see Albert, 1979; Peters, 1980). Quinine is highly active as a blood schizontocide against all forms of human malaria (Desjardins *et al*, 1988).

Mepacrine was introduced in 1932, and primary reports showed good suppressive activity against *P. relictum* in canaries (Kikuth, 1932). Also, mepacrine was tested by Fairley (1946) in human volunteers. Early investigations by Green (1932) and Hoops (1932) with mepacrine in human malaria showed it to be better than quinine in suppressive therapy. Although James (1932) and Soesilo (1934) had suggested that large doses of mepacrine may be active against sporozoite-induced infections, Fairley's (1945) findings showed that this antimalarial compound cannot eradicate an infection of *P. vivax*, nor could it be used as causal prophylactic. Mepacrine was subsequently replaced by 4-aminoquinolines chloroquine and amodiaquine. In 1944 chloroquine and amodiaquine underwent extensive clinical studies. Both were highly effective and they remained the best therapeutic and suppressive antimalarials for over 25 years.

The antifolates proguanil and pyrimethamine were also developed in the 1940's. Both are inhibitors of parasite dihydrofolate reductase (Peters,

1982), and were used for clinical cure in all forms of malaria and radical cure in most cases of falciparum malaria (Bruce-Chwatt *et al*, 1981). The advent of resistance to pyrimethamine (McGregor & Smith, 1952; Chakravarty & Chaudhuri, 1953; Avery, 1954; Maberti, 1960), prompted the use of the combination of pyrimethamine with the sulphonamide sulphadoxine (Fansidar). Sulphonamides and sulphones, first were described as antibacterial agents (Trefouel *et al*, 1935), but following discovery of their high efficacy against the asexual blood forms of *P. falciparum* they were employed as antimalarial drugs. Many authorities considered that Fansidar could be employed fairly extensively and successfully, but such optimism did not live long, and resistance to Fansidar became widespread (Peters, 1987).

The 8-aminoquinoline pamaquine was synthesised in 1926 (Roehl, 1926; Horlein, 1926). This compound was identified from screening studies against *P. praecox* in canaries. The action of pamaquine against established human malaria infections was first investigated by Soili (1926) and Muhlens (1926). They found that pamaquine was effective on the asexual erythrocytic forms of *P. vivax* and *P. malariae*, but that against *P. falciparum* the drug action was limited mainly to the gametocytes. Other investigators suggested that although pamaquine was highly active against avian plasmodia, it suffered disadvantages of toxicity and relatively poor schizonticidal activity in human malaria (Roehl, 1926; Albert, 1979; Peters, 1980). Pamaquine was later replaced by primaquine, a compound with similar action but much less toxic effect. Primaquine was introduced in 1950 as the drug of choice for radical cure of *P. vivax* (Edgecomb *et al*, 1950). At present, it is employed as a gametocytocidal

compound in falciparum malaria. It is also the most effective available antimalarial drug for the elimination of the hypnozoites of relapsing malaria.

Following the development of resistance to existing synthetic antimalarial drugs, particularly chloroquine, mefloquine was tested and released by the United States Army in the early 1970's; since 1976 it has been developed jointly by Walter Reed Army Institute of Research, World Health Organisation and Hoffman-La Roche (WHO, 1984). Mefloquine is highly blood schizontocide and its clinical trials started in 1972 mostly against multidrug-resistant strains of *P. falciparum*.

Halofantrine was also developed in the Walter Reed Army Institute of Research programme. It has potent blood schizontocidal action and is active against both chloroquine-resistant and chloroquine-sensitive stains of *P. falciparum* (Cosgriff *et al*, 1982). Halofantrine appears to be a useful alternative to mefloquine.

Qinghaosu, a novel antimalarial drug, was isolated from *Artemisia annua*, a plant used in Chinese traditional systems of medicine for centuries (Qinghaosu Antimalaria Co-ordinating Research Group, 1979; Anand, 1984). Qinghaosu has most recently been employed as a blood schizontocide. It is now in the clinical development phase.

1.3.2 The control of malaria

1.3.2.1 Vector control

Malaria was originally carried from country to country by slaves and trading (Bruce-Chwatt, 1970). The spread of malaria has continued up to the present day with widespread airline traffic and improved communication systems.

As the life cycle of malaria shows (Fig. 1.2.1) control of malaria can be exerted by attacking the mosquito or by curing the disease in man. Between 1955 and 1970 considerable efforts were made towards eradication of malaria based on killing of vector anophelines with DDT (dichloro-diphenyl-trichloroethane). DDT was first synthesised by Zeidler in 1874 as a chemical substance. Sixty-five years later, Muller discovered the insecticidal efficacy of DDT. Subsequently, in 1942 the insecticide was produced industrially as a 5% powder by J.R. Geigy and S.A. Basel. Initially, DDT was employed against louse infestations, typhus and relapsing fever. In 1944 this insecticide was used in the field against *Anopheles labranchiae* in Italy, then the application of DDT was followed by US and British Armies (Gamiccia & Beales, 1988) and spread very soon in malarious areas.

In 1955 the worldwide programme of malaria eradication was declared by the Eighth World Health Assembly (Bruce-Chwatt, 1985). Just before the eradication of malaria programme started, it was estimated that 1070 million out of 2650 million world population in that time were living in malarious areas (Russell, 1956). Also at that time about 250 million cases with 2.5 million deaths were estimated for the global annual incidence of malaria per year (Pampana & Russell, 1955). By 1970, as a result of intensive efforts 727 million people had been freed from the risk of malaria. Also, malaria disappeared from the whole of Europe and USA, most of the Asian parts of Russia, Brunei, Hong Kong, Japan, Singapore, Macao, Taiwan, some parts of North and South America, most of the Caribbean and some areas of western Asia (Bruce-Chwatt, 1978). Optimism as to the worldwide eradication, however, was short lived and the resurgence of malaria was reported from several countries in Latin America, Asia

and the Asian part of Turkey. The number of reported cases of malaria throughout the world had risen to 10,742,000 in 1977, in comparison with 3,315,000 cases in 1972. This resurgence was the result of several factors including the development of resistance to organochlorines insecticides, particularly DDT, which most recently has extended to organophosphate compounds and carbamates in many species of anophelines (Bruce-Chwatt, 1985). At present comprehensive vector control methods such as environmental modification and manipulation, chemical and biological larvicides, insecticide space spraying and residual insecticide spraying are employed in the most of malarious areas.

1.3.2.2 The use of drugs for malaria control

Although the malaria eradication programmes were initiated by spraying residual insecticides in the malarious areas, mass chemotherapy or mass chemoprophylaxis was also proposed by some authorities (WHO, 1957). Therefore, the use of antimalarial drugs was considered as a substitute for, or a complement to, residual insecticides in the malaria eradication programmes (Beales, 1988). Thus, many of antimalarial compounds were being prescribed for chemoprophylaxis. Proguanil and pyrimethamine, which are classified as antifol compounds, were essentially used for prophylaxis, acting against the pre-erythrocytic forms, in particular of *P. falciparum*. Primaquine, a compound of the 8-aminoquinoline group, was also effective against the pre-erythrocytic schizonts and employed as a chemoprophylactic.

Some authors postulated that the antimalarial drugs were employed to achieve two aims: the interruption of transmission and the complete

elimination of the parasite reservoir (Beales, 1988), but the emergence of drug-resistance in the strains of *P. falciparum* ended such optimism. Although many fixed drug combinations including Fansidar (pyrimethamine and sulphadoxine) were administered instead of single drug in areas where chloroquine-resistant *P. falciparum* was endemic (Howells, 1982, 1986; Win *et al*, 1985; Peters, 1987), resistance to Fansidar was reported soon after administration of the drug (Peters, 1987). At present the chemoprophylaxis and chemotherapy of malaria at *P. vivax, P. ovale* and *P. malariae* is achievable, but for *P. falciparum* in the most of malarious areas still remains unclear.

WHO finally recognised that the worldwide eradication of malaria was not an attainable goal and the Thirty-first World Health Assembly declared the new control of malaria programme based on adjusting antimalarial action to local conditions and available resources (WHO, 1978). The prevention of mortality and reduction of the human suffering associated with the malaria disease in all areas, is the first aim of the new programme. Moreover, investigation to discover new antimalarial drugs with novel mechanism of action is encouraged by the new programme (WHO, 1984).

1.4 Currently employed antimalarials

In recent years, the chemotherapy of malaria has assumed a great role in the prevention of mortality and suffering of malaria in malarious areas. A few doses of an effective antimalarial drug can produce a rapid improvement in the patient's clinical situation. Since a number of terms have been used over the past decades in connection with usage of antimalarial drugs, we need a clear

understanding of their meaning. The main use of antimalarial drugs are (Bruce-Chwatt *et al*, 1981, Bruce-Chwatt, 1985; Wernsdorfer and McGregor, 1988):

1. Prophylactic use (protective). This implies that the drug is used before tissue or blood infection occurs, with the aim of preventing either the occurrence of the infection or its clinical manifestation. Absolute prevention is the term used for destruction of the inoculated sporozoites. There are no known drugs effective for this purpose. In causal prophylaxis the early stages of the parasite, while it is still confined to the liver, are destroyed by antimalarial drug. Clinical prophylaxis (or suppressive treatment) implies the prevention or elimination of clinical symptoms by the early destruction of erythrocytic parasites.

2. Therapeutic (curative) use. Therapeutic use implies that the antimalarials are used as curative drugs after infection is established either in terms of clinical symptoms, parasitaemia or both. Therefore, therapeutic use comprises the treatment of the acute attack, radical treatment and antirelapse treatment.

3. Prevention of transmission. This refers to the use of antimalarial drugs to prevent infection of mosquitoes and implies either action on gametocytes in the peripheral blood of the human host or interruption of sporogony in the mosquito. Finally, when antimalarial drugs are administered to individuals for prevention of the infection, this refers to individual protection or individual prophylaxis. When such drugs are given to a whole community or

a well-defined proportion of a population it is termed collective drug protection.

Although all schizonticides can be used for treatment of malaria in sensitive plasmodia, drug treatment of malaria is being undermined by the spread of drug resistant parasites in malarious areas.

1.4.1 Quinine

Quinine [6-methoxy-α-(5-vinyl-2-quinuclidinyl)-4-quinoline methanol] is the most important antimalarial alkaloid found in cinchona bark. The three other alkaloids, cinchonidine, quinidine and cinchonine, all possess strong antimalarial efficacy similar to that of quinine (MacGilchrist, 1915). Although quinine is the oldest known antimalarial in Europe and the Americas, its use did not become widespread until cultivation of cinchona was established in Java and India. In 1923 Stephens concluded that regardless of the type of treatment, quinine could not prevent the occurrence of relapses in *Plasmodium vivax* (Stephens, 1923). Moreover, some investigators found that quinine was unable to prevent sporozoite-induced infection (Yorke & Macfie, 1924; Hanschell, 1924). Subsequent experiments by Berliner *et al* (1946) and Findlay (1951), showed that quinine has no action on either the sporozoites or tissue phase of malaria, its major action being on the asexual erythrocytic forms. Although quinine is an effective gametocytocide in *P. vivax, P. ovale* and quartan malaria, it has little activity against gametocytes of *P. falciparum* (Bruce-Chwatt *et al*, 1981). Therefore quinine cannot be used in causal prophylaxis, suppressive cure, as a gametocytocidal agent in *P. falciparum* and cannot effect a radical cure in *P. vivax* when used alone. Although quinine, owing to its relative toxicity, has been

replaced by chloroquine and other 4-aminoquinolines for a long time, however, as a result of the widespread occurrence of both chloroquine and antifolate drug resistance in *P. falciparum*, quinine is now the last line of defence in the treatment of cerebral and complicated malaria (Anand, 1984; Bruce-Chwatt *et al*, 1986). Side effects such as giddiness, headache, impaired hearing, ringing in the ears (tinnitus) and nausea may be seen following quinine treatment using the daily dosage of 600-1500mg. Moreover, other transient adverse effects such as tremors, depression and blurred vision may occur during the first days of treatment (Bruce-Chwatt *et al*, 1981).

1.4.2 Chloroquine

Chloroquine [7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline] is amongst the most important antimalarials in use today. This antimalarial was used in therapy for the first time in 1945 (Peters, 1970) and in 1963 was fully described by Coatney. Chloroquine is inactive against the sporozoites and the primary and secondary exoerythrocytic stages, and is highly effective against the asexual blood stages of all four human malaria parasites except in areas where chloroquine-resistant strains occur. Although chloroquine acts as gametocytocidal drug against *P. vivax, P. ovale* and *P. malariae*, it is only effective against the immature gametocytes of *P. falciparum* (Anand, 1984; Bruce-Chwatt *et al*, 1981). The action of chloroquine is rapid and usually reduces the febrile illness after 24 hours. Therefore, chloroquine produces clinical cure of all types of human malaria and radical cure of falciparum malaria. Moreover, chloroquine can be used as a good suppressive drug against all species. If the response in falciparum is slow or incomplete, the presence of chloroquine-

resistant parasites should be considered.

The toxicity of chloroquine is minimal in the usual antimalarial doses. Nausea and vomiting may occur if the drug is taken on an empty stomach. Long term administration of the drug especially in large doses may show other side effects such as headache, bleaching of hair, electrocardiographic changes, weight loss, skin eruptions and visual disturbances (Canfield, 1980; Bruce-Chwatt *et al*, 1986). However, the symptoms are reversible on withdrawal of the drug. At present, with the widespread occurrence of chloroquine-resistance in *P. falciparum*, the usefulness of this drug has been seriously limited.

1.4.3 Amodiaquine

Amodiaquine [7-chloro-4-(3-diethylaminomethyl-4-hydroxyanilino) quinoline] is another 4-aminoquinoline similar to chloroquine. Recently, amodiaquine has been put forward as an alternative to chloroquine for the prevention and treatment of chloroquine-resistant infections (Deloron *et al*, 1988; Childs *et al*, 1989). Studies on the pharmacokinetics of amodiaquine in humans showed that, following oral administration, amodiaquine was rapidly metabolised to desethylamodiaquine. These investigations also demonstrated that the metabolite had between 33 and 100% of the antimalarial potency of the parent compound, depending on the strain of parasite tested (Churchill *et al*, 1985; Pussard *et al*, 1985). Amodiaquine is somewhat more active than chloroquine (Peters, 1987), but it is also more expensive. Although amodiaquine has (similar to chloroquine) no activity against the sporozoites and the primary and latent exoerythrocytic stages, it is highly effective against the asexual blood stages of all the four human malaria parasites.

Overall, amodiaquine has a similar spectrum of activity to chloroquine. In addition, the toxicities associated with the therapeutic administration of amodiaquine are similar to those of chloroquine.

1.4.4 Proguanil and proguanil analogues

Proguanil [1-(P-chlorophenyl)-5-isopropylbiguanide] and its analogues all exhibit considerable antimalarial activity in human malaria. The first studies with proguanil were accomplished during the second world war. A few years later chlorproguanil, which is thought to be a longer-acting proguanil analogue, came into use (Peters, 1987). Investigations with proguanil in man showed good activity against *P. vivax* infections and falciparum malaria (Adams *et al*, 1945; Maegraith *et al*, 1945). The work of Fairley *et al* (1946) demonstrated that proguanil can be used as a true causal prophylactic against *P. falciparum*. Moreover, a number of investigations in chicks showed both a suppressive and causal prophylactic activity against *P. gallinaceum* (Curd *et al*, 1945). Proguanil and chlorproguanil [1-(3, 4-dichlorophenyl)-5-isopropylbiguanide] are inactive against sporozoites but highly active against the primary exoerythrocytic forms of *P. falciparum*. The inhibitory action of proguanil on the primary exoerythrocytic stages of *P. vivax* is transient and on *P. malariae* is unknown. Proguanil and chlorproguanil are inactive against the hypnozoite stages and hence are not recommended for the radical cure of vivax malaria (Bruce-Chwatt *et al*, 1986). Proguanil and its analogues show considerable activity against asexual blood forms of all species of human malaria parasites, but they are not recommended for the treatment of acute attacks of malaria (Peters & Richards, 1984; Bruce-Chwatt *et al*, 1986).

Soon after proguanil was discovered, it was found that the drug was inactive against *P. gallinaceum* and *P. cynomolgi in vitro* (Tonkin, 1946; Hawking, 1947), but when the dosed monkeys' sera were employed against *P. cynomolgi in vitro* the parasites were affected by the sera (Hawking, 1947). These findings suggested that proguanil was activated *in vivo* (Ferone, 1984). Later on it was accepted by most investigators that proguanil and probably chlorproguanil act after conversion to a triazine metabolite (cycloguanil) (Ferone, 1984).

Toxicity of proguanil at the usual prophylactic dose is very low. Gastrointestinal symptoms may be caused by single doses of the order of 0.8-1.0g daily, but were completely reversible when the drug was discontinued (Bruce-Chwatt *et al*, 1986; Desjardins *et al*, 1988).

1.4.5 Pyrimethamine

In 1948 Hitchings and co-workers reported a possible structural analogy between proguanil and a number of pyrimidines which were considered folic acid antagonists on the basis of their inhibition of the growth of *Lactobacillus casei*. According to this finding, Falco *et al* (1949) suggested that compounds of this type might possess antimalarial activity. Later on, the above suggestion was confirmed by Goodwin (1949) and Falco *et al* (1951), when they employed the compounds experimentally against *P. berghei, P. gallinaceum* and *P. cynomolgi*.

Pyrimethamine [2, 4-diamino-5-p-chlorophenyl-6-ethylpyrimidine] was first administered by Archibald (1951) in clinical trials in Nigeria. The results of these trials demonstrated the effect of pyrimethamine against the erythrocytic forms of human malaria. Subsequently a number of investigations were accomplished by Goodwin (1952) and Robertson *et al* (1952). They found that

pyrimethamine could completely prevent the sporozoite-induced infection with *P. falciparum*. Although Ferone (1984) and Anand (1984) have suggested that pyrimethamine can be used as sporontocidal agent, Bruce-Chwatt *et al* (1986) doubt this. The effectiveness of pyrimethamine on the primary exoerythrocytic forms has not yet been determined, although Peters *et al* (1975) demonstrated such an action of the drug and several other dihydrofolate reductase inhibitors against *P. berghei*. Because pyrimethamine is inactive on the hypnozoites of *P. vivax*, this drug is not applicable for the radical treatment of vivax malaria. Also, pyrimethamine has no action on the production of gametocytes (Anand, 1984).

In the usual antimalarial doses adverse effects related to pyrimethamine are very low, frequent administration of high doses of the drug for a long term may culminate in a megaloblastic anaemia. Duch and co-workers (1979) reported that pyrimethamine also could inhibit the enzyme histamine N-methyl transferase.

1.4.6 Sulphonamides and sulphones

The first reports of the action of sulphonamides on plasmodia were published as early as 1938 by Coggeshall (1938) and Chopra & Das Gupta (1938). These reports contained a number of experiments which demonstrated activity of sulphanilamide and soluseptazine against *P. knowlesi* in rhesus monkeys. The activity of sulphones was described by Coggeshall *et al* (1941), who used rhesus monkeys infected with *P. cynomolgi*.

The first report of the clinical administration of sulphonamides in human malaria was made by Diaz de Leon (1937), who successfully treated 15 cases of vivax malaria with Ribiazol (6-carboxy-4-sulphamide-2-4-diaminoazo-

benzene). In the same year Hill & Goodwin (1937) and Van der Wielen (1937) reported their experience with Prontosil for curative treatment of *P. falciparum* and *P. malariae* infections, respectively. In 1941 dapsone was used as an antimalarial agent by Coggeshall and co-workers against *P. knowlesi, P. cynomolgi* and *P. inui*, and later Archibald & Ross (1960) used this drug for treatment of falciparum malaria.

Sulphonamides and sulphones are thought to be comparatively slow acting antimalarial drugs. They are inactive against sporozoites, primary exoerythrocytic stages and hypnozoites. In contrast, the drugs are highly effective against the asexual blood stages and they are more effective against *P. falciparum* than against *P. vivax*. Although the effectiveness of sulphonamides and sulphones on falciparum gametocytes is not definitely proven, drug exposed gametocytes are not infective to mosquitoes (Bruce-Chwatt *et al*, 1986).

The administration of sulphonamides and sulphones in the correct dosage usually is well tolerated, but the drugs may produce side effects in certain individuals. The most frequent and serious side effects associated with sulphonamides use involve skin reactions in the form of urticaria. According to Bruce-Chwatt *et al* (1986) Stevens-Johnson syndrome and toxic epidermal necrolysis may occur following gross over-dosage of the drugs. On the other hand, it has been reported that such reactions are not necessarily dose related, and likely depend on individual idiosyncrasy or allergy (Desjardins *et al*, 1988). The most serious side effects related to sulphones are haematological problems such as methaemoglobinemid and severe haemolytic anaemia (Desjardins *et al*, 1988). However, both sulphonamides and sulphones can cause severe haemolysis

in individuals who are G6PD-deficient (Scholer *et al*, 1984; Bruce-Chwatt *et al*, 1986).

The chemical structure of the most important sulphonamides and sulphones are as follows: sulfadiazine [N'-2-pyrimidinylsulfanilamide], sulfadoxine [N'- (5, 6-dimethoxy-4-pyrimidinyl)-sulfanilamide], sulfalene [N'-(3-methoxy-2-pyraxinyl)-sulfanilamide], dapsone [4, 4'-diaminodiphenylsulfone] and acedapsone [4, 4'-diacetyldiaminodiphenylsulfone].

1.4.7 The 8-aminoquinolines

Pamaquine, the first antimalarial compound of the 8-aminoquinoline series was first used against human malaria by Sioli (1926). After a few years a new 8-aminoquinoline primaquine [6-methoxy-8-(4'-amino-1'-methylbutylamino) quinoline] replaced pamaquine. Primaquine is the most effective of the 8-aminoquinolines. Primaquine has a broad spectrum of activity on all stages of the parasites in appropriate concentrations. Quinocide [6-methoxy-8-(4'-amino-4'-methylbutylamino) quinoline], an analogue of primaquine, is similar to primaquine in terms of activity except it is somewhat more toxic and therefore rarely used (Anand, 1984). Primaquine and quinocide are both active against the primary exoerythrocytic stages of *P. vivax* and *P. falciparum*, and highly active against hypnozoites in vivax malaria. These drugs are highly gametocytocidal against all species of human malaria parasites. They are inactive against sporozoites.

Although at the recommended dosage the toxicity of primaquine is very low as a rule, the administration of increased doses may manifest side effects such as gastrointestinal complaints, weakness and uneasiness in the chest.

Additionally, there may be remarkable effects on the formed elements of the blood and the bone marrow. Administration of primaquine also may cause production of cyanosis as a result of conversion of haemoglobin to methaemoglobin. Primaquine can induce haemolysis in individuals with the enzyme glucose-6-phosphate dehydrogenase (G6PD)-deficiency (Desjardins *et al*, 1988).

1.4.8 Naphthoquinones

Menoctone [2-(8-cyclohexyloctyl)-3-hydroxy-1, 4-naphthoquinone] a naphthoquinone derivative, was tested in non-immune volunteers infected with the Malayan (camp) strain of *P. falciparum* by Rieckmann and co-workers (WHO, 1973). Although menoctone in early investigations in animals showed encouraging activity as a gametocytocidal agent and was active against pre-erythrocytic schizonts of plasmodium, the drug did not show any gametocytocidal or sporontocidal action against the strain mentioned previously or chloroquine-sensitive Uganda I strain. This failure has been ascribed to poor gastrointestinal absorption of the drug (Peters, 1987). A number of hydroxynaphthoquinones have been evaluated against *P. falciparum* by Hudson *et al* (1985). Desjardins and his associates (1979a) demonstrated that BW58C, a hydroxynaphthoquinone, was active *in vitro* against the Wellcome (FCRI/Nigeria) strain of *P. falciparum*.

The toxicity of hydroxynaphthoquinone to man is not clear, but because of their potent effects on isolated mammalian mitochondrial enzymes presumably some of these compounds would be toxic to man. However, at least five hydroxynaphthoquines, including menoctone, have been reported to be without any serious side effects to man (Hudson, 1984). Some others (eg.

hydrolapachol) showed only transient side effects such as nausea, anorexia and a pink skin coloration in some patients (Hudson, 1984).

1.4.9 Mefloquine

Between 1963 and 1976 nearly three hundred 4-quinolinemethanol derivatives were investigated by the US Army Medical Research and Development Command. Mefloquine [WR142, 490; di-erythro-α-(2-piperidyl)-2, 8-bis (trifluoromethyl)-4-quinolinemethanol] was originally synthesised by Ohnmacht and co-workers (1971). Synthesis has subsequently been improved by Grethe & Mitt (1978). The extensive development and assessment of this compound was initiated by the US Army; since 1976 the drug has been developed by WHO, accompanied by the Walter Reed Army Institute of Research and Hoffman-La Roche (WHO, 1984). The action of mefloquine on animal and human plasmodia has been extensively investigated by a number of authors (see Schmidt *et al*, 1978a; Rozman & Canfield, 1979; WHO, 1984; Sweeney, 1984; Peters, 1987).

In 1985, mefloquine was marketed for the first time in Switzerland for treatment and prophylaxis in children aged over 2 years and adults excepting pregnant women (Peters, 1987). Mefloquine is a potent schizontocide with a long serum half-life (approximately 20-30 days), perhaps the longest among the available antimalarial drugs (Anand, 1984). The suppressive activity of mefloquine against sporozoite-induced falciparum and vivax malarias was investigated by Clyde *et al* (1976). They demonstrated that administration of 250mg of mefloquine hydrochloride in a single dose at weekly intervals, 500mg at intervals of 2 weeks and 1000mg at intervals of 4 weeks to men bitten by

mosquitoes heavily infected with a chloroquine- and pyrimethamine-resistant strain of *P. falciparum* can suppress the transmission of the infections. Also they elucidated that sporozoite-induced *P. vivax* infections were halted by single doses of 250mg of the drug given at weekly intervals.

Clinical trials of mefloquine to date have shown that the drug is well tolerated in man. Symptoms encountered during the long term administration of mefloquine have included headache, dizziness, nausea, vomiting, abdominal pain and diarrhoea (Canfield, 1980; Bruce-Chwatt *et al*, 1986; Desjardins *et al*, 1988). These side effects appeared to be reversible.

1.4.10 Qinghaosu (Artemisinin)

Qinghaosu (artemisinin) was isolated in 1972 from the traditional Chinese herb Qinghao (*Artemisia annua*), long used in China as a febrifuge (Qinghaosu Antimalaria Co-ordinating Research Group, 1979; Bruce-Chwatt, 1982). It has been shown to be an endoperoxide of a sesquitepenoid lactone, and bears no structural resemblance to any other antimalarial agent (Qinghaosu Antimalaria Co-ordinating Research Group, 1979; Warburton, 1984). The activity of qinghaosu depends on the presence of the endoperoxide bridge (Gu *et al*, 1980). The drug has been employed in both animal and human studies. Warburton (1984) has stated that the administration of artemisinin against *P. berghei* in mice subcutaneously was more effective than oral dosing. Qinghaosu is only sparingly soluble in water and oil, but its derivatives, artemether and artesunate, are soluble in oil and water respectively (WHO, 1984; Anand, 1984). The half-life of qinghaosu and its derivatives is extremely short (approximately 4 hours) and it is generally well tolerated at clinical dosage. The drug and its

derivatives are effective against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* as well as *P. vivax* malaria. These drugs are highly active against blood schizonts, but have no tissue schizontocidal action against either *P. falciparum* or *P. vivax*. The drugs are inactive against gametocytes (Jiang *et al*, 1982). Qinghaosu can be administered either orally or intramuscularly, but the administration of the drug intramuscularly is more effective than oral dosing. The recommended medication is a single dose of 300mg daily intramuscularly in adults for three consecutive days (Warburton, 1984).

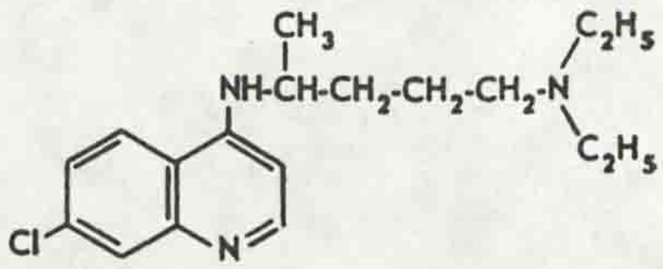
The toxicity studies of qinghaosu in mice showed that the drug was well tolerated. The results also demonstrated that adverse effects (eg. liver damage) may occur only when very large doses are given (WHO, 1984). Chawira and colleagues (1987) suggested that qinghaosu is not recommended for prophylaxis because of the embryotoxic effects of the drug.

1.4.11 Halofantrine

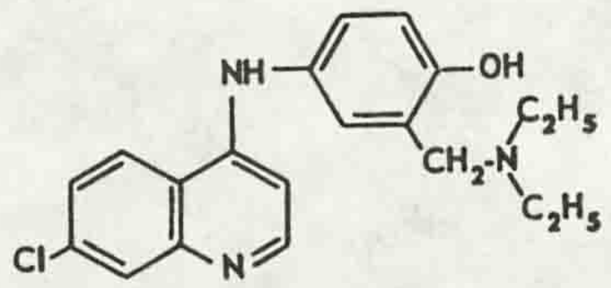
Halofantrine {WR171,669; 1,3-dichloro-α-[2-(dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol} is among the most promising antimalarial drugs to date. The drug was originally synthesised by Colwell *et al* (1972) as one of a series of arylaminopropanols, which subsequently was examined in depth as an antimalarial agent. At the beginning the activity of halofantrine was widely proved against the animal models of plasmodia (see Oselene *et al*, 1967; Colwell *et al*, 1972; Schmidt *et al*, 1978b). Halofantrine (WR171, 669) is highly active against blood schizonts of both chloroquine-sensitive and -resistant strains of *P. falciparum* (Schmidt *et al*, 1978b; Cosgriff *et*

Structural formulae of antimalarial drugs

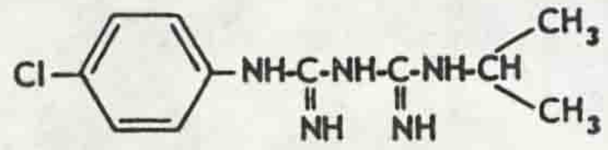
Chloroquine
7-chloro-4-(4′-diethyl-amino-1′-methylbutyl-amino)quinoline



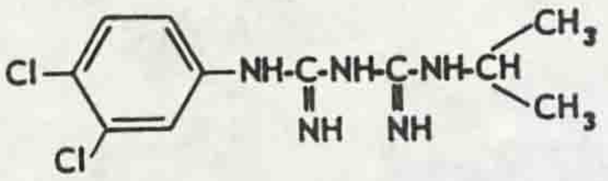
Amodiaquine
7-chloro-4-(3′-diethylamino-methyl-4′-hydroxyanilino)-quinoline



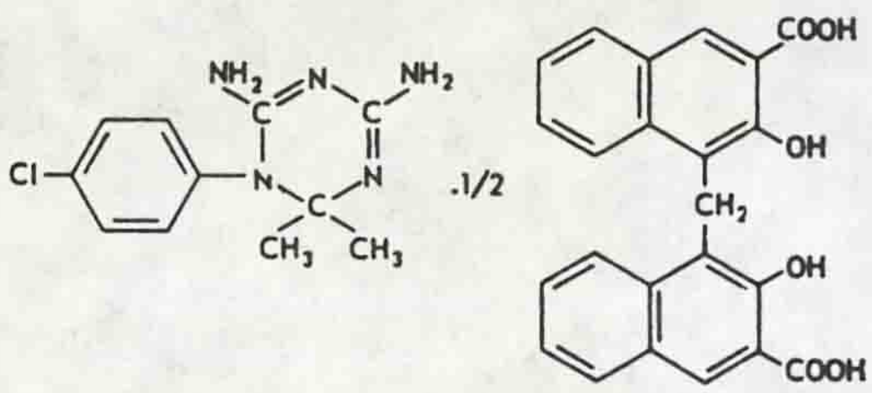
Proguanil
N^1(*p*-chlorophenyl)-N^5-iso-propyldiguanide



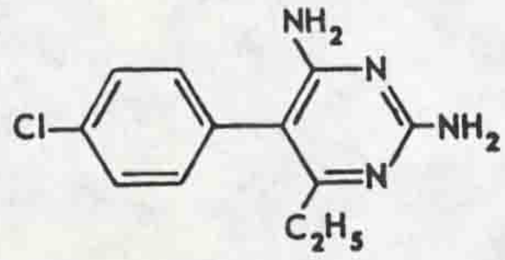
Chlorproguanil
N^1-(3,4-dichlorophenyl)-N^5-iso-propyldiguanide



Cycloguanil embonate
4,6-diamino-1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine with 4,4′-methylene-bis(3-hydroxy-2-naphthoic acid (2:1)

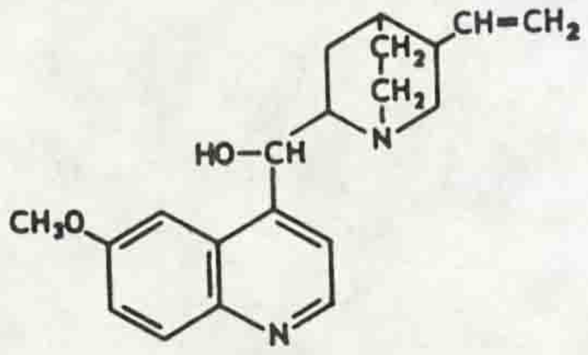


Pyrimethamine
2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine

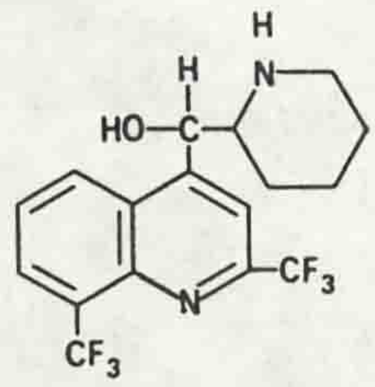


Primaquine
6-methoxy-8-(4′-amino-1′-methylbutylamino)quinoline

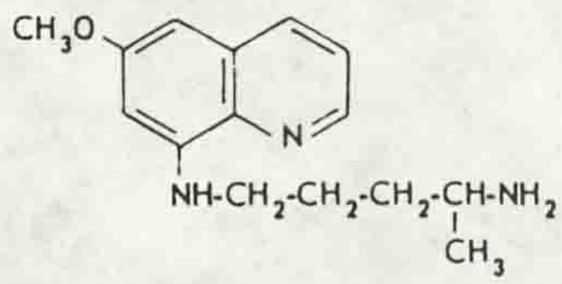
Quinine
6-methoxy-α-(5-vinyl-2-quinuclidinyl)-4-quinolinemethanol



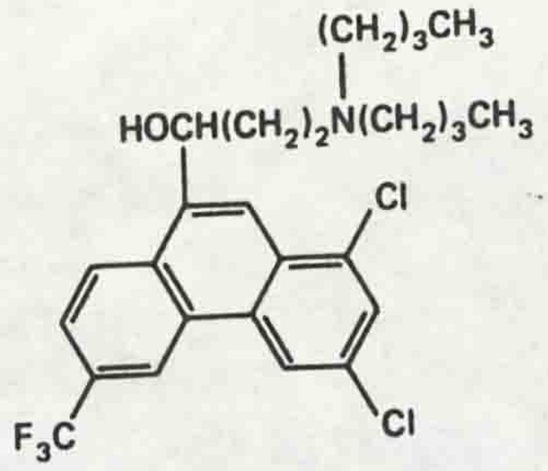
Mefloquine (WR 142,490)
α-(2-piperidyl)-2,8-bis(trifluoro-methyl)-4-quinolinemethanol



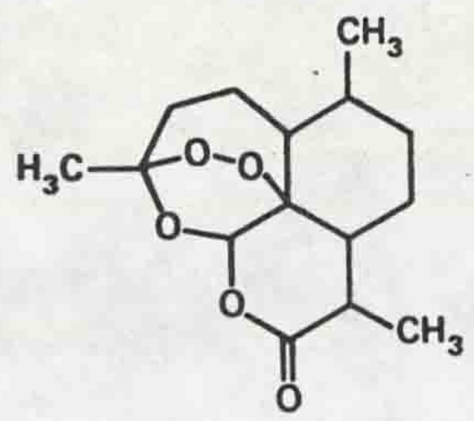
Quinocide
6-methoxy-8-(4′-amino-4′-methylbutylamino)quinoline



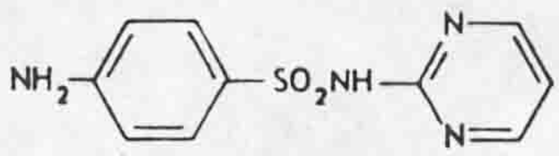
Halofantrine
1,3-dichloro-α-[2-(dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol



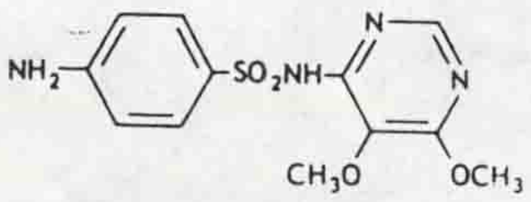
Qinghaosu
(artemisinine)



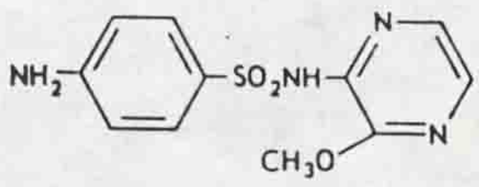
Sulfadiazine
N'-2-pyrimidinylsulfanilamide



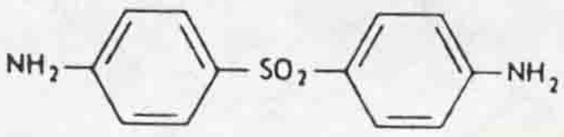
Sulfadoxine
N'-(5,6-dimethoxy-4-pyrimidinyl)-sulfanilamide



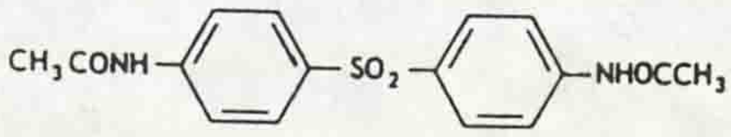
Sulfalene
N'-(3-methoxy-2-pyrazinyl)-sulfanilamide



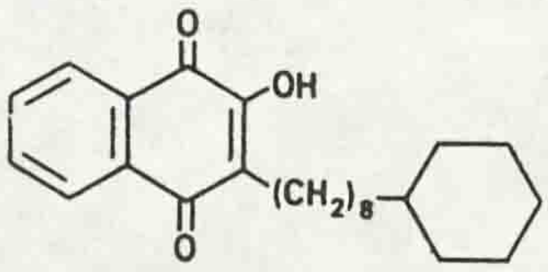
Dapsone
4,4'-diaminodiphenylsulfone



Acedapsone
4,4'-diacetyldiaminodiphenylsulfone



Menoctone (WR 49,808)
2-(8-cyclohexyloctyl)-3-hydroxy-1,4-naphthoquinone



(WHO, 1984 and Bruce-Chwatt et al., 1986)

al, 1982). The drug and its activity will be discussed in more detail elsewhere (Chapter 5). The toxicity of halofantrine also was extensively examined by some investigators. Lee *et al* (1972) reported that the acute oral LD_{50} of the drug in male rats was 3400mg/kg and the acute intraperitoneal LD_{50} was 2050mg/kg. Moreover, the probable toxic symptoms included nasal and ocular discharge, anorexia, weight loss and inactiveness; the clinical studies indicated that halofantrine was well tolerated in the malaria-infected patients. Gastrointestinal symptoms may rarely occur in some patients (Richard-Lenoble *et al*, 1989). For further studies see Hodgson *et al* (1977) and Lee *et al* (1972).

1.5 Antimalarial drug combinations

Experiments carried out by a number of investigators (see Peters, 1970, 1980, 1982, 1987; WHO, 1984; Wernsdorfer, 1984), indicate that the use of antimalarial drug combinations may slow down, if not prevent, the development of resistance to them by *P. falciparum*. The rationale for this approach comes from the genetic basis of drug resistance. The probability of multiple genetic mutations in strains of *P. falciparum* which result in resistance to a variety of drugs with different mechanisms of action being very low (Li *et al*, 1984). The potential of antimalarial drug combinations was summarised by Peters (1987). He also has described that the employment of antimalarial compounds in combination has many advantages such as the exploration of novel and rational approaches of producing drug synergism, overcoming resistance to one or other partner of the combined drugs and prolonging the useful life of each member of the combination (Peters, 1984a, b).

It is very important for a useful combination that the pharmacokinetics of the components especially absorption rate and elimination half-lives, should be as well matched as possible.

In recent years a number of combinations of antimalarial drugs such as Maloprim (pyrimethamine and dapsone), Fansidar (pyrimethamine and sulphadoxine) and Fansimef (mefloquine, sulphadoxine and pyrimethamine) have been employed for prophylaxis of malaria (Howells, 1986; Desjardins *et al*, 1988). Today, the administration of the above combinations has encountered problems; resistance to Fansidar is widespread, and Maloprim and Fansimef carry the risk of serious side effects (Howells, 1986; Peters, 1987; Desjardins *et al*, 1988). Although a number of new combinations of mefloquine or pyronaridine with Fansidar have been suggested (Merkli *et al*, 1980; Guo *et al*, 1988; Kollaritsch *et al*, 1988; Anh *et al*, 1990), the prolonged usefulness of such combinations is still doubted (Peto *et al*, 1985). More recently the combination of chloroquine with several non-antimalarial agents has been proven against both chloroquine-sensitive and -resistant strain of *P. falciparum in vitro* (Martin *et al*, 1987; Bitonti *et al*, 1988; Peters *et al*, 1989; Bitonti & McCann, 1989), which will be discussed in Chapter 4.

1.6 The development of drug resistance in the field

The natural variations in the drug-sensitivity of strains of *Plasmodium falciparum* from different parts of the world has been recognised for many years (WHO, 1965; Peters, 1969), but the emergence of drug-resistance as a direct result of the widespread use of drug chemoprophylaxis and chemotherapy

displayed a new and perilous status in the drug treatment of malaria (Fig. 1.6.1). An exhaustive review of this topic up to 1970 was covered by Peters (1970) and updated by Peters (1987). Reports from most malarious areas make it clear that *P. falciparum* is becoming resistant to most available antimalarial drugs. Moreover, the recent reports from Thailand indicate that all existing alternative treatments are powerless against *P. falciparum* (Cowman & Foote, 1990). The onset of drug resistance in falciparum malaria observed in the early 1960's has led to an extensive effort to synthesise and test new drugs.

Drug resistance was defined by the WHO (1963) as the "ability of a parasite strain to multiply or to survive in the presence of concentration of a drug that normally destroys parasites of the same species or prevent their multiplication. Such resistance may be relative (yielding to increased doses of the drug tolerated by the host) or complete (withstanding maximum doses tolerated by the host)".

In 1967 the WHO Scientific Group on Chemotherapy of Malaria defined three degrees of drug-resistance in man to a 1.5g oral dose of chloroquine (Table 1.6). The levels of resistance were graded from primary resistance (RI), in areas where resistance was widespread (Bruce-Chwatt *et al*, 1986). These three degrees of drug-resistance; RI, RII, RIII, were later modified to the form shown in Fig. 1.6.2 (WHO, 1973).

1.6.1 Quinine and other aminoalcohols

According to the above definitions the resistance of *P. falciparum* to quinine was the first case of complete resistance to an antimalarial drug reported from Brazil as early as 1910 by Nocht & Werner. However, in spite of

the widespread and extensive use of quinine the reports of quinine-resistance are still quite rare (WHO, 1984; Ambroise-Thomas & Rossignol, 1986). A number of authorities suggest that resistance could be a result of a short course of treatment or inadequate plasma levels of the orally administered drug (Hall, 1972; WHO, 1984). Bastien (1987) has reported two cases of probable quinine-resistance from Vanuatu. Both were found in male Melanesian children (aged 7 and 10). Bastien's report thus shows poor response of *P. falciparum* to oral quinine treatment. Jaroonvesama *et al* (1974), reviewed three cases of *P. falciparum* in Thai patients of which two gave clinical RIII response to quinine, but all patients were cured with the standard pyrimethamine-sulphadoxine regimen. Anand (1984) stated that a 7 days treatment of falciparum malaria with quinine in south east Asia showed a high level of recrudescence, but the disease was treated with a combination of quinine and tetracycline using a 7 days treatment.

Although mefloquine is restrictively used in areas where chloroquine resistance is a major problem (Pearlman *et al*, 1980; Bruce-Chwatt *et al*, 1986), recently a number of cases of poor response or resistance of falciparum malaria to mefloquine has been reported from Thailand and numerous African countries (Boudreau *et al*, 1982; Bygbjerg *et al*, 1983; Oduola *et al*, 1987; Brasseur *et al*, 1988; Karwacki *et al*, 1989; Kilimali *et al*, 1989; Kremsner *et al*, 1989). Oduola and co-workers (1987) have shown that west African isolates of *P. falciparum* were more sensitive to chloroquine than to mefloquine, the converse of south east Asia isolates. These findings suggest that *P. falciparum* may be inherently resistant to mefloquine in the above mentioned areas (Oduola *et al*, 1987). Since mefloquine

belongs to the quinolinemethanols, and is related structurally to quinine (Sweeney, 1984) some authorities believe that there might be cross-resistance between quinine and mefloquine (WHO, 1984).

1.6.2 Resistance to mepacrine and 8-aminoquinolines

Mepacrine was never used extensively except during the second World War (Bruce-Chwatt, 1986). However, the first and perhaps unique report of primary mepacrine resistance in falciparum malaria was made by Fairley (1946). Resistant strains were isolated from patients undergoing mepacrine treatment. There was some evidence to suggest that resistance to mepacrine was apparently lost from one of the resistant strains after four passages in untreated volunteers (Peters, 1987).

The 8-aminoquinoline, primaquine, is the only drug of this class currently in use in malaria chemotherapy. Although there have not been reported any clear cases of primaquine-resistance in *P. falciparum*, resistance, or at least poor response, to this agent has frequently been shown in the erythrocytic stages in association with resistance to chloroquine (Moor & Lanier, 1961). A study by Powell *et al* (1963) demonstrated that 45mg primaquine and 300mg chloroquine base as prophylactic doses could not fully protect against *P. falciparum* whether this reflects primaquine resistance is very questionable.

1.6.3 Resistance to chloroquine and other 4-aminoquinolines

The first reports of chloroquine-resistance in *P. falciparum* were made by Moor & Lanier (1961) from Colombia, and Harinasuta *et al* (1962) from Thailand, and it has been shown that the resistance was stable to cyclical transmission (Peters, 1987). Young & Johnson (1972) quoted that the first

documented case of chloroquine-resistance in falciparum malaria may have been as early as 1956. Also, Young (1961) earlier demonstrated a cross-resistance between chloroquine, amodiaquine and hydroxy-chloroquine in a chloroquine-resistant strain of *P. falciparum*. Strains of *P. falciparum* resistant to chloroquine have since been found in the most malarious areas (WHO, 1984; Fox *et al*, 1985; Ekanem, 1985; Kyronseppa *et al*, 1984; Sansonetti *et al*, 1985; Edrissian & Shahabi, 1985) and more recently, Delfini (1989) has reported the first case of *P. falciparum* resistant to chloroquine treatment from Afghanistan.

The degree and frequency of chloroquine-resistant cases of *P. falciparum* depends on the length of use and the intensity of drug pressure, in addition to the extent of malaria transmission after the initial selection and introduction of resistant parasites in the affected areas. In support of these contributing factors a high frequency and high degree of chloroquine-resistance in *P. falciparum* has been observed in Kampuchea, Thailand and Vietnam (WHO, 1984). In Thailand about 90% of falciparum malaria infections have been classified as highly chloroquine resistant (RII or RIII) (Wernsdorfer, 1984; Thaithong *et al*, 1983). The emergence of chloroquine-resistant strains of *P. falciparum* was somewhat delayed in Africa, probably owing to the semi-immune status of African individuals (Peters, 1980) and dose-response relationship of African isolates to chloroquine (Peters, 1969). Geographical distribution of chloroquine-resistance is presented in Fig. 1.6.3.

In spite of evidence that amodiaquine has given a higher cure rate than chloroquine in many regions, it has not been used as extensively as chloroquine (WHO, 1984; Peters, 1987). In 1982, the number of RIII responses

to amodiaquine were significantly less compared to chloroquine in Colombia (WHO, 1984). Nevertheless, Young & Johnson (1972) in a comparative study in Panama demonstrated that the recrudescence rates of falciparum malaria in a number of patients was similar with both chloroquine and amodiaquine. Several investigators also recorded resistance to amodiaquine in *P. falciparum* (Galvao *et al*, 1961.1962; Silva & Lopes, 1964; Glew *et al*, 1974; Hall *et al*, 1975; Campbell *et al*, 1983; Childs *et al*, 1989). Vasconcelos & Rosario (1983) however, observed that some strains such as Brazilian strains were more sensitive *in vitro* to amodiaquine than to chloroquine. The *in vivo* and *in vitro* studies conducted by Watkins *et al* (1984) in Kenya, demonstrated that amodiaquine was still effective for the treatment of chloroquine-resistant falciparum malaria, but chloroquine had considerably lost its capability for treatment of the infection in that area. For further studies see Peters (1987).

1.6.4 Resistance to proguanil, pyrimethamine, sulfonamides and sulfones

The first field cases of proguanil resistance in *P. falciparum* were reported from Malaya and India (Field & Edeson, 1949: Chaudhuri & Chaudhuri, 1949). *P. vivax* and *P. malariae* resistance to proguanil was identified in New Guinea (Gunther *et al*, 1952) and in Indonesia (Van Goor & Lodens, 1950). Schneider *et al* (1948), however, has shown that proguanil is more effective against *P. falciparum* compared to *P. vivax* and *P. malariae*. Covell *et al* (1949) made the first significant report of the failure of proguanil prophylaxis in an African strain of *P. falciparum*. Peters (1984c) suggested that proguanil is not a good choice as a prophylactic agent against *P. falciparum* and *P. vivax* since widespread proguanil resistance occurs, but proguanil is the drug of choice for

malaria prophylaxis in the UK (Dr S A Ward, personal communication and Phillips-Howard *et al*, 1988).

Pyrimethamine-resistance has, more or less, followed a similar pattern to that of proguanil resistance. In 1953, Chakravarty & Chaudhuri reported that a number of patients from Calcutta infected with falciparum malaria failed to respond to pyrimethamine even at higher than recommended doses. Coatney *et al* (1952), Young (1957) and Burgess & Young (1959) succeeded in producing pyrimethamine-resistant strains of *P. vivax*, *P. malariae* and *P. falciparum* in human volunteers. Some authors proposed that some strains of *P. falciparum* show a natural resistance to pyrimethamine (Wilson, 1952; McGregor & Smith, 1952; Avery-Jones, 1954, 1958). At present, pyrimethamine-resistance in *P. falciparum* is widespread and has become a major problem in all areas where *P. falciparum* is endemic (Bruce-Chwatt *et al*, 1986).

Resistance to sulphonamides and sulphones has been developed experimentally in *P. berghei* and *P. gallinaceum* (Bruce-Chwatt *et al*, 1986). Scholer *et al* (1984) suggested that resistance to the drugs has not been observed with human plasmodia, but a certain percentage of treatment failures has been reported by a number of investigators. Laing (1974) reported that using sulphadimethoxine in a three days treatment (50-100mg daily) only eliminated parasitaemia in three of 14 semi-immune Gambian malaria-infected patients. Ramos & Cabrera (1972) also reported two failures in twenty-two Philippine patients with chloroquine-resistant falciparum infection after being given 80mg/kg sulphamonomethoxine. Trenholme and co-workers (1975) suggested that such failures may occur owing to individual variations in drug metabolism instead of

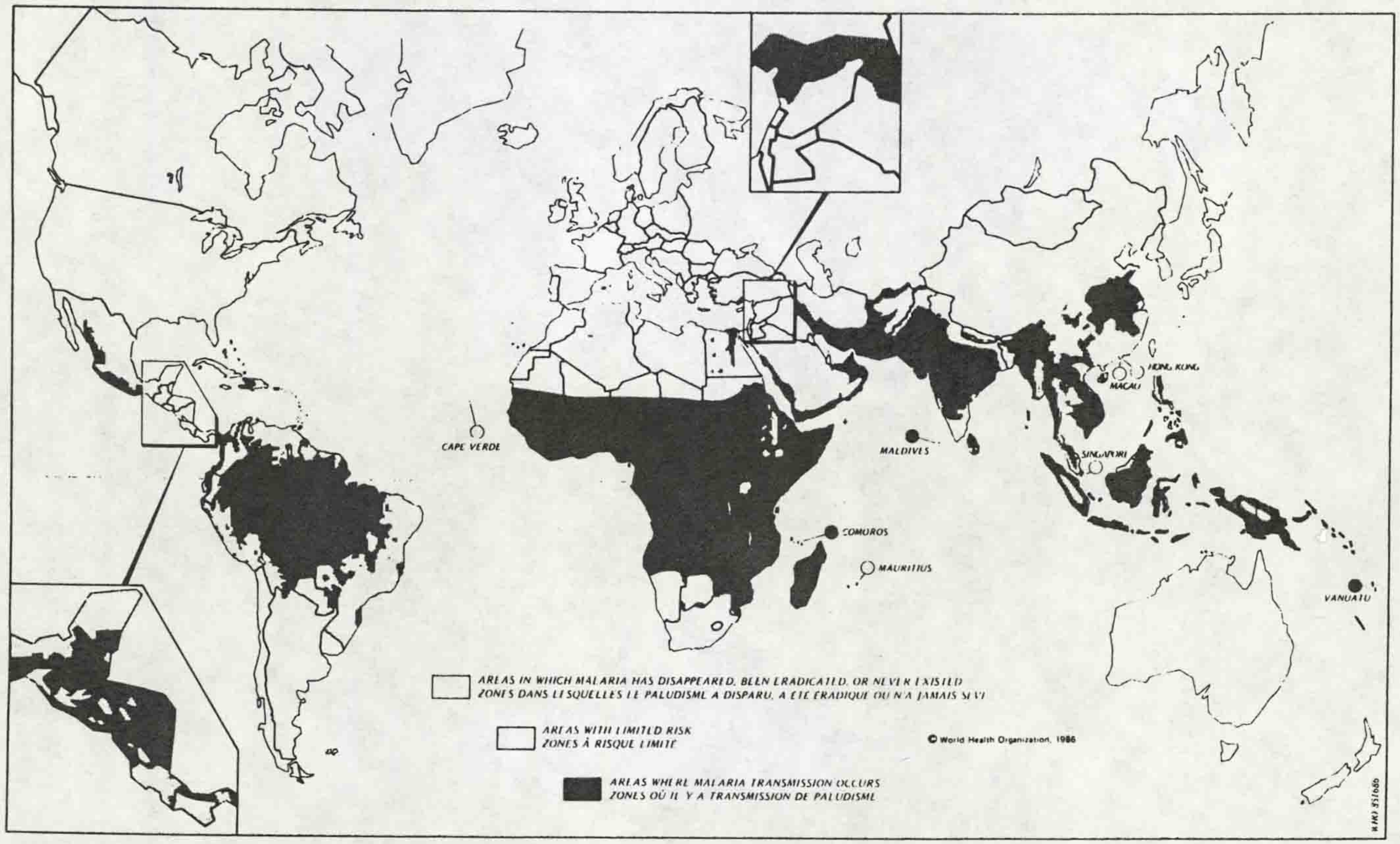
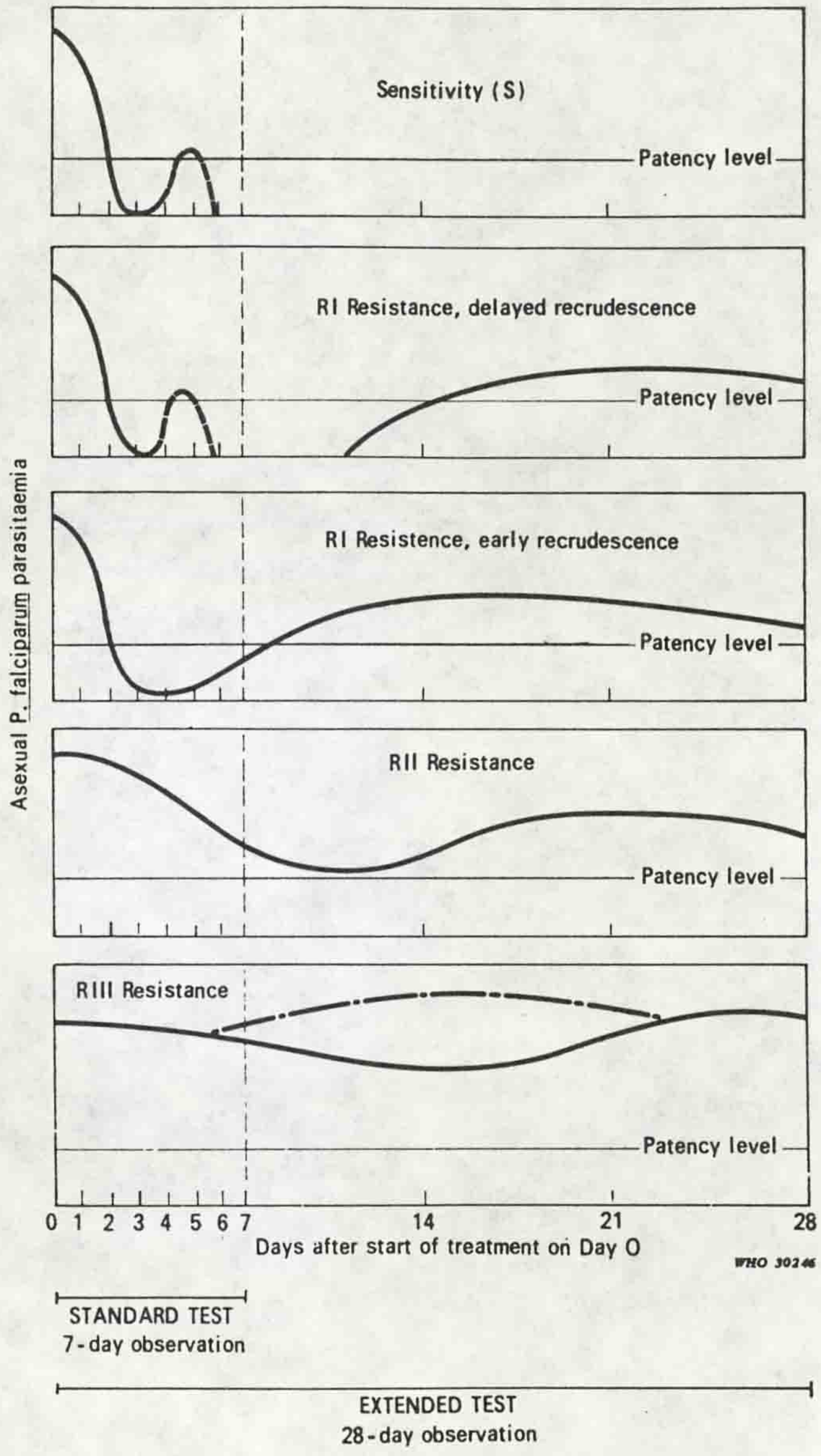


Fig. 1.6.1 **Geographical distribution of malaria in 1984. (Reproduced courtesy of World Health Organisation) (Wernsdorfer and McGregor, 1988)**

Table 1.6 Grading of resistance of asexual parasites (*P. falciparum*) to schizontocidal drugs (4-aminoquinolines) (WHO, 1973)

Response	Recommended Symbol	Evidence
Sensitivity	S	Clearance of asexual parasitaemia within 7 days of initiation of treatment, without subsequent recrudescence
Resistance	RI	Clearance of asexual parasitaemia as in sensitivity, followed by recrudescence
	RII	Marked reduction of asexual parasitaemia, but no clearance
	RIII	No marked reduction of asexual parasitaemia

Fig. 1.6.2 **Response to field test for sensitivity of falciparum malaria to chloroquine***



***Chloroquine administered during the first 3 days.**

---------- Patent parasitaemia may reappear by day 5; in sensitive (S) strains, however, it is absent on and after day 6.

- - - - - There may be an increase in parasitaemia in RIII resistance.

Note that it is not possible to distinguish between S and RI in the standard (7-day) test.

(WHO, 1973)

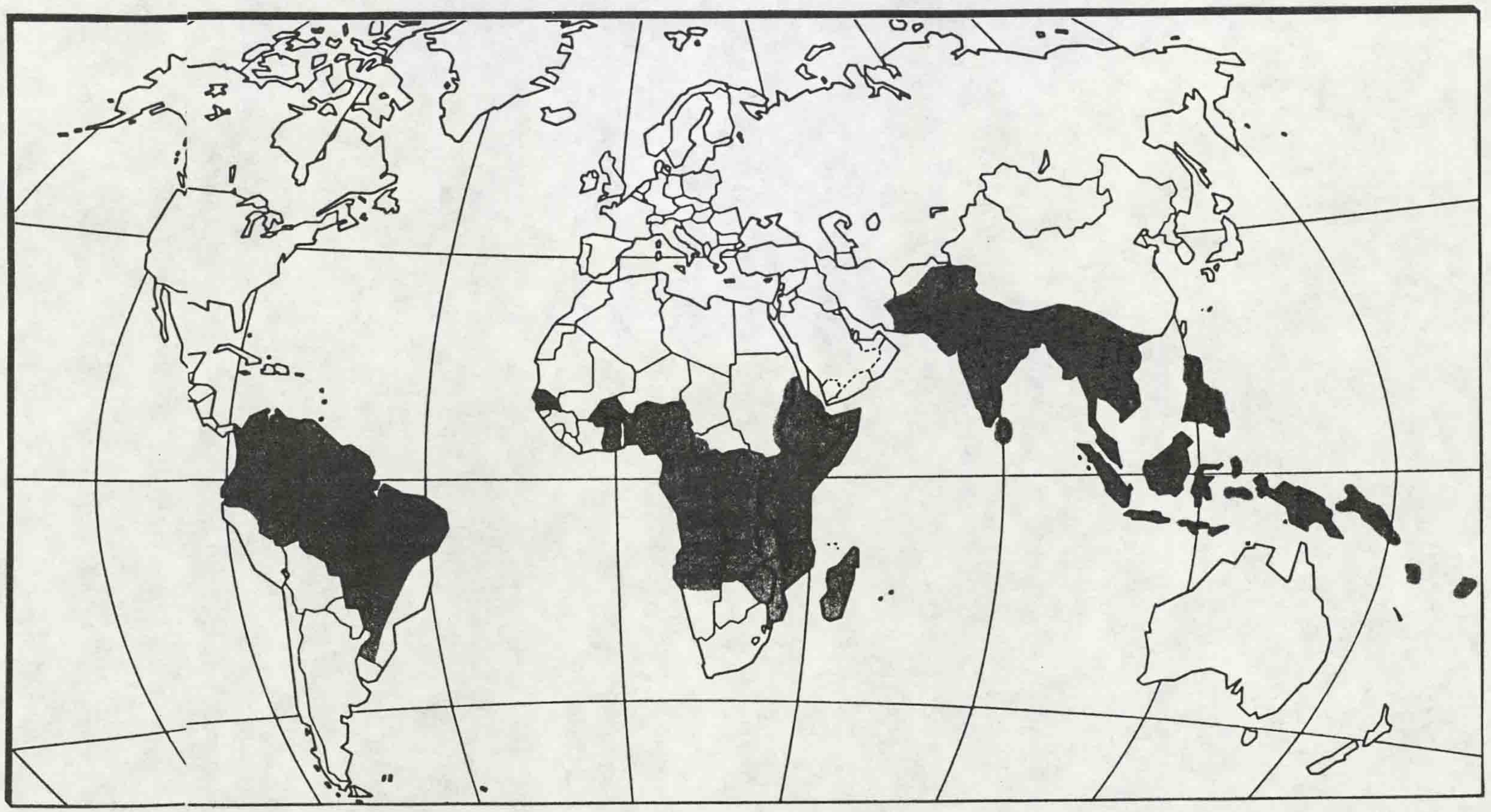


Fig. 1.6.3 **Distribution of chloroquine resistant *Plasmodium falciparum* in the world (status in 1987) (Payne, 1987)**

resistance. However, Milhous *et al* (1984) (quoted in Peters, 1987) reported two Indochina isolates of *P. falciparum* which showed considerable levels of resistance to sulphadoxine, sulphamethoxazole, sulphalene and dapsone *in vitro*.

The combination of pyrimethamine and sulphadoxine has been extensively employed as an alternative drug to chloroquine for treatment of chloroquine-resistant falciparum malaria. Pyrimethamine is a competitive inhibitor of the enzyme tetrahydrofolate dehydrogenase (dihydrofolate reductase, EC 1.5.1.3), but sulphadoxine competes with para-aminobenzoic acid (PABA) for the active site on the enzyme dihydropteroate synthase (EC 2.5.1.15) (Desjardins *et al*, 1988). Therefore, the potentiation between pyrimethamine and sulphadoxine against chloroquine-resistant falciparum infections can be explained on the basis of different sites of action in the parasites. However, the pyrimethamine-sulphadoxine combination Fansidar was also widely administered as a prophylactic drug, and this possibly accelerated the emergence of resistance to the combination (WHO, 1984). Fansidar resistance has now been reported in Thailand and Kampuchea (Hurwitz *et al*, 1981; Chongsuphajaisiddhi & Sabchareon, 1981; Johnson *et al*, 1982; Childs *et al*, 1986), East Africa (Stahel *et al*, 1982; Herzog *et al* 1982; Markwalder & Meyer, 1982), Colombia, Brazil and Vietnam (Wernsdorfer, 1984).

Recently the combination of mefloquine, pyrimethamine and sulphadoxine (Fansimef, containing 250mg mefloquine, 25mg pyrimethamine and 500mg sulphadoxine) has been used in the areas where chloroquine and Fansidar resistant strains of *P. falciparum* exist (Win *et al*, 1985; Kollaritsch *et al*, 1988; Anh *et al*, 1990). The use of Fansimef in Thailand in 1985 showed that this triple drug

combination can give rise to severe skin reactions in the malaria-infected patients (Desjardins *et al*, 1988), but there is no report about Fansimef-resistance in plasmodia to date. The combination has been discontinued.

1.7 The development of drug resistance in the laboratory (*in vitro*)

The production of experimentally induced drug-resistance in the laboratory provides the opportunities for investigators to study the nature and genetics of drug resistance mechanisms to a given agent, patterns of cross-resistance, the mode of action of drugs and evaluation of the antimalarial potential of new compounds (Beale *et al*, 1978; Rollo, 1968; Peters & Porter, 1976). Experimental techniques for the production of drug resistance have been exhaustively reviewed by Peters (1984d, 1987). The continuous cultivation of the blood stages of *P. falciparum in vitro* opened a new way for the selection or induction of drug resistance in malaria parasites. The earliest attempts were made by Nguyen-Dinh & Trager (1978), who successfully produced a chloroquine-resistant line of *P. falciparum* from an isolate originating in the Gambia in West Africa (FCR-3). They maintained a line of FCR-3 in continuous culture by the petri dish-candle jar method in a medium containing progressively higher concentrations of chloroquine (0.01-0.16μg/ml). The developed resistance was stable and comparable *in vitro* to the highly chloroquine-resistant field strain (FVO) that was isolated from a patient infected in Vietnam. Jensen *et al* (1981) demonstrated that a chloroquine-sensitive strain of *P. falciparum* (FCR-3) after nearly 4 years of continuous culture without chloroquine pressure became resistant to chloroquine both *in vitro* and *in vivo*. Golenser *et al* (1981) obtained

an aminopterin-resistant strain of *P. falciparum* by employment of microplates as culture plates. Parasites were grown on microplates in the presence of aminopterin. Strains FCR-8 and Richards were used for the above studies. While the FCR-8 strain died within a week of treatment with aminopterin, a few parasites of the Richards strain survived treatment showing normal growth and multiplication. In 1981, Brockelman and co-workers reported that they produced a mefloquine-tolerant strain of *P. falciparum* from a mefloquine-sensitive (FCK) strain. They used an intermittent drug exposure method. Cultures were exposed to an appropriate concentration of mefloquine for 48 hours. Cultures that showed healthy ring forms were subcultured with fresh non-infected erythrocytes and drug-free medium, and maintained for several generations. Cultures then were exposed to mefloquine at a 2-4 fold higher concentration and the process repeated. After three months the IC_{50} (50% inhibitory concentrations) value increased from 8nmol/ml of mefloquine to 128nmol/ml, however the stability of this resistance was not determined. Lambros & Notsch (1984) also produced two mefloquine-resistant strains of *P. falciparum* using continuous exposure. The multidrug-resistant Vietnam Smith and Malayan camp strains were employed as starting points. At the end of continuous cultivation in the presence of drug (12μg/l) the ID_{50} (50% inhibitory dose) values for mefloquine increased 4-fold from 3μg/l to 12μg/l. Both strains remained resistant to mefloquine when grown in a drug-free culture medium or after storage in liquid nitrogen.

The application of mutagenic agents to induce drug resistance in *P. falciparum* has been reported by a number of investigators. Inselburg (1984) selected three mutants resistant to aphidicolin, an inhibitor of DNA polymerase

α, to cycloheximide, an inhibitor of protein synthesis, and to sinefungin, an inhibitor of methylation. He explored the use of the potent mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NG) using highly synchronous cultures. It has been documented that NG exerts its mutagenic effect on replicating DNA. The parasites were exposed to 1.5μg/ml aphidicolin, 0.15μg/ml cycloheximide and 0.75 μg/ml sinefungin, concentrations equivalent to approximately twice the concentrations that inhibited at least 90% of parasite growth. In another investigation Inselburg (1985) isolated two temperature-sensitive (ts) mutants. A culture of parasites was exposed to N-methyl-N-nitrosoguanidine (NG). The isolated mutants lost their ability to multiply at 39-39.5°C. In 1986, Banyal & Inselburg reported that they had selected a number of pyrimethamine-resistant mutants on the basis of either standard methods (using continuous exposure) or by the use of mutagenic agents. The mutants exhibited resistance to between 10 and 200 times higher concentrations of drug than the original parasite strains. They concluded that the pyrimethamine-resistant mutant is due neither to an increased amount of dihydrofolate reductase [DHFR; 5, 6, 7, 8-tetrahydrofolate: $NADP^+$ oxidoreductase (EC 1.5.1.3)] nor an alteration in the interaction between DHFR and pyrimethamine, but was due to a reduced permeability of the parasite to pyrimethamine.

Oduola and co-workers (1988b) obtained two mefloquine-resistant strains of *P. falciparum* after continuous drug exposure. The two cloned strains of *P. falciparum* W-2 and M1, both chloroquine resistant, were continuously cultured in drug treated medium. After nearly two years the mefloquine-resistant strain (W2-mef) derived from the W-2 strain was four to six-fold less sensitive to

mefloquine than the parent strain. After a further three years of continuous culture in drug-free medium W2-mef was still resistant to mefloquine.

1.8 Mode of action of antimalarial drugs and parasite resistance mechanism

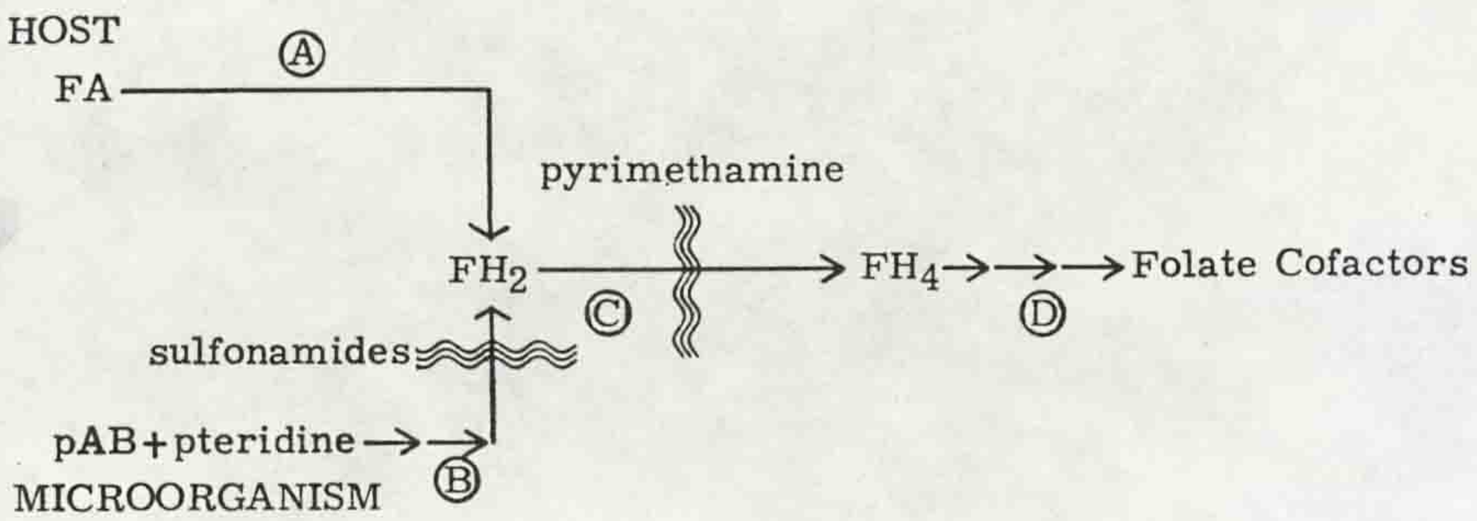
1.8.1 Antifols and PABA-antagonists

1.8.1.1 Mode of action

Malaria parasites are dependent on synthesised purines for their growth, and folate co-enzymes are essential for this synthesis. Folate co-enzymes are synthesised as follows; the condensation of P-aminobenzoic acid (PABA) with pteridine gives pteroate, going on to dihydrofolate, which then results in tetrahydrofolate by the mediation of the enzyme dihydrofolate reductase (DHFR). The inhibition of either of these two enzymes, dihydropteroate synthetase by PABA-antagonists or dihydrofolate reductase by DHFR-inhibitors, culminates in an inhibition of purine and pyrimidine biosynthesis (Fig. 1.8.1.1) (Thompson & Werbel, 1972; Warhurst, 1973; Ferone, 1984; WHO, 1984; Anand, 1984; Hyde, 1990).

Pyrimethamine exerts its antimalarial activity by antagonising folic acid metabolism, and is a potent inhibitor of dihydrofolate reductase. Although the block of folic acid metabolism by pyrimethamine is common to both parasite and host cells, the affinity of parasite dihydrofolic acid reductase for pyrimethamine is significantly higher than the affinity of the host enzyme. In addition, the host cells unlike plasmodia can utilise exogenous folate obtained from dietary sources (Thompson & Werbel, 1972). Milhous and colleagues (1985)

Fig. 1.8.1.1 **Folate cofactor biosynthesis pathways. (A) folate reductase; (B) PABA + pteridine condensing system; (C) dihyrdofolate reductase; (D) systems for additions of 1-carbon units to FH_4. Wavy lines indicate *loci* of action of inhibitors. See Hitchings (1960) for general review of the pathways outlined here (Ferone & Hitchings, 1966; Peters, 1970).**



developed a number of systems to test the *in vitro* susceptibility of *P. falciparum* to antifol antimalarial drugs. They have reported that their observations do not confirm the suggestion that plasmodia are unable to utilise intact exogenous folates and in addition they could successfully maintain three of four isolates of *P. falciparum* in a culture medium depleted of both folic acid and P-aminobenzoic acid. However, the mode of action of pyrimethamine has been well documented by a lot of investigators. Most of them believe that pyrimethamine exerts its antimalarial activity by inhibiting dihydrofolate reductase in plasmodia (see Peters, 1987). Cycloguanil, the active metabolite of proguanil, is though to have the same mechanisms of action as pyrimethamine, ie. inhibition of DHFR. Sulphonamides and sulphones exert their antimalarial activity by preventing the formation of folic acid. The fact can be explained by inhibition of P-aminobenzoic acid incorporation into folic acid, which has been discussed by a number of investigators (Woods, 1940, 1962; Thompson & Werbel, 1972; also see Scholer *et al*, 1984).

1.8.1.2 The mechanism of resistance

The potential mechanism(s) of drug resistance in micro organisms which apply equally to antimalarial resistance in plasmodia are as follows (WHO, 1984).

- modifications of drug transport mechanisms
- gene amplification leading to an increase in the synthesis of target enzymes
- production of mutant enzymes with reduced drug affinity
- increase in drug-inactivating enzymes
- use of alternative metabolic pathways

It has been demonstrated that the 50% inhibitory concentration of pyrimethamine on extracted dihydrofolate reductase from a highly pyrimethamine-resistant line of *P. berghei* was 30 times that for the enzyme from parent pyrimethamine sensitive strain. Additionally the specific activity of dihydrofolate reductase from a pyrimethamine-resistant strain of *P. berghei* to pyrimethamine was higher than that from a drug-sensitive strain (Thompson & Werbel, 1972; Warhurst, 1973). In 1964, Jacobs reported that pyrimethamine-resistant *P. berghei* had an increased requirement for PABA. Milhous and co-workers (1985) suggested, on the basis of their investigations, that resistance of *P. falciparum* to antifol antimalarial agents is a complex phenomenon. Based on their suggestion the resistance to antifols depends on either the de novo synthesis of active folate co-factors or the ability to utilise exogenous intact folate in various forms. Dieckmann & Jung (1986a) by using radiolabelled pyrimethamine (^{14}C-pyrimethamine), demonstrated that the dihydrofolate reductase (DHFR) from the pyrimethamine-resistant strain of *P. falciparum* was 300 times less sensitive to inhibition by pyrimethamine than DHFR from the pyrimethamine-sensitive strain. They also proposed "altered properties of plasmodial DHFR are apparently the only mechanism responsible for pyrimethamine resistance in the strain of *Plasmodium falciparum* (W2) studied". Recently, Banyal & Inselburg (1986) selected a number of pyrimethamine-resistant mutants of *P. falciparum* in vitro, and found that pyrimethamine-resistance of their mutant parasites is neither due to an increased level of dihydrofolate reductase nor to a remarkably altered enzyme interaction with pyrimethamine, but a reduced permeability of the parasites to pyrimethamine may be the main reason for pyrimethamine resistance in the mutants. However,

how this relates to field isolates is questionable. More recently, Hyde (1990) has emphasised the possible role of gene amplification to produce pyrimethamine-resistance, by the cloning of the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene, in malaria parasites.

Sulphonamide resistance in *P. falciparum* may develop as a result of a complex change in parasite metabolism (Peters, 1987). It has been found that para-aminobenzoic acid (PABA) is not an essential nutrient for sulphadoxine-resistant *P. falciparum* (Brockelman & Tan-ariya, 1982a). This finding was confirmed by Tan-ariya & Brockelman (1983) by using Waymouth (P-aminobenzoic acid-deficient) medium and pyrimethamine-resistant strains of *P. falciparum*. In a series of experiments with [35S] sulfadoxine and using W2 strain of *P. falciparum*, Dieckmann & Jung (1986b) suggested that three mechanisms may be involved in sulfadoxine-resistance in the parasites; reduced drug uptake, reduced synthesis of the toxic analogue of dihydropteroate, and an increased ability of the resistant parasites to synthesise PABA de novo. On the basis of the third mechanism of resistance, Dieckman & Jung (1986b) stated that by means of their de novo biosynthesis of PABA, the respective parasites do not need to take up PABA, even if it is present in the surrounding medium.

1.8.2 4-aminoquinolines

1.8.2.1 Mode of action

The 4-aminoquinolines have a variety of biological effects. The widely employed drug of this group of agents is chloroquine. Thompson and co-workers (1948) demonstrated that chloroquine caused pigment clumping and induced several cytoplasmic and nuclear degenerations in *P. lophurae*. Later on,

a number of investigators reported an interaction between chloroquine and nucleic acids (Parker & Irwin, 1952; Hahn *et al*, 1966), suggesting a mechanism of action related to DNA replication and RNA transcription (see Peters, 1970, 1978). Warhurst & Williamson (1968, 1970) and Warhurst (1969) showed that exposure of *P. knowlesi* to chloroquine culminated in the breakdown of parasite ribosomal RNA. Warhurst & Hockley (1967) reported an ultrastructural change related to the *in vivo* action of chloroquine. In this report they showed that the aggregation of haemozoin granules was the first step of the action. Some authors observed that chloroquine was concentrated up to 600 times more in the infected erythrocytes by *P. knowlesi, P. berghei* and *P. falciparum* compared to the plasma concentrations (Polet & Barr, 1968a; Fitch, 1969, 1970). At present, there are two theories which have been used to explain the mode of action of chloroquine; the Ferriprotoporphyrin IX (FPIX) complexation theory, and Lysosomotropic Weak Base theory. Studies of chloroquine-resistance in rodent malaria focussed on the relationship of haemoglobin digestion to drug susceptibility. A number of investigations into the induction of resistance in rodent malaria made it clear that some chloroquine-resistant lines digest haemoglobin and produce plenty of haemozoin when they are not exposed to chloroquine. In contrast, during exposure to chloroquine the production of haemozoin is decreased. Certain strains of chloroquine-resistant *P. berghei* produce no haemozoin under any situation. However, when chloroquine-resistant rodent malaria parasites revert to chloroquine susceptibility they invariably revert to pigment production (see Chou *et al*, 1980; McChesney & Fitch, 1984). It has been documented that malaria pigment contains abundant amounts of ferriprotoporphyrin IX (FP)

(Fitch, 1986). The toxicity of FP was extensively investigated in both rodent malaria parasites and *P. falciparum* (see McChesney & Fitch, 1984). It has been found that FP, apparently, exists only transiently and then it is sequestered in haemozoin in a complex that makes it innocuous for the parasite (Fitch & Chevli, 1981). The foregoing process of sequestration keeps the levels of FP at very low or undetectable level, which are non-toxic to parasites. It has been proposed that chloroquine exerts its action by inhibition or delaying the sequestration of ferriprotoporphyrin IX into malaria pigment, and thereby allows FP to exert its intrinsic cellular toxicity (Fitch, 1986). The second proposed mechanism of action of chloroquine is interference with lysosomal function which has been considered by several investigators. They have reported that chloroquine and related drugs act as lysosomotropic agents accumulating extensively in lysosomes when their concentrations in the cytoplasm reach, apparently, the range of 10^{-6}-10^{-4}M (Allison & Young, 1964; de Duve *et al*, 1974; Wibo & Poole, 1974). It has been suggested that this concentration in lysosomes is related to a substance associated with the lysosomal membranes which binds nitrogenous bases (Dingle & Barrett, 1969). One alternative mechanism suggests that chloroquine accumulates by means of ion trapping via a proton gradient into the acidic lysosomes resulting in large amounts of unbound, diprotonated chloroquine in the lysosomes (see McChesney & Fitch, 1984). Warhurst (1986) has propounded a modification of the lysosomotropic hypothesis which involves the existence of a permease which can facilitate accumulation. This theory is as follows:

"chloroquine and mepacrine could be imported into the parasite cytoplasm on a membrane carrier (permease) under the influence of a proton

gradient maintained by an ATP-dependent pump, transferring protons from the parasite cytoplasm into the erythrocyte cytoplasm. Presumably from this cytoplasmic location a relatively high concentration of chloroquine are further concentrated in the lysosomes of the parasite, which can be demonstrated to be acidic by uptake of neutral red from external 100μM concentration, and have recently been measured to have internal pH around 5.0".

1.8.2.2 The mechanism of resistance

It has been well proven that chloroquine-resistant *P. falciparum* (Fitch, 1970; Krogstad *et al*, 1987) and *P. berghei* (Macomber *et al*, 1966; Fitch, 1969) accumulate less chloroquine than sensitive parasites. Krogstad *et al* (1987) showed that the efflux of chloroquine from chloroquine-resistant *P. falciparum* is 40-50 times more rapid than that from chloroquine-sensitive parasites. They also observed that the initial rate of chloroquine accumulation was the same in the chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* studied. The efflux of chloroquine from *P. falciparum* is supposed to be the basis of chloroquine-resistance. According to the Ferriprotoporphyrin IX Complexation Theory, because the accumulation of chloroquine is owing to the binding of the drug to FP, therefore the efflux of chloroquine should be associated with inaccessibility or absence of FP. In other words, chloroquine-resistant parasites would produce little or none of the FP-chloroquine complex (Fitch *et al*, 1982; McChesney & Fitch, 1984). The studies of several authors indicate that chloroquine accumulates in the acid vesicular compartment of the malaria parasites (*P. falciparum*) (Yayon *et al*, 1984, 1985; Krogstad *et al*, 1987; Ginsburg, 1988). Homewood *et al* (1972) suggested that drug accumulation was confined

to the acidic food vacuole regulated by the weak base effects of the drug. On the basis of the foregoing suggestion, the antimalarial activity was attributed to alkalinisation of the vacuole by the accumulation of drug, which is accompanied by interference with the digestive processes. Some doubts have been expressed by Yayon *et al* (1985) about the idea that the accumulation of chloroquine at the therapeutic dose culminates in the alkalinisation of the food vacuole. The idea that chloroquine-resistance of malaria parasites could simply be due to an increase in the pH of the food vacuoles has been supported by Yayon *et al* (1985) and Ginsburg (1988). The development of drug-resistance as a result of any alteration in the permeability of parasite membranes to protons or halting secretion of protons has been proposed by Warhurst (1986).

1.8.3 Quinine and drugs with quinine-like action

1.8.3.1 Mode of action

Although quinine has been used for a long time as an antimalarial drug, the mechanism of action of the drug has not been explained as fully as that of the antimetabolites. Studies on *P. gallinaceum* showed that quinine inhibited lactate formation from glucose and certain glycolytic enzymes in the parasite (Silverman *et al*, 1944; Marshall, 1948). Clarke (1952) reported that ^{32}P incorporation into RNA and DNA in *P. gallinaceum* was prevented by quinine. The potential interaction between quinine and nucleic acids was studied by a number of investigators (Allison *et al*, 1965; Hahn *et al*, 1966; Allison, 1968). Further studies in this context were accomplished by Hahn (1979). Several authors proposed that the chloroquine-induced pigment clumping of malaria

parasites can be competitively inhibited by quinine and other cinchona alkaloids (Warhurst *et al*, 1972). Ferriprotoporphyrin IX as a receptor for quinine has been suggested by a number of investigators (Chou *et al*, 1980). However, the relevance of the binding of quinine to FP and the chemotherapeutic effects is unclear (Hofheinz & Merkli, 1984).

The findings of several authors indicate that mefloquine has a high affinity for erythrocytic membranes (Sweeney, 1984; Chevli & Fitch, 1981). Jearnpipatkul *et al* (1980) also suggested that *P. berghei* haemozoin binds mefloquine and might act as a drug-binding site in the parasite cytoplasm. In 1975, Davidson *et al* reported that mefloquine, unlike chloroquine and quinine, does not significantly intercalate with calf thymus DNA. Fitch *et al* (1979) carried out an exhaustive study in mouse erythrocytes infected with either chloroquine-resistant or -sensitive *P. berghei*. They reported that chloroquine competitively inhibits mefloquine accumulation and vice versa. Therefore, they concluded that there are identical receptors for the drugs. Chevli & Fitch (1982) expressed their doubt, at least partly, to the foregoing suggestion because the accumulation of mefloquine in infected and uninfected erythrocytes greatly exceeds the accumulation of chloroquine in the erythrocytes. They suggested that mefloquine interacts both with ferriprotoprohyrin IX (FP) and with certain phospholipids. They also suggested that binding of mefloquine to the phospholipids might be the main effect of the drug in the treatment of chloroquine-resistant malaria.

The precise mode of action of halofantrine on *P. falciparum* is not clear. Most of the relevant investigations have been focussed on *P. berghei*. The data obtained by a number of investigators indicated that there is a similarity in

mode of action of halofantrine to that of quinine, mefloquine and WR122, 455 (Peters *et al*, 1987). Additionally, halofantrine can inhibit chloroquine-induced haemozoin clumping *in vivo* and also to induce some mitochondrial damage (Einhaber *et al*, 1976; Peters *et al*, 1987).

1.8.3.2 The mechanism of resistance

Although resistance to quinine and to mefloquine (see 1.6.1) has been increasingly reported, the mechanism of resistance is not clear. It has been suggested that chloroquine, quinine and mefloquine share a similar [at least in FP and phospholipid (Lullmann *et al*, 1980)] receptor and therefore it has been postulated that the drug-resistance is via the same mechanism for all the drugs. Genetics mechanisms have also been investigated by some authorities (see WHO, 1984). They suggested that the drug-resistance might occur as a result of multiple mutations at one or more gene loci. Furthermore, resistant mutants probably arise in nature at a relatively high frequency. Since resistance to halofantrine has not been independently reported to date, the mechanism of resistance to halofantrine has not been investigated. Only cross-resistance between a number of antimalarial drugs and halofantrine has been studied in the laboratory developed halofantrine-resistant strains of *P. berghei* (Peters *et al*, 1987) and *P. falciparum* (see Chapter 5).

1.8.4 8-aminoquinolines and naphthoquinone

Primaquine is the most effective 8-aminoquinoline. It was observed that there are no morphological effects in *P. berghei berghei* after two hours exposure to primaquine *in vivo* (Howells *et al*, 1969). However, Aikawa & Beaudoin (1969) showed that primaquine induced swelling in the mitochondria

of exo-erythrocytic stages of *P. gallinaceum* after 24 hours. In 1970, Howells and co-workers reported that the parasite mitochondrion is the primary site of action of primaquine. Twenty-four hours of treatment by primaquine did not produce any nuclear change, and after forty-eight hours the cytoplasmic membrane showed a degree of degeneration. On the other hand, Whichard *et al* (1968) and Morris *et al* (1970) suggested that primaquine binds to DNA and other nucleic acids. However, it has been known for a long time that the activity of primaquine is due to a metabolite, the identity of which is unknown (see Anand, 1984).

The classical examples of naphthoquinones which have antimalarial activities are menoctone (WR 49, 808) and hydroxynaphthoquinones including BW58C. The activity of the drugs is variable depending on the strains of *P. falciparum* (Peters, 1987). The mode of action of the hydroxynaphthoquinones was investigated by numerous authors. The group of Folkers suggested that the drugs exert their antimalarial effects by inhibiting the parasite's respiratory system (see Hudson, 1984). Howells *et al* (1970) demonstrated that the action of menoctone shows some similarity to that of primaquine (see above).

1.8.5 Qinghaosu (artemisinin)

1.8.5.1 Mode of action

Studies on the mechanisms of antimalarial action of qinghaosu have implicated a variety of biological processes. Although the site of action of the drug is not clear (Jiang *et al*, 1985), it has been implied from numerous investigations that the drug affects a number of different organelles (Qinghaosu Antimalaria Co-ordinating Research Group, 1979). Jiang *et al* (1985) have suggested that qinghaosu primarily induces swelling of *Plasmodium inui*

mitochondria. On the other hand, it has been suggested that the drug causes initial deformation of the food vacuole of the trophozoites of *P. berghei* (Klayman, 1985). In another study, ultrastructural changes in *P. berghei* were followed by Ellis and co-workers (1985). Their observations indicated that artemisinin induced changes in the limiting and other membranes of the parasite thirty minutes after administration of the drug, but no changes occurred in the digestive vacuoles or pigments. They also stressed that the drug caused alterations in ribosomal organisation and the endoplasmic reticulum. Moreover, in a supplementary study with *P. falciparum* by the foregoing researchers, the correlation of morphological changes in ribosomes with the depression in protein synthesis was observed in the parasite. At twelve to fourteen hours after administration of the drug to mice infected with *P. berghei*, the critical alterations appeared in the parasites and at 20-24 hours after treatment, there was marked impairment of development of the inner structures of most of the trophozoites (Qinghaosu Antimalaria Co-ordinating Research Group, 1979; Klayman, 1985). Some studies indicate that qinghaosu appears unable to induce clumping *in vitro*. In addition, the drug, apparently, inhibits chloroquine-induced pigment clumping (Klayman, 1985; Peters *et al*, 1986). Gu *et al* (1983) focussed their investigations on protein synthesis in *P. falciparum* as a target of qinghaosu. They employed $[^3H]$ isoleucine as an index of protein synthesis. Their results indicated that even low concentrations of the drug or qinghaosu-like compounds inhibit protein synthesis after 1 hour incubation. Li and co-workers (1983) demonstrated that although the action of qinghaosu against *P. falciparum in vitro* using radiolabelled hypoxanthine was relatively rapid, dihydroquinghaosu and artemether were more active than quinghaosu. Other studies showed that P-aminobenzoic acid (PABA)

did not antagonise the antimalarial activity of qinghaosu (Qinghaosu Antimalaria Co-ordinating Research Group, 1979; Klayman, 1985).

1.8.5.2 The mechanism of resistance

Although the mode of action of qinghaosu in plasmodia is not precisely understood, it has been extensively investigated. The mechanism of qinghaosu-resistance however has received less attention. This promising antimalarial has encountered frequent recrudescences of malaria (Qinghaosu Antimalaria Co-ordinating Research Group, 1979; Li *et al*, 1984; Myint & Shwe, 1986, 1987; Naing *et al*, 1988). While the reasons for recrudescences have not yet been precisely determined the short half life of qinghaosu or incorrect dosing of the drug have been suggested as possible reasons (Qinghaosu Antimalarial Co-ordinating Research Group, 1979; Myint & Shwe, 1986). According to QACRG (1979) with the use of oil suspension or water suspension of the drug, the recrudescence rate might be reduced. Chawira and co-workers (1986) have postulated a number of mechanisms for the mechanism of resistance to qinghaosu. According to their suggestion, changes in membrane composition or in lipid composition may confer resistance to the drug. However, further studies would be required to verify that, although at present resistance to qinghaosu is not really a problem.

1.9 Aims of the present study

In view of the importance of control and treatment of malaria, particularly in tropical and subtropical areas, intensive studies in the field of chemotherapy and drug resistance are essential. We have attempted to improve our understanding of resistance mechanisms and the mode of action of selected antimalarials with the following principal aims:

1. Interactions between standard antimalarial drugs and a number of non-antimalarial compounds, in particular, calcium channel blockers (eg. Verapamil), calmodulin antagonists (eg. chlorpromazine), and serotonin inhibitors (eg. fluoxetine) on *P. falciparum* in vitro, in order to further investigate the role of drug transport on *P. falciparum* resistance as implicated by other workers.

2. Induction of halofantrine-resistance in *P. falciparum* in vitro. In this category two methods of selection of halofantrine-resistant strains of *P. falciparum* have been compared, intermittent versus continuous exposure to the drug. Moreover, cross-resistance patterns were compared between HF-resistant strains and parent strains. Halofantrine (HF) was selected as it is a new antimalarial with clinical promise. However, the main concern of the study was to determine the ease with which laboratory resistance could be acquired using two different approaches.

3. Interactions between halofantrine and verapamil, fluoxetine and penfluidol on HF-resistant and -sensitive strains have been evaluated to determine if the laboratory derived HF resistance shared common characteristics with chloroquine resistance in *P. falciparum*.

4. Finally, we have studied the uptake of [14-C] halofantrine by HF-resistant strains of *P. falciparum* to confirm a role for drug transport in HF resistance.

Furthermore, cultivation of asexual stages of *P. falciparum* and semi-automated microdilution technique with some improvements are described in detail.

CHAPTER 2 : MATERIALS AND METHODS

CHAPTER 2 Page

Materials and methods

2.1 *In vitro* asexual culture techniques

2.1.1 Introduction

The establishment of a continuous culture for malaria parasites was achieved in 1976 (Trager & Jensen; Haynes *et al*). Of the four species of human malaria parasites, only *P. falciparum* can be maintained in culture for long periods of time. This technique provided a wonderful opportunity for investigations into the biochemistry, immunology and molecular biology of *P. falciparum*.

The current technique of continuous culture was made via a modified "flow vial" method [Harvard medium (Geiman *et al*, 1946) was flowing slowly over a settled layer of *P. falciparum*-infected cells] (Trager, 1971) with RPMI 1640 medium supplemented with HEPES buffer and 10% human serum. In 1977, the foregoing technique was simplified and adapted for use with a candle-jar (Jensen & Trager, 1977). At present, this technique with minor modifications is widely employed for the maintenance of *P. falciparum* in vitro. For further information see Trager & Jensen, 1978; Trager, 1982, 1987. In this study we have employed the technique of Jensen & Trager (1977) with some modifications.

2.1.2 The culture of *Plasmodium falciparum*

2.1.2.1 Aseptic technique

The work relating to the culture of *P. falciparum* and the sensitivity tests were accomplished under strict aseptic conditions. Sterile operations were conducted under a microbiological safety cabinet (Envair, UK Limited); all disposable plasticware was prepared pre-sterilised. Glassware and some of the solutions were autoclaved at 120^{o}C for 20 minutes at 30p.s.i. Solutions were sterilised through a 0.2μm filter (Acrocap and Acrodisc, Gelman Sciences). All

waste liquid was discharged into a beaker in the hood. During the sterile work hands were treated with a 70% ethanol solution in water.

2.1.2.2 Medium preparation

The RPMI 1640 stock solution was prepared in a 1 litre volume by dissolving 10.4gm of RPMI 1640 powdered medium (Gibco Laboratories; Sigma), 0.2% (w/v) sodium bicarbonate (BDH; Sigma) and 50mg hypoxanthine (Sigma) (Ifediba & Vanderberg, 1981) to glass-distilled water. The solution was allowed to dissolve for at least six hours on a magnetic stirrer and then sterilised by pumping (Millipore, model 7015.72) the solution through a 0.2μm Acrocap (Gelman Sciences) filter. The filtered stock solution was stored in 500ml aliquots at 4^{o}C in tightly capped bottles for not more than four weeks.

2.1.2.3 Serum and red blood cells

The serum for these studies was obtained from the Blood Transfusion Centre, Liverpool. Fresh frozen human AB blood serum was provided in 100-200ml aliquots. Prior to use, the samples of sera were thawed at 37^{o}C in a water bath, pooled and divided into 10-20ml aliquots in sterile plastic universal tubes (Sterilin) and stored at -20^{o}C. Frozen serum was rethawed and used for the preparation of complete culture medium.

Human O^{+} blood was obtained in citrate phosphate dextrose-adenine from the Blood Transfusion Centre, Liverpool. This blood was sterile and assumed to be free from either antimalarial drugs or anti-malaria parasite antibodies. The blood was, under aseptic conditions, aliquoted into autoclaved glass bottles and stored at 4^{o}C for 4 weeks.

The phosphate buffered saline (PBS) was made by dissolving 8.5gm sodium chloride, NaCl (BDH; Sigma), 1.07gm disodium hydrogen phosphate, Na_2HPO4 (BDH) and 0.39gm sodium dihydrogen orthophosphate, NaH_2PO42H_2O (BDH) into one litre of distilled water, pH 7.1 (Osisanya *et al*, 1981). The PBS was sterilised through a 0.2μm Acrocap filter and stored in 500ml aliquots at 4^{o}C.

The red blood cells were prepared for culture by spinning of whole blood at 5000rpm for 10 minutes in a Minor centrifuge (MSE), the buffy coat was discarded and the packed cells were washed twice in PBS for 10 minutes and once in complete medium. After last wash the supernatant was removed and the packed red blood cells were suspended in an equal volume of complete medium, giving a 50% haematocrit. The washed erythrocytes were stored at 4^{o}C for up to one week.

2.1.2.4 Culture maintenance

Complete culture medium was made up by adding to the stock solution of RPMI 1640, 25mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Gibco; Sigma), 10% human AB type serum and 10μg/ml gentamicin (Gibco) (as a precaution against bacterial contamination). Complete medium was stored at 4^{o}C for not more than a week.

Cultures were maintained in 50 and 200ml flasks (Nunc, Denmark). 50ml flasks were used most often. Each 50ml flask contained about 0.5ml of infected erythrocytes and 9.5ml of complete medium (5% haematocrit). The haematocrit used with the 200ml flasks varied between 5-6.5%. Medium from the cultures was changed daily. The old medium was discarded by use of sterilised and cotton plugged glass pasteur pipette (Bilbate) without disturbing the settled

mono layer of cells and the volume of medium removed was replaced with pre-warmed fresh medium. Parasitaemia was monitored daily using Giemsa-stained thin blood smears as follows: a drop of infected erythrocyte suspension was spread on a glass slide and allowed to dry. The slide was fixed with absolute methanol and stained with a 10% solution of Giemsa stain in buffered distilled water (pH 7.2) for 20 minutes. Dried blood slides were examined with a light microscope at 100x magnification under oil immersion.

The cultures were usually diluted to a parasitaemia of 1% every 2-3 days, or when the parasitaemia exceeded about 8%. The procedure of subculture was as follows: for routine cultures, 0.5ml washed red blood cells were added to a new labelled flask and a few drops of the old culture (as seed) was dropped into the new flask. Fresh medium was then added until the desired haematocrit was achieved. Subsequently, new cultures were flushed with a gas mixture consisting of 3% O_2, 4% CO_2 and 93% N_2 (BOC, special gases). All cultures were regassed daily after changing the medium. Flasks were gassed via a silicone rubber tubing fitted with a 0.2μm Acro 50A filter and connected to the cylinder of gas. This line was terminated by a second 0.2μm Acrodisc sterile 19g needle (Terumo). A new sterile needle was fitted to the line when doing each individual strain. The Acrodisc filter was changed once a week. 50ml flasks were gassed for 30-45 seconds and 200ml flasks for 1 minute.

2.1.2.5 Decontamination of the cultures

During the present studies, some cultures were occasionally contaminated with noxious organisms (bacteria, yeasts and filamentous fungi). For routine cultures, 10μg/ml gentamicin was included in the complete medium.

When such cultures became contaminated, the bacterial contamination was eliminated with a mixture of 100 units/ml benzylpenicillin sodium, 1,000,000 units (Glaxo) and 100mg/ml streptomycin sulphate, 1g (Evans). The fungal contamination was eradicated by 50 units/ml mycostatin (Nystatin), 500,000 units (Squibb). The drugs were added (in addition to 10μg/ml gentamicin) to the contaminated cultures until the noxious organism had disappeared, then treatment was continued for a further three days.

2.1.2.5.1 Antibiotic resistance during decontamination

During the elimination of bacterial contamination from the cultures, three cases of antibiotic-resistance have been observed as follows: one parasite line (K_1HF_4strain) was contaminated by low levels of bacteria, about two bacteria per twenty erythrocytes on a giemsa stained slide. This culture was, as usual, treated with 100 units/ml benzylpenicillin plus 100μg/ml streptomycin. Twenty-four hours after treatment the number of bacteria decreased to about one bacterium per 150 red blood cells, despite prolonged treatment for over 48 hours, the amount of bacteria increased to 10 bacteria per 100 erythrocytes. Although this culture was exposed to a mixture of 200 units/ml benzypenicillin plus 200μg/ml streptomycin for 24 hours, the bacteria persisted in the culture. Eventually the contamination was eradicated using 300 units/ml benzylpenicillin plus 300μg/ml streptomycin over 48 hours. While at the beginning of treatment parasitaemia was 1.55%, it dropped throughout the treatment to about 0.44% and then, 24 hours after removing the antibiotics, increased up to 0.80%. In another contaminated culture affected by bacteria, the contamination persisted even against 300 units/ml benzylpenicillin and 300μg/ml streptomycin. Increased drug

concentrations killed both the bacteria and parasites. In the third case of contaminated cultures, when treatment with 300 units/ml benzylpenicillin plus 300μg/ml streptomycin failed to eliminate the bacterial contamination, chloramphenicol (CAM) was successfully employed for decontamination (Yayon *et al*, 1984a). According to Vazquez (1974) and Pongs (1979) chloramphenicol inhibits protein synthesis in prokaryotes at <3mg% and at high concentration produces deleterious effects in eukaryotic organelles such as mitochondria and chloroplasts. The procedure of decontamination was as follows: the medium was discarded and the culture washed twice in gentamicin-free medium for 5 minutes. After the final wash, cells were recultured with gentamicin-free medium containing 0.1mg/ml chloramphenicol (Sigma) incubated at 37°C for four hours, then the medium was decanted and replaced with fresh gentamicin-free medium containing 0.1mg/ml chloramphenicol. The culture was incubated at 37°C for an additional 24 hours. The last process was repeated and after washing the culture, it was resuspended in the normal growth medium.

2.1.2.6 Synchronisation of cultures

Two procedures were employed for synchronising parasite growth. In the first method, sorbitol was used which caused lysis of all stages except ring stage. This method was carried out as described by Lambros & Vanderberg (1979), with minor modifications. The method was as follows: the culture was centrifuged at 2000rpm in a Minor centrifuge (MSE) for 5 minutes, the supernatant was discarded and the pellet suspended in five volumes of 5% sorbitol in PBS (W/V) pre-filtered through a 0.2μm Acrodisc. The suspension was incubated at 37°C for 10 minutes. After the incubation period and

centrifugation as above, the supernatant was discarded and the pellet washed once with complete culture medium and then returned to normal culture conditions. The second method of synchronisation was based on that described by Inselburg (1984) using aphidicolin as a DNA synthesis inhibitor. In this procedure late trophozoites and schizonts were separated from ring stage parasites using a Percoll cushion (see 2.1.2.7). Trophozoites and schizonts were introduced into culture and incubated for 30 hours with one change of medium after 24 hours. After this incubation period 1-1.5μg/ml aphidicolin (Sigma) was added to the culture and incubated for an additional 15 hours. The culture was then washed once in complete medium free of aphidicolin and reintroduced into normal culture with fresh medium. The culture was allowed to proceed normally and synchronously.

2.1.2.7 Fractionation of parasites using Percoll

The different stages of *P. falciparum* were separated from each other by using a Percoll cushion. The method was performed as described by Kutner *et al* (1985). 90% sterile percoll in ten times concentrated RPMI 1640 solution was diluted to 80%, 70% and 60% in serum free complete medium. Four ml of the 90% dilution was allowed to settle in a 15ml sterile centrifuge tube (Sarstedt) and overlaid successively with 3ml 80%, 2ml 70% and 2ml of 60% dilutions. Two ml of infected erythrocytes (25% haematocrit) in the complete medium with a 10-15% parasitaemia was gently applied over the top layer. The tube containing the gradient was centrifuged at 10,000rpm (Minor centrifuge, MSE) for 20 minutes at room temperature. The top zone contained percoll and culture medium, the next layers contained schizonts, trophozoites, mature rings

and young rings plus uninfected cells, respectively.

2.1.2.8 Cryopreservation of parasites

The procedure used in these studies was as described by Wilson and co-workers (1977). Twenty percent dimethyl sulfoxide (DMSO), as a cryoprotectant, was prepared in PBS (w/v), filter-sterilised and stored at 4°C. The parasite culture with >8% parasitaemia (ring stages) was centrifuged at 500g for 5 minutes and the supernatant decanted. The pellet was then suspended in an equal volume of complete medium and two volumes of ice-cold 20% DMSO. The mixture was dispensed into sterile cryotubes (Nunc, 1.8ml), which were then plunged into liquid nitrogen. For recovery two methods were employed; in the first procedure, the cryotubes containing frozen parasites were thawed for 1-2 minutes at 37°C and centrifuged at 500g for 2 minutes. The supernatant was discarded and the pellet suspended gently into 1ml of a 10% sterile sorbitol solution which had been previously prepared in PBS. The suspensions were recentrifuged as described above and the supernatant removed. The procedure was repeated with a 5% sorbitol solution followed by a single wash in complete medium. The pellet was finally placed in fresh culture medium. In the second method (Rowe *et al*, 1968; with some modifications), 3.5% NaCl in glass distilled water (w/v) was used instead of sorbitol. The procedure was as follows: the contents of a frozen cryotube were thawed at 37°C for 1-2 minutes and centrifuged at 500g for 2 minutes. The supernatant was removed and 1ml of 3.5% NaCl solution added to the pellet. The cells were then centrifuged as before and washed once in complete medium. Finally, the pellet was introduced into fresh complete medium and cultured as usual.

2.1.2.9 The laboratory strains of *P. falciparum*

Several strains of *P. falciparum* which has been adapted to culture in other laboratories were employed throughout these studies. The strains FCR_3, K_1, $T_{9.96}$, W2-mef and CH150-R_4 were maintained appropriately for this study. Details of the strains can be found in Table 2.1.2.9. In addition, two strains K_1HF and $T_{9.96}$HF were adapted in this study (see Chapter 5).

2.1.2.10 Problems in culture maintenance

Although cultures were performed under aseptic conditions and the laminar flow cabinet was completely sterilised at least once a month, cultures occasionally became contaminated with various micro-organisms. It cannot be attributed to a single cause, but it seems the most frequent cause of contamination was connected with screwing the top onto bottles and flasks. However, occasional contamination is not avoidable. During these studies a number of cultures clotted after receiving new batches of either serum or blood. Moreover, it has been found that using RPMI 1640 from different sources resulted in a failure to support the growth of parasites.

2.2 *In vitro* drug sensitivity testing

2.2.1 Introduction

The development of *in vitro* techniques for the culture of *P. falciparum* provided a wonderful opportunity for quantitative studies around this fatal parasite. Assessment of antimalarial activity against *P. falciparum* using *in vitro* techniques has undergone various modifications to date. The first drug sensitivity test was reported by Rieckmann and co-workers (1968). The method

Table 2.1.2.9 The strains of *P. falciparum* which were employed during these studies

Strain[+]	FCR3
Origin and isolation of strain	It was isolated from a Gambian patient
Drug sensitivity of strain	The strain was initially sensitive to CQ*, one line of this strain was experimentally made resistant to CQ (Nguyen-Dinh & Trager, 1978). In addition, another CQ-sensitive line of the strain became CQ-resistant as a result of nearly 4 years of continuous culture without chloroquine pressure (Jensen *et al*, 1981).
Strain	K_1
Origin and isolation of strain	Isolated in Kanchanaburi, Thailand by Thaithong & Beale (1981).
Drug sensitivity of strain	Resistant to chloroquine and pyrimethamine (Thaithong & Beale, 1981).
Strain	$T_{9.96}$
Origin and isolation of strain	The strain was cloned from the T_9 strain which was isolated in Thailand by Rosario (1981).
Drug sensitivity of strain	Sensitive to chloroquine and pyrimethamine (Thaithong *et al*, 1984).
Strain	W_2-mef

Origin and isolation of strain	W_2 was cloned from the chloroquine-resistant Indochina III/CDC strain by Oduola *et al* (1988a). One line of W_2 strain was experimentally made resistant to mefloquine and termed W_2-mef (Oduola *et al*, 1988b).
Drug sensitivity of strain	Resistant to chloroquine, quinine and mefloquine (Oduola *et al*, 1988b).
Strain	CH150-R4
Origin and isolation of strain	The strain was cloned from CH150-D21 strain which was isolated in the border of Thailand and Kampuchea by Webster *et al* (1985).
Drug sensitivity of strain	Resistant to chloroquine and mefloquine (Webster *et al*, 1985).
Strain	K_1HF
Origin and isolation of strain	K_1, CQ-resistant strain, was experimentally made resistant to halofantrine by Nateghpour *et al* (in press).
Drug sensitivity of strain	Resistant to halofantrine, quinine, pyrimethamine and mefloquine. For further information see Chapter 5.
Strain	$T_{9.96}$HF
Origin and isolation of strain	The strain was derived from chloroquine-sensitive $T_{9.96}$ strain (Nateghpour *et al*, in press).
Drug sensitivity of strain	Resistant to halofantrine and mefloquine.

+ In this study both cloned and uncloned parasites are termed as strains
*** CQ : chloroquine**

was simple and based on collecting small samples of venous blood from infected patients which were maintained in screw-cap, flat-bottomed glass vials containing 5mg glucose. Some of the vials were treated with various amounts of drug and some other left untreated as controls. In 1973, Richards & Williams reported the advantages of *in vitro* studies as follows:

1. Drug activities may be assessed more quickly and cheaply *in vitro*.
2. The concentrations of drug attainable *in vitro* are often much higher than those possible for plasma levels *in vivo* which enables less active compounds to be quantitatively assessed. This is of importance in drug-design studies.
3. The complications of metabolism, excretion and toxicity of the drug are largely absent which allows the intrinsic antimalarial activities of novel compounds to be determined.
4. Very small quantities of compound are required which is particularly valuable when, for example, only limited amounts of a drug or its metabolite are available.

Rieckmann and co-workers introduced a microtechnique for sensitivity testing in 1978, which has been extensively employed in the field. They used the flat-bottomed wells of microtitre plates which previously treated with various quantities of chloroquine diphosphate (except for controls). Each well received 50μl of culture medium containing RPMI 1640 supplemented with 2mg/ml sodium bicarbonate, 6mg/ml HEPES buffer and 4μg/ml gentamicin sulphate. Furthermore, a 5μl of parasitised blood was dispensed to each well. The plates were covered with lids, stirred and gassed inside a glass desiccator using the

candle-jar method. The desiccator was incubated at 38-39°C for 24-30 hours. After the incubation period, thick blood films were prepared, stained and schizonts per 500 white blood cells were counted. In 1980, Nguyen-Dinh & Trager developed a modified 48 hour sensitivity test. In this method 500μl of either normal medium or drug treated medium were dispensed to each flat-bottomed 16mm well. Twenty microlitres of infected erythrocytes then were added to the wells so that the final parasitaemia reached 2%. The plates were incubated at 37°C for 48 hours with replacement of the gas phase using the candle-jar method after 24 hours. Yisunsri and Rieckmann (1980) also described an *in vitro* microtechnique for sensitivity testing of *P. falciparum* which used a similar process to Nguyen-Dinh & Trager's method. In 1982, a visual *in vitro* sensitivity test was employed by Rieckmann, who showed that by adding a mixture of sodium hydroxide and sodium chloride to cultures, at the end of the incubation period, there will be observed a dark precipitate in the non-drug treated wells. It was suggested that these dark deposits are produced in virtue of complexation between the alkali and the malaria pigment. Therefore, in the presence of effective quantities of antimalarial drugs because rings cannot mature to schizonts the precipitates are not observed. Other *in vitro* drug sensitivity test methods have been described by several investigators (Richards & Maples, 1979; Brockelman & Tan-ariya, 1982b). The early studies on the incorporation of nucleic acid precursor into DNA and RNA (Polet & Barr, 1968b; Gutteridge & Trigg, 1970), the incorporation of fatty acids into lipids (Rock 1971) and the incorporation of amino acids into protein (Canfield *et al*, 1970; Butcher & Cohen, 1971) led several investigators to employ these biochemical criteria for assessment

of parasite activity by using radiolabelled materials. Radiolabelled leucine and isoleucine have been used by a number of authors in antimalarial drug sensitivity tests (Richards & Williams, 1973; Iber *et al*, 1975; Gu *et al*, 1983; Gershon & Howells, 1984). At present, the use of 3H-hypoxanthine as a marker of drug effect is applied for most *in vitro* antimalarial drug assays. In these studies the majority of drug assays have been performed on the basis of semi-automated microdilution techniques (Desjardins *et al*, 1979b) with some modifications. Moreover, the 48 hour drug sensitivity test in *P. falciparum* (Nguyen-Dinh & Payne, 1980) with some variations was employed for a number of experiments. In Table 2.2.1 the more important *in vitro* drug sensitivity assays in *P. falciparum* have been compared.

2.2.2 Drug stock solution

The drugs employed in this study were amodiaquine, chloroquine, halofantrine, mefloquine, mepacrine, pyrimethamine, quinine, quinidine, qinghaosu, chlorpromazine, fluoxetine, penfluridol, praziquantel and verapamil. All the drugs were obtained in powder form. Stock solutions were prepared in 70% ethanol (glass distilled water) except for pyrimethamine. The drugs were dissolved in the solvent to a concentration of 10^{-2}M. Pyrimethamine was dissolved as a 10^{-2}M concentration in 5% (V/V) mixture of dimethyl sulfoxide (DMSO) and ethanol, sterilised through a 0.2μm acrodisc. For the sensitivity tests all the drugs were diluted with complete culture medium to achieve the appropriate concentrations. It was observed that storage of the stock solutions at 4^{o}C for long periods resulted in an increase in the concentrations, presumably caused by evaporation of ethanol. Therefore, the stock solutions were kept at

Table 2.2.1 Comparison between different *in vitro* drug sensitivity assays for *P. falciparum*

Type of assay	Investigators	Assay vessel	Incubation period	Criteria of drug effect
A simple *in vitro* method	Rieckmann *et al* (1968)	Flat-bottomed glass vials	24hr	Inhibition of maturation of rings to schizonts
An *in vitro* micro-technique	Rieckmann *et al* (1978)	96 well plate	24hr	Inhibition of maturation of rings to schizonts
A modified 48-hour test	Nguyen-Dinh & Trager (1980)	24 well plate	48hr	Reduction of parasitaemia in the drug treated wells
Visual *in vitro* micro-technique	Rieckmann (1982)	96 well plate	24-32hr	Observation of the dark precipitates in the test control wells and lack of them in the drug treated wells
Semi-automated technique	Desjardins *et al* (1979)	96 well plate	48hr	The incorporation of 3H-hypoxanthine into parasites' nucleic acid

Table 2.2.2 The source, stock solution and solvent of either drugs or compounds used in this study

Drug/compound	Source	Stock solution	Solvent
Amodiaquine	Ch. Lab.[a]	10^{-2}M	70% ethanol
Chloroquine diphospate	Sigma[b]	"	"
Halofantrine hydrochloride	S K & F[c]	"	"
Mefloquine hydrochloride	Ch. Lab.	"	"
Mepacrine hydrochloride	Sigma	"	"
Pyrimethamine	Ch. Lab.	"	50% DMSO in ethanol (v/v)
Quinine hydrochloride	Sigma	"	70% ethanol
Quinidine hydrochloride	Ch. Lab.	"	"
Qinghaosu	Ch. Lab.	"	"
Chlorpramazine hydrochloride	Sigma	"	"
Fluoxetine hydrochloride	E.L.C.[d]	"	"
Penfluridol	J.R.F.[e]	"	"
Praziquantel	Bayer	"	"
Verapamil hydrochloride	Sigma	"	"

a The Malaria Chemotherapy Laboratory of the Liverpool School of Tropical Medicine

b Sigma Chemical Company

c Smith Kline & French Laboratories Ltd

d Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana

e Janssen Research Foundation, Belgium

-20°C except for halofantrine and qinghaosu which were precipitated by low temperature. The stock solutions of the latter drugs were stored at 4°C and renewed every two to three weeks. All the stock solutions were agitated for fifteen minutes by ultrasonicator (Decon FS100) immediately after preparation and prior to use. The source of drugs is described in Table 2.2.2

2.2.3 Assessment techniques

Assays were accomplished on the basis of two methods:

2.2.3.1 Incorporation of [3H] hypoxanthine

This method was performed using microtitre plates consisting of 96 flat-bottomed wells (Nunclon, Nunc). The stock solution (10^{-2}M) of the desired drug was diluted in the complete culture medium to give 10^{-4}M or 10^{-5}M concentrations. The successive dilutions were expediently made from the latter concentrations. The dilutions of drug were arranged in triplicate wells starting from lowest towards highest. Two-fold triplicate control wells were set up near the centre of the plate. In addition, three wells on the top of the plate were used as a background containing non-infected erythrocytes. One hundred microlitres of drug free medium was dispensed to each of the control and background wells by means of a pipetman pipette (Gilson) and 100μl aliquots of drug treated medium was placed in the remaining wells. Each test well received 10μl of a 50% infected erythrocyte suspension producing a 4.5% haematocrit at 1% parasitaemia (mostly ring stages). The background wells received 10μl of a 50% uninfected red blood cells suspension per well. After preparation as above, the plates were covered, shaken gently, then placed in a modular incubator chamber (Billups-Rothenberg) and flushed with a gas mixture of 3% O_2, 4% CO_2 and 93% N_2, the

gas chamber then was incubated at 37°C for 24 hours. After the first incubation period, 0.5μci of [G-3H] hypoxanthine (Amersham) was added to each well. The preparation of [G-3H] hypoxanthine was as follows: the content of a single ampoule containing 1.0mci of the isotope was dissolved in 2ml of 50% ethanol in sterile glass distilled water under aseptic conditions. The prepared stock solution was kept at -20°C and just before use, a 0.2ml sample was withdrawn from the stock solution and placed in a sterile bijou (Sterilin). The ethanol was then evaporated and the remaining material diluted ten times in complete culture medium. Subsequently, 5μl aliquots of the diluted isotope containing 0.5μci were dispensed to the wells. The radiolabelled plates were then briefly shaken and returned in the modular incubator chamber. The chamber was regassed and incubated at 37°C for an additional 20-22 hours. After the second incubation period, the contents of the wells were collected onto a glass fibre mat (Skatron Ltd) using a Titertek cell harvester (Skatron, AG). The cell harvesting was as follows: the plates were removed from the chamber and agitated gently. The cells were sucked up by means of a vacuum tube connected to the cell harvester and lysed with a rinse of distilled water for 15-20 seconds. The same process was employed to suck air to dry the mat, which finally was left to dry entirely at room temperature within a few hours. After drying individual discs were placed into scintillation vials (LIP Ltd), 4ml aliquots of optiphase 'safe' scintillation fluid (LKB) was added to the vials and prepared for scintillation counting. Radioactivity was determined by means of a scintillation counter (LKB, 1219 Rack Beta) linked to an Olivetti personal computer M24 which was programmed to calculate DPMs (disintegration per minute). The calculation and percentage

of DPMs were carried out as follows: DPMs were calculated from CPMs (counts per minute) of each tube. The mean value in DPMs for the control tubes was determined, then this process was followed on for each set of triplicate drug treated or background tubes. Subsequently, the mean value of the background tubes was subtracted from the mean value of either the control tubes or each set of the drug treated tubes. Finally, the results were expressed as a percent of the control. All of the foregoing calculations were conducted by the computer which was programmed by Mr Pat Bray. The IC_{50} (50% inhibitory concentration) and IC_{90} values were determined visually on the basis of the graph of the log drug concentration against incorporation of radiolabelled hypoxanthine by parasites (as a percentage of control value). In 72 hour incubations the plates were regassed every 24 hours, and either drug treated or drug free medium replaced after 48 hours. The radiolabelled hypoxanthine was also dispensed to the wells after 48 hours. The plates were regassed and incubated for an additional 20-22 hours. The cell harvesting, radioactivity determinations and the calculation of IC_{50} values was the same as in the 48 hour assay.

2.2.3.2 Microscopic examination method

In this method, assays were also performed in 96-well microtitre plates. Two wells were set up for either the control or individual concentration of drug. 133μl aliquots of normal culture medium was added to the control wells, and then the same volume of drug treated medium was inoculated into the test wells. Seven microlitres of parasitised erythrocytes at a 1% parasitaemia was then added to each of the wells. The final erythrocyte suspension was 5%. The plates were slightly agitated and placed in a modular incubation chamber (Billups-

Rothenberg). This chamber was gassed as before (see 2.2.3.1) and incubated at 37°C for 24 hours. After the 24 hour incubation period the plates were briefly shaken, regassed and incubated for an additional 20-22 hours. After the last incubation period, a thin blood film was prepared from each well, stained with Giemsa stain (see 2.1.2.4), and examined under the light microscope. The parasitaemia was determined per 3000 erythrocytes, and expressed as a percentage of the control. The IC_{50} values in this method were determined graphically as described earlier.

In preliminary experiments performed via either radiolabelled or microscopic examination methods, it was found that the growth of parasites in the wells situated at the edges of the plate was significantly poorer than others. Therefore, the wells in the outermost columns were left empty but the problem remained unsolved. The problem was overcome when the outermost columns of wells were filled up with complete culture medium. This adaptation was employed in all subsequent experiments.

2.3 Uptake of [14C] halofantrine by *P. falciparum*-infected and uninfected erythrocytes

2.3.1 Parasite cultivation

Cultivation of parasites was conducted as described in Chapter 2.1.2. Parasites synchronised by sorbitol lysis (see Chapter 2.1.2.6) were used at the trophozoite or early schizont stages. Cultures with high parasitaemia but mixed stages were fractionated by using Percoll (see Chapter 2.1.2.7), then the trophozoite stages were separated for immediate experimentation. Parasitaemias varied from 8 to 12%.

2.3.2 Radiolabelled halofantrine

[14C] halofantrine (25.82μCi/mg; SK & French Lab. UK) dissolved in absolute methanol. A 50μl aliquot was removed and the methanol was evaporated by flushing with a gas mixture of 3% O_2, 4% CO_2 and 93% N_2 (BOC, special gases) and 15μl ethanol added to a one μci residual halofantrine. The solution was sonicated for 10 minutes with an ultrasonicator (Decon FS100) and diluted with 10ml of complete culture medium. The final amount of [14C] halofantrine was 0.1μCi per millilitre in the dilution.

2.3.3 Drug stock solution

The non-radioactive halofantrine and penfluridol were prepared as described in Chapter 2.2.2. The concentrations used correspond to the IC_{50} values for halofantrine against the K_1 (2.2nmol/l) and $T_{9.96}$ (6.6nmol/l) strains, respectively and for penfluridol against the $T_{9.96}$ (493.3nmol/l) strain.

2.3.4 Sample preparation

Cultures mostly at the trophozoite stages (more than 80%) were selected for experiments. The parasitised erythrocyte suspensions were centrifuged at 5000rpm for 5 minutes in a Minor centrifuge (MSE), the supernatant was discarded and the packed erythrocytes were diluted to give 2 x 10^5 cells per 0.1 millilitre in complete culture medium. The red cell counting methods have been described in detail by Baker & Silverton (1976), in brief: a haemocytometer including a counting chamber, a coverglass, pipettes for diluting blood and a rubber tube with a plastic mouthpiece used for such. Diluting fluids also are required for diluting red blood cells to the desired concentrations. In this study complete culture medium was used as a diluting fluid. Some erythrocytes were drawn from the sample into a red cell pipette up to the level of 0.5 which

was marked on the pipette. The outside of the pipette was wiped off with a piece of clean gauze and complete culture medium drawn up to the 101 mark. The pipette was withdrawn from the medium and again wiped off the outside of the pipette. The pipette was rotating during the processes. The suspension was mixed thoroughly for 3-4 minutes and about a quarter of the mixture taken off. Some of the remaining suspension was inserted between the cleaned and matched counting chamber and coverglass by holding the pipette at an angle of 45 degrees. The fluid was not allowed to overflow into the channels (Fig. 2.3.4.1). The chamber was examined under a light microscope using a x40 objective and x10 eyepieces. The central square millimetre (containing 400 small squares) of the counting chamber was used for counting, and the red blood cells situated within 80 of the 400 small squares were counted. The final result after a simple mathematical calculation culminated in an equation as follows: $1mm^3$ of diluted red blood cells = N x 10,000 cells where, N = number of cells counted in 80 small squares.

A five percent haematocrit was made by suspending the infected erythrocytes in complete culture medium containing 0.1μCi/ml of [14C] halofantrine. The suspension was incubated at 37^{o}C for 10, 60 and 120 minutes. Triplicate (600μl) samples containing 300μl of dibutylphthalate (Sigma) and 300μl of the incubated parasite suspension were prepared in 1.5ml microcentrifuge tubes (Sarstedt, W. Germany) after each incubation period. The samples were centrifuged at 12000rpm for 30 seconds in a microcentrifuge (Micro Centaur, MSE) and each pellet was, after removal of supernatant and two rapid washes, kept frozen at -30^{o}C for the next process.

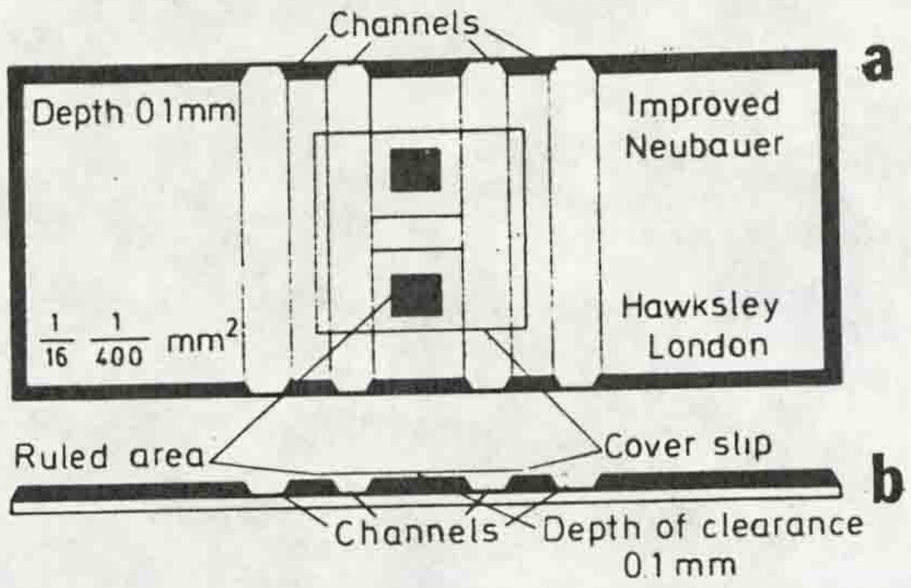
2.3.5 Determination of radioactivity

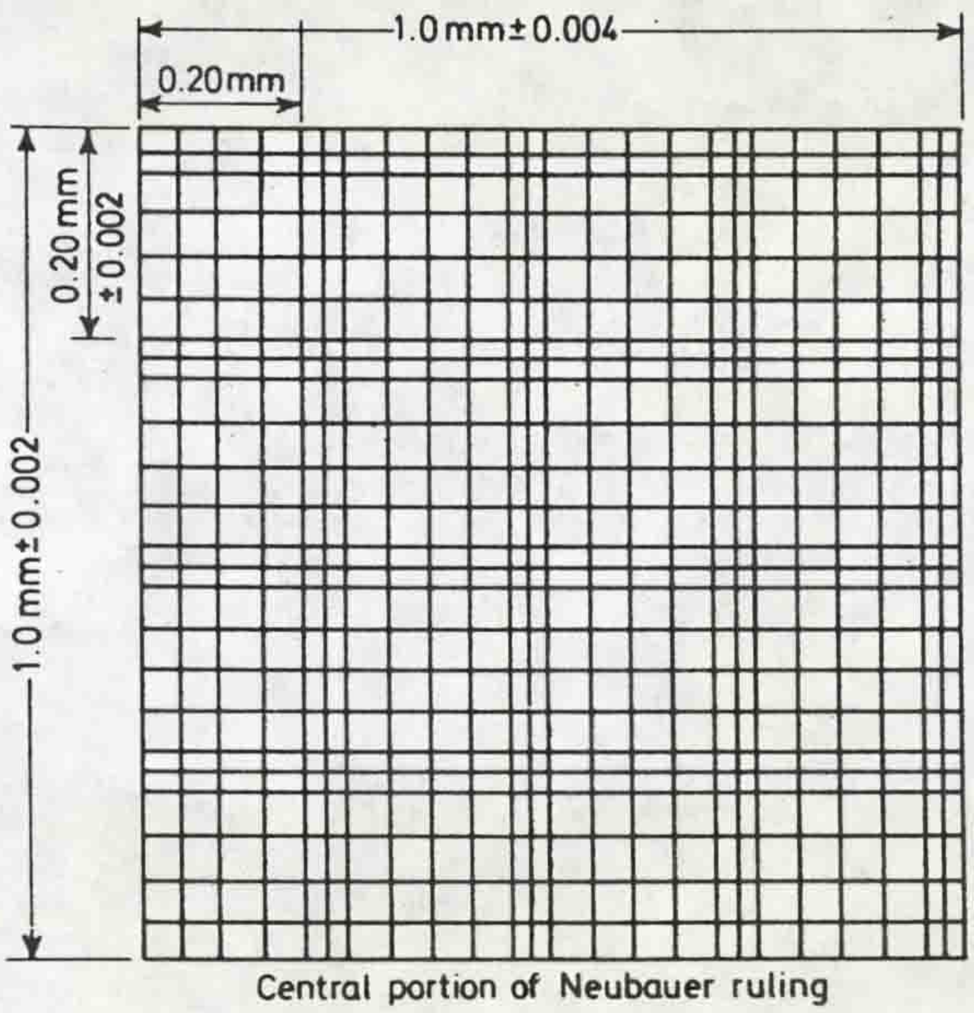
The surface of frozen tubes (containing prepared samples) was decontaminated because of presumable radioactive contamination by 5% Decon 90 (Decon Laboratories Ltd) in water. The tubes were then dried at room temperature and kept frozen for an additional 30 minutes. At the end of second freezing period the tubes were cut from the top of pellets and each pellet was put into a scintillation vial (LIP Ltd). A 15μl volume of medium of each sample was previously pipetted into separate scintillation vials. Four millilitre aliquots of scintillation fluid (Optiphase 'safe', LKB) were added to the vials containing medium and made ready for counting. 0.5ml aliquots of distilled water were pipetted into the vials containing pellets and the content of vials were agitated for 30 seconds by means of a vortex (Chiltern Scientific). Subsequently, 0.5ml of Scintran Tissue Solubiliser (BHD Chemicals Ltd) were added to each vial. The vials were shaken for an additional 30 seconds and incubated at 70^{o}C for one hour. At the end of the incubation period 4ml aliquots of scintillation fluid were added to the vials and kept for determination of radioactivity. At the same time, uninfected erythrocytes underwent the same process as stated above, as control groups.

Radioactivities were determined as mentioned in Chapter 2.2.3.1, except the programme A1 (adapted for 14C radiolabelled materials) was used instead of the programme A2 (adapted for 3H radiolabelled materials). The mean values of DPMs for each group of triplicate infected and uninfected samples were calculated, then the mean values of uninfected samples were subtracted from the mean values of relevant infected samples. The comparative results were plotted on the basis of DPM versus time course of uptake of [14C] halofantrine.

2.3.6 Combination of [14C] halofantrine with non-radioactive halofantrine and penfluridol

The experimental process was the same as that described above for [14C] halofantrine. Concentrations of 2.2 and 6.6nmol/l halofantrine alone or in combination with a concentration of 493.3nmol/l penfluridol, as well as [14C] halofantrine were simultaneously added to the infected and uninfected erythrocytes. The amount of radiolabelled halofantrine was 0.1μci/ml in all experiments.





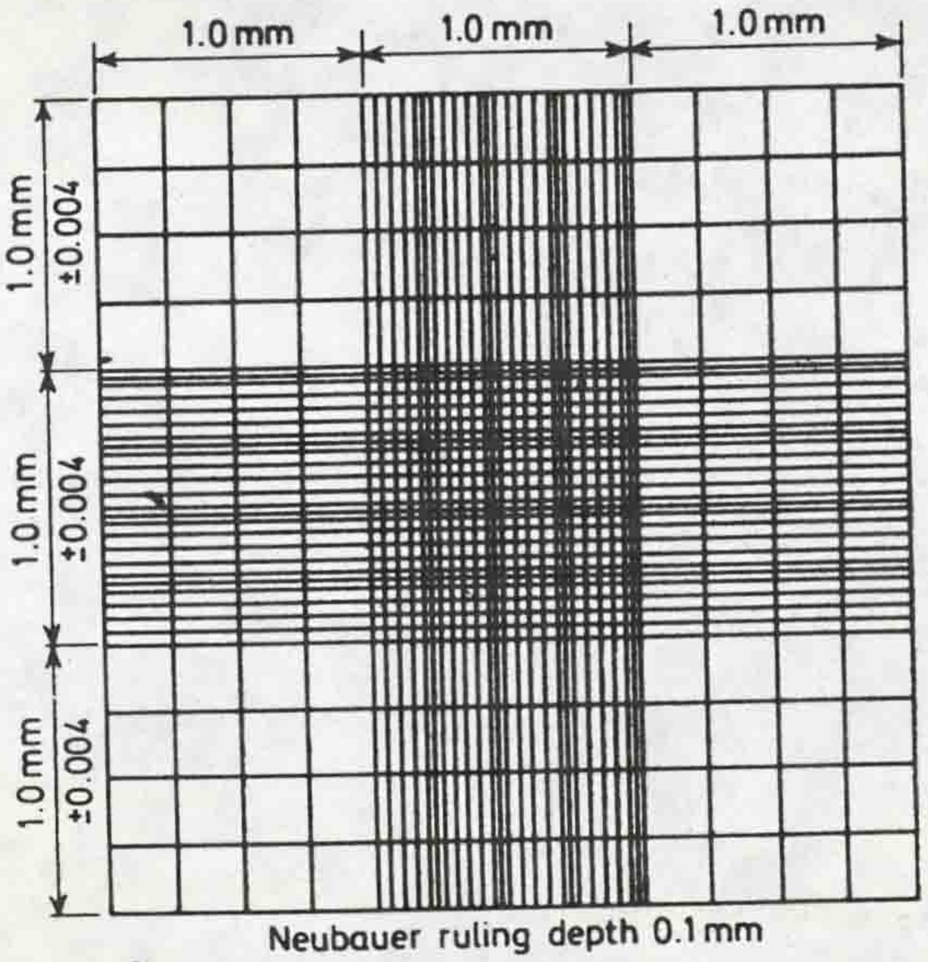


Fig. 2.3.4.1 **Counting chamber and coverglass (Baker and Silverton, 1976).**

(a) **double-sided counting chamber**
(b) **side view of chamber**
(c, d) **Neubauer ruling (coverglass)**

CHAPTER 3 : THE DETERMINATION OF BASELINE DRUG SENSITIVITIES OF *P. FALCIPARUM IN VITRO*, USING SINGLE AGENTS

CHAPTER 3 Page

The determination of baseline drug sensitivities of *P. falciparum* *in vitro*, using single agents

3.1 Introduction

Over the past two decades the problem of antimalarial drug resistance in *Plasmodium falciparum* has continued to increase. At present, new drugs are urgently needed for treatment of the malignant tertian malaria parasites which are resistant to several antimalarial drugs. Such necessities have prompted a number of authorities to evaluate various antimalarial drugs in combination with some reversing agents against *P. falciparum* both *in vivo* and *in vitro*.

In these studies we were interested to evaluate various classes of pharmacological agents with the potential of reversing chloroquine-resistance *in vitro*. However, before we could assess these compounds it was important to obtain baseline values for each drug including antimalarial drugs against the selected strains of *P. falciparum in vitro*.

3.2 Materials and methods

The antimalarial drugs were selected from aminoalcohols (quinine, quinidine, mefloquine and halofantrine), 4-aminoquinolines (chloroquine and amodiaquine) and dihydrofolate reductase inhibitors (pyrimethamine), as well as mepacrine and qinghaosu. As such the non-antimalarial compounds were selected from calcium channel blockers (verapamil) (Snyder & Reynolds, 1985), calmodulin antagonists (chlorpromazine and penfluridol) (Scheibel *et al*, 1987) and serotonin inhibitors (fluoxetine and praziquantel) (Pax *et al*, 1979 ; Harder *et al*, 1987). The sensitivity tests were conducted as described in Chapter 2.2. Comparison of [3H] hypoxanthine incorporation between K_1 and $T_{9.96}$ strains of *P. falciparum* in 48 hour assay was performed as follows; the mean values of DPMs for control wells in ten experiments as described in Chapter 2.2 for each

strain were individually determined and compared.

3.3 Results

3.3.1 A comparison of [3H] hypoxanthine incorporation between K_1 and $T_{9.96}$ strains of *P. falciparum* in the absence of drug pressure

The results of this comparative study using chloroquine-resistant (K_1) and chloroquine-sensitive ($T_{9.96}$) strains are presented in Table 3.2.1. As the results show the growth and multiplication of the K_1 strain (as determined by [3H] hypoxanthine incorporation) is significantly more rapid than the chloroquine-sensitive strain in 48 hour culture.

3.3.2 Comparison of drug susceptibility of K_1 and $T_{9.96}$ strains in 48-hour assay as measured by [3H] hypoxanthine incorporation

3.3.2.1 Antimalarial drugs

The IC_{50} and IC_{90} values of chloroquine, mefloquine, halofantrine, quinine, quinidine, mepacrine, amodiaquine, qinghaosu and pyrimethamine against K_1 and $T_{9.96}$ strains have been illustrated in Tables 3.2.2.1a to 3.2.2.1f. As anticipated, the K_1 strain was resistant to chloroquine, quinine and pyrimethamine with the IC_{50} values of 340nmol/l (SD of $\pm$ 151.2), 320nmol/l (SD of $\pm$ 132.2) and > 1000nmol/l respectively. The IC_{90} for chloroquine and quinine were 635nmol/l (SD of $\pm$ 111.2) and 780nmol/l (SD of $\pm$ 200) in this study. On the other hand, the $T_{9.96}$ strain was sensitive to chloroquine, quinine and pyrimethamine with the IC_{50} values of 27nmol/l (SD of $\pm$ 1.7), 77nmol/l (SD of $\pm$ 4.2) and 36.5nmol/l and with the IC_{90} values of 46nmol/l (SD of $\pm$ 2), > 100nmol/l and 400nmol/l, respectively. Both K_1 and $T_{9.96}$ strains were sensitive

to quinidine, mepacrine, amodiaquine, mefloquine, halofantrine and qinghaosu. Surprisingly we found that $T_{9.96}$ is slightly resistant to mefloquine and halofantrine with the IC_{50} values of 58.3nmol/l (SD of $\pm$ 4.7) for mefloquine and 6.6nmol/l (SD of $\pm$ 1.2) for halofantrine, while the IC_{50} values against K_1 were 23nmol/l (SD of $\pm$ 2.5) and 2.2nmol/l (SD of $\pm$ 0.32) respectively. The ratios of IC_{50} (K_1)/IC_{50} ($T_{9.96}$) and IC_{90} (K_1)/IC_{90} ($T_{9.96}$) are summarised in Table 3.2.2.1. The table shows that halofantrine is more effective than mefloquine and qinghaosu on K_1 and $T_{9.96}$ strains. Values of 2.36, 3.2, 1.18 and 1.6 were obtained with quinidine, mepacrine, amodiaquine and qinghaosu respectively, indicating that $T_{9.96}$ is more sensitive to these drugs than K_1. In contrast, the ratios of 0.40 and 0.33 obtained for mefloquine and halofantrine indicate that $T_{9.96}$ is less sensitive to these drugs. The susceptibility of K_1 and $T_{9.96}$ strains of *P. falciparum* to the antimalarials tested are shown graphically in Figs. 3.2.1 to 3.2.4.

3.3.2.2 Non-antimalarial drugs

The IC_{50} and IC_{90} values for verapamil, chlorpromazine, fluoxetine, praziquantel and penfluridol against K_1 and $T_{9.96}$ strains are tabulated in Tables 3.2.2.2a to 3.2.2.2d. As the IC_{50} and IC_{90} values for the compounds demonstrate these agents have a negligible effect on either the K_1 or $T_{9.96}$ strains. Among the non-antimalarial compounds, penfluridol with an IC_{50} of 370nmol/l for K_1 and 470nmol/l for $T_{9.96}$ is slightly active against both strains. The ratio of 0.78 (Table 3.2.2.1) which has been obtained with penfluridol indicates that the drug is more effective against the K_1 strain than the $T_{9.96}$ strains, whilst the ratios of 1.63, 1.14, 1.17 and 1.13 obtained for verapamil, chlorpromazine, fluoxetine and praziquantel respectively show that there is no significant difference between the effectiveness

of these drugs against either strain. Dose response curves for non-antimalarial drugs are represented graphically in Figs. 3.2.5 and 3.2.6.

3.3.3 Comparative dose response relationship against CH150-R4, FCR_3 and W2-mef strains of *P. falciparum*

3.3.3.1 Antimalarial drugs

Chloroquine, amodiaquine, mefloquine and halofantrine were employed against the FCR_3 strain. As anticipated the FCR_3 strain was resistant to chloroquine with an IC_{50} values of 182nmol/l (SD of $\pm$ 55.4), whilst the strain demonstrated a good level of sensitivity to amodiaquine, mefloquine and halofantrine with IC_{50} values of 36, 29 and 8.2nmol/l respectively. The results showed that halofantrine was more effective against FCR_3 than the two other drugs. W2-mef and CH150-R4 strains were only tested with chloroquine and mefloquine. The results indicated that both strains remained resistant to chloroquine and mefloquine with IC_{50} values of 205 and 95nmol/l (SD of $\pm$ 31.2) for W2-mef and 150 and 34nmol/l for CH150-R4. The comparative IC_{50} and IC_{90} values are tabulated in Tables 3.2.2.1a and 3.2.2.1b. In addition the dose response curves of chloroquine, amodiaquine, mefloquine and halofantrine against FCR_3, W2-mef and CH150-R4 strains are plotted in Figs. 3.2.7, 3.2.9 and 3.2.12.

3.3.3.2 Non-antimalarial drugs

Four non-antimalarial drugs, verapamil, chlorpromazine, fluoxetine and penfluridol were tested against FCR_3 and W2-mef strains. The IC_{50} and IC_{90} values indicated that all the drugs remained relatively inactive against the strains used. The comparative IC_{50} and IC_{90} values of verapamil, chlorpromazine, fluoxetine and penfluridol are shown in Tables 3.2.2.2a to 3.2.2.2d and presented

graphically in Fig. 3.2.8.

3.3.4 Response of K_1 and $T_{9.96}$ strains to halofantrine, verapamil, chlorpromazine and fluoxetine in a 72-hour assay, employing [3H] hypoxanthine incorporation

The sensitivity of K_1 and $T_{9.96}$ strains to halofantrine was determined in a 72-hour assay. The ratio of IC_{50} value for halofantrine in 48-hour assay/IC_{50} value in 72-hour assay (2.82) for the K_1 strain shows that the effectiveness of halofantrine on the strain over 72-hours is significantly greater than after 48-hours. In contrast, the ratio of 1.53 indicates that there is no significant difference between 48-hour and 72-hour assays of halofantrine on $T_{9.96}$ strain. The results are tabulated in Tables 3.2.4a and 3.2.4b. Verapamil, chlorpromazine and fluoxetine also were employed against K_1 strain in this 72-hour assay. The results indicate that there is no significant difference between 48-hour and 72-hour assays for chlorpromazine and fluoxetine against K_1 strains but the IC_{50} value for verapamil decreased from 7017nmol/l in the 48-hour assay to 3800nmol/l in 72-hour assay, the results are presented in Table 3.2.4c. All results are graphically illustrated in Figs. 3.2.14 and 3.2.15.

3.4 Discussion

The investigations carried out and described in this Chapter are an essential prerequisite for the work which is to follow providing baseline sensitivity data for those drugs used extensively throughout the project. The use of semi-automated microdilution technique and incorporation of radiolabelled hypoxanthine allows for accurate quantitation of sensitivity and avoiding the time

consuming processes which are necessary for microscopic counting of parasites numbers in blood stained smears. Although the feasibility and accuracy of employing the uptake of [3H] hypoxanthine to assay antimalarial activity *in vitro* has been proved by a number of investigators such as Desjardins *et al* (1979b), Golenser *et al* (1981), Lambros & Notsch (1984) and Chawira & Warhurst (1987), some investigators have noted significant differences in drug sensitivities as assessed by the two methods (ie. microscopic examination and radioisotope utilisation). Desjardins and co-workers (1979b) reported that there was good correlation between the ID50 values for chloroquine and mefloquine determined by the microscopic examination and isotopic method. Similarly, the same procedure was examined with chloroquine, mefloquine, amodiaquine, quinine and pyrimethamine on Viet Nam Smith, Malayan Camp, MR-Smith and MR-Camp strains by Lambros & Notsch (1984). The results obtained from their experiments showed good correlation between the ID50 values for chloroquine, amodiaquine, quinine and pyrimethamine by both methods, but the ID50 values for mefloquine determined by morphological examination were significantly greater than those obtained using isotopic method. However, we have shown that there was no significant difference between microscopical techniques and isotopic incorporation methods for the determination of the IC_{50} values of halofantrine against K_1, K_1HF and $T_{9.96}$ strains of parasites, but morphological examination and radioisotope utilisation showed different IC_{50} values for halofantrine against $T_{9.96}$HF parasites.

[3H] hypoxanthine incorporation by K_1 and $T_{9.96}$ strains has been compared. The results indicate that uptake of radiolabelled hypoxanthine by the

K_1 parasites is significantly greater than in $T_{9.96}$. Although the reason is not clear, it is possible that growth and multiplication of an uncloned strain (K_1) with heterogeneous parasite populations may in general terms be greater than that in cloned strain ($T_{9.96}$) with homogeneous parasite populations. Alternatively the increased growth in the K_1 parasites may reflect the increased vigour which has been reported by some investigators to be associated with chloroquine resistance. However, further studies would be required to clearly define the basis of these differences.

The results presented in Table 3.2.2.1 clearly illustrate the cross-resistance of K_1 to both chloroquine and quinine. The $T_{9.96}$ strain was 12.6 and 4.15 times more sensitive to chloroquine and quinine respectively, compared to K_1 parasites. Although the $T_{9.96}$ strain was 2.36 times more susceptible to quinidine than the K_1 strain, both were within the sensitive range to the drug. In contrast, K_1 was 2.53 and 3 times more sensitive to mefloquine and halofantrine than the $T_{9.96}$ parasites, although halofantrine was highly active against both strains. The $T_{9.96}$ strain showed slight resistance to mefloquine. The low susceptibility of chloroquine-sensitive strains to mefloquine also have been demonstrated by Gershon (1985), Lambros & Notsch (1984) and Chawira & Warhurst (1987) with IC_{50} values of 94, 11 and 51.2nmol/l for the TZ, Camp and NF54, chloroquine-sensitive strains versus 31, 8.4 and 39.4nmol/l for the K_1, Smith and K_1, chloroquine-resistant strains. Furthermore, Oduola *et al* (1987) reported that in West Africa, where mefloquine has only recently been employed for the treatment of falciparum malaria, *P. falciparum* isolates are less sensitive to mefloquine than isolates from southeast Asia. In another investigation,

Brasseur and co-workers (1988) reported that in northern Cameroon, where mefloquine has never been used, the local isolates of *P. falciparum* responded poorly to the drug. The chloroquine-sensitive strain used in this study, namely $T_{9.96}$, was isolated from Thailand (Rosario, 1981) where even before the drug was released for used mefloquine-resistant strains could be found in the area (Boudreau *et al*, 1982). Although it is still unclear, inherent resistance to mefloquine in some parasites has been suggested by Oduola *et al* (1987). On the other hand, Brasseur and co-workers (1988) believe that such resistance to mefloquine may occur as a result of cross-resistance between different antimalarial drugs. However, further studies are needed to verify the facts in detail.

Although the IC_{50} values for quinine and pyrimethamine indicated that the drugs were, more or less, active against the $T_{9.96}$ strain, the IC_{90} values were more than 100nmol/l for both drugs, indicating slight resistance (Fig. 3.2.13). Quinidine, a diastereoisomer of quinine, was more active on $T_{9.96}$ than quinine with the IC_{50} and IC_{90} values of 33 and 84nmol/l (Fig. 3.2.3). Quinidine is used as an antiarrhythmic drug, but it is also an effective antimalarial drug against falciparum malaria being slightly more potent than quinine (White *et al*, 1981). This drug more recently has been successfully employed in combination form with quinine and cinchonine for the treatment of acute falciparum malaria (Sowunmi *et al*, 1990) and in the treatment of chloroquine resistant falciparum malaria in Thailand (Bunnag *et al*, 1989). The results in this study indicate that the activity of amodiaquine and mepacrine against $T_{9.96}$ is similar in magnitude to chloroquine with the IC_{50} and IC_{90} values of 24 and 49nmol/l for amodiaquine,

25 and 61nmol/l for mepacrine, respectively (Fig. 3.2.4).

Comparison between blood schizontocidal drugs and non-antimalarial compounds such as verapamil, chlorpromazine, fluoxetine, praziquantel and penfluridol indicates that there is only a small difference between the concentrations required to kill 50 or 90% of sensitive parasites for most of the standard antimalarial drugs, ie. only a relatively small increase in concentration is required to move from IC_{50} to IC_{90} (ie. dose response curve is very steep). In contrast, for the non-antimalarial drugs there is a wide spread in the concentrations required to produce a minimal effective and fully active concentrations (see related figures). And these concentrations are generally orders of magnitude greater than effective concentrations of true antimalarials and in most cases unattainable in man.

Both K_1 and $T_{9.96}$ strains showed a similar response to non-antimalarial compounds verapamil, chlorpromazine, fluoxetine and praziquantel. These compounds remained inactive against either strain even in very high concentrations (Table 3.2.2.1) but penfluridol showed slight activity against K_1 and $T_{9.96}$ with the IC_{50} values of 370 of 470nmol/l, respectively (Table 3.2.2.1). The above compounds will be discussed in more detail in Chapter 4.

We have demonstrated that $T_{9.96}$ is more sensitive to qinghaosu than K_1 with an IC50 value of 15nmol/l for $T_{9.96}$ versus 24nmol/l for K_1 strain. A similar result also has previously been obtained by Chawira & Warhurst (1987) with the IC_{50} values of 23.7 and 40.7nmol/l for NF54 and K_1 strains, respectively. At present, it is hoped that qinghaosu or its derivatives will provide new alternatives for the treatment of both chloroquine and multidrug-resistant strains

of *P. falciparum*, although the reduced sensitivity of chloroquine resistant strains to qinghaosu may point to the potential for resistance to develop against the action of these drugs. Recently, a qinghaosu-resistant strain has been isolated from *P. yoelii* (NS) in mice (Chawira *et al*, 1986). Data in rodents showed that half-life of qinghaosu was very short, about 30 minutes following i.v. injection, but pharmacokinetic data in man are still unavailable (Peters, 1987). Qinghaosu is a very fast acting blood schizontocide, but with a high recrudescence rate (Anand, 1984; Li *et al*, 1984). However, these recrudescences may result from non-parasite dependent processes and to date clinical trials with qinghaosu and related compounds have proved successful. So our concerns over resistance to these agents may be overly pessimistic and unwarranted.

Our results indicated that the FCR_3 strain remained resistant to chloroquine, but sensitive to mefloquine with K_1 parasites being 1.86 fold more resistant to chloroquine than FCR_3. Verapamil, chlorpromazine and fluoxetine were inactive against FCR_3 with the IC_{50} values of 9500, 22000 and 17000nmol/l, respectively. There was no significant difference between the activity of the non-antimalarial compounds against K_1 and FCR_3 strains.

As mentioned beforehand, W2-mef and CH150-R4 strains were as expected, shown to be resistant to chloroquine and mefloquine. W2-mef initially was more resistant to chloroquine with an IC_{50} value of 150.74ng/ml but the level of resistance to chloroquine decreased as mefloquine resistance was selected (Oduola *et al*, 1988a, b). The IC_{50} value for mefloquine on W2-mef determined in this laboratory was 1.9 fold greater than the IC_{50} value obtained by Oduola and co-workers (1988b) against the same strain.

Although three non-antimalarial compounds, verapamil, chlorpromazine and fluoxetine, remained inactive against W2-mef, penfluridol did show some activity with an IC_{50} value of 1600nmol/l. In a study conducted by Martin and co-workers (1987), testing verapamil against two chloroquine-resistant, Indochina (W-2) and Brazil (IEC-30b) strains and one chloroquine-sensitive, West Africa (D-6) strain, they showed that the drug remained more or less inactive against all strains with IC_{50} values of 15700, 8000 and 11500 nmol/l for D-6, W-2 and IEC-30b, respectively. Scheibel *et al* (1987) also obtained an ED50 (50% effective dose) value of 4500nmol/l for chlorpromazine against FCB_{K+}, chloroquine-resistant, strain. These data would support our conclusion that verapamil exhibits very little inherent antimalarial activity against *P. falciparum* independent of resistance states.

The CH150-R4 strain was only treated with chloroquine and mefloquine. The results in this study indicated that CH150-R4 was more resistant to chloroquine than to mefloquine (Table 3.2.2.1a), similar results were obtained previously by Webster and colleagues (1985).

Comparative dose response curves of K_1 and $T_{9.96}$ parasites to halofantrine in a 72-hour assay indicated that chloroquine-resistant strain (K_1) was more sensitive to this drug than the chloroquine-sensitive strain ($T_{9.96}$). This may be related to the heterogeneity of the uncloned K_1 population and indicate a lack of cross-resistance between chloroquine and halofantrine. Experiments designed to induce halofantrine resistance indicated that halofantrine resistance was associated with an enhanced chloroquine sensitivity in the strain derived from originally chloroquine resistant parasites but not in the strain derived from

chloroquine sensitive parasites (Nateghpour & Howells, 1990).

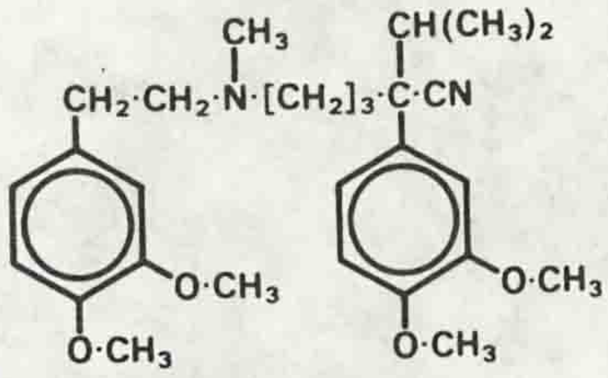
The K_1 strain showed different response to verapamil, chlorpromazine and fluoxetine in the 72-hour assays (Table 3.2.4c), compared to the 48-hour test. However, verapamil and fluoxetine still showed very low activity against the strain with the IC_{50} values of 3800 and 1650nmol/l, respectively, while chlorpromazine with an IC_{50} of 15500nmol/l remained inactive. The results reported by Scheibel and co-workers (1987) demonstrated that there was no significant difference between the ED50 values for verapamil 7700nmol/l and chlorpromazine 4500nmol/l against FCB_{K+}, chloroquine resistant isolates of *P. falciparum* in a 72-hour assay.

The work described in this Chapter has provided accurate quantitative data on the sensitivity of culture adapted strains of *P. falciparum* to an extensive selection of common antimalarials and agents which have been implicated as capable of reversing drug resistance in various cellular systems including *P. falciparum*. This data will provide the basis for the design of those studies described in subsequent chapters.

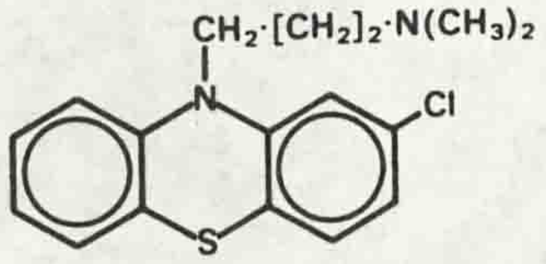
Abbreviations:

AM = Amodiaquine	CQ = Chloroquine
CZ = Chlorpromazine	FX = Fluoxetine
HF = Halofantrine	MF = Mefloquine
MP = Mepacrine	PF = Penfluridol
PQ = Praziquantel	PYR = Pyrimethamine
QD = Quinidine	QHS = Quinghaosu
QN = Quinine	VP = Verapamil

Structural formulae of non-antimalarial drugs

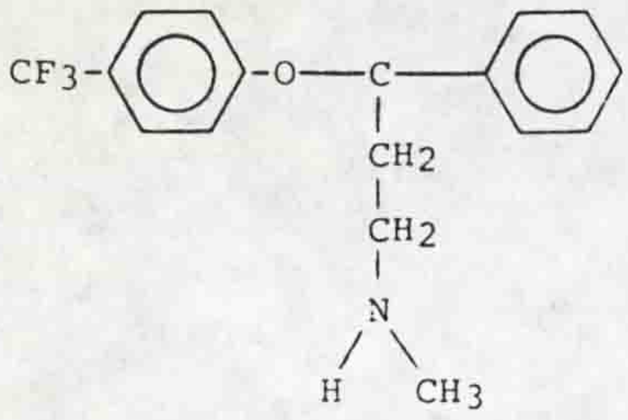


Verapamil

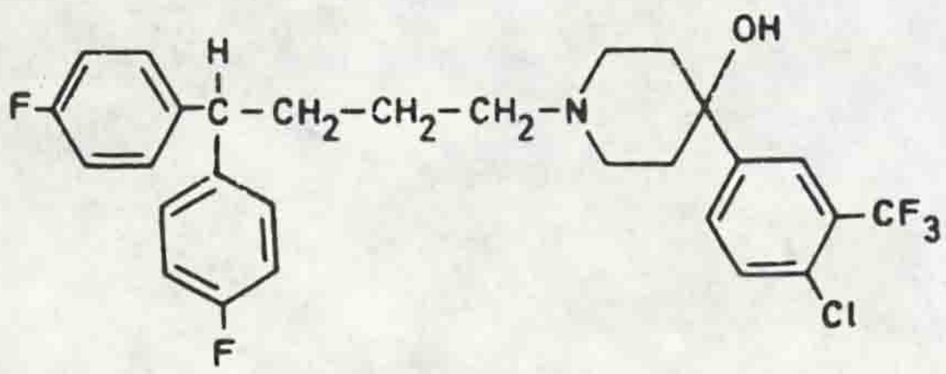


Chlorpromazine

Praziquantel



Fluoxetine



Penfluridol

(Janssen et al.,1970 ; Wong et al.,1974 ;Pharmaceutical Codex,1979 and Gustafsson et al.,1987)

Table 3.2.1 **Comparison of [3H] hypoxanthine incorporation between K_1 and $T_{9.96}$ strains of *P. falciparum* in 48-hour assay**

	Strains	
	K_1	$T_{9.96}$
	49463.96*	36772.74
	36472.22	53448.33
	56506.10	23939.87
	70378.38	24851.88
	43307.12	22499.17
	44435.87	21270.43
	30281.32	27791.37
	61896.48	21274.24
	37219.02	28433.60
	28243.07	15635.63
Mean values	45820.35	27521.72
SD	$\pm$13780.12	$\pm$10662.48

T = 3.30 p = 0.0039 df = 18

parasitaemia : 1%

*DPM (disintegration per minute)

Table 3.2.2.1 **The ratios of IC_{50} (K_1)/IC_{50} ($T_{9.96}$) and IC_{90} (K_1)/IC_{90} ($T_{9.96}$) for the drugs evaluated in the 48-hour assay, employing [3H] hypoxanthine incorporation**

Strain / Drug	K_1 Mean IC_{50} (nmol/l)	$T_{9.96}$ Mean IC_{50} (nmol/l)	IC_{50} (K_1) / IC_{50} ($T_{9.96}$)	K_1 Mean IC_{90} (nmol/l)	$T_{9.96}$ Mean IC_{90} (nmol/l)	IC_{90} (K_1) / IC_{90} ($T_{9.96}$)
Chloroquine	340	27	12.6	635	46	13.80
Quinine	320	77	4.15	780	>100	-
Quinidine	78	33	2.36	110	84	1.30
Mepacrine	80	25	3.2	109.5	61	1.79
Amodiaquine	28.5	24	1.18	57	49	1.16
Mefloquine	23	58.3	0.40	47.3	>100	-
Halofantrine	2.2	6.6	0.33	5.5	21.2	0.25
Qinghaosu	24	15	1.6	49	43.3	1.13
Pyrmethamine	>1000	36.5	-	> 1000	400	-
Verapamil	7017	4300	1.63	>10000	56000	-
Chlropromazine	24000	21000	1.14	55000	46500	1.18
Fluoxetine	21667	18500	1.17	75667	41500	1.82
Praziquantel	108667	95500	1.13	653333.3	>100000	-
Penfluridol	370	470	0.78	3500	6100	0.57

Table 3.2.2.1a **Chloroquine IC_{50} and IC_{90} values for K_1, FCR_3, $T_{9.96}$, W2-mef and CH140-R4 strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	220		560	
	270	340 ± 151.2	580	635 ± 111.2
	310		600	
	560		800	
FCR_3	120		400	
	150		490	
	160	182 ± 55.4	300	550 ± 221
	230		760	
	250		800	
$T_{9.96}$	28		45	
	28	27 ± 1.7	44	46 ± 2
	25		48	
W2-mef	110	-	280	-
	300		580	
CH150-R4	140	-	520	-
	160		560	

Table 3.2.2.1b **Mefloquine IC_{50} and IC_{90} values for K_1, FCR_3, $T_{9.96}$, W2-mef and CH150-R4 strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	23		50	
	25	23 ± 2.5	48	47.3 ± 3
	20		44	
FCR_3	29	-	50	-
$T_{9.96}$	53			
	60	58.3 ± 4.7	>100	-
	62			
W2-mef	70		300	
	85	95 ± 31.2	400	407 ± 110.1
	130		520	
CH150-R4	30	-	82	-
	38		85	

Table 3.2.2.1c — **Halofantrine IC_{50} and IC_{90} values for K_1, FCR_3 and $T_{9.96}$ strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	1.7 2.2 2.3 2.3 2.6	2.2 ± 0.32	4.3 5 5.6 6 6.4	5.5 ± 0.82
FCR_3	8.2	-	9.8	-
$T_{9.96}$	5.6 6.2 8	6.6 ± 1.2	20 9.8 34	21.2 ± 2.1

Table 3.2.2.1d **Quinine, Quinidine and mepacrine IC_{50} and IC_{90} values for K_1 and $T_{9.96}$ strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Drug	Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
		Individual	Mean ± SD	Individual	Mean± SD
Quinine	K_1	220 270 470	320 ± 132.2	610 730 1000	780 ± 200
	$T_{9.96}$	72 78 80	77 ± 4.2	>100	-
Quinidine	K_1	78	-	110	-
	$T_{9.96}$	32 34	-	80 88	-
Mepacrine	K_1	74 86	-	99 120	-
	$T_{9.96}$	21 29	-	53 69	-

Table 3.2.2.1e **Amodiaquine IC_{50} and IC_{90} values for K_1, FCR_3 and $T_{9.96}$ strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	18 21 37 38	28.5 ± 10	42 44 70 72	57 ± 16.2
FCR_3	22 50	-	61 75	-
$T_{9.96}$	18 19 34	24 ± 9	41 45 60	49 ± 10

Table 3.2.2.1f **Qinghaosu and pyrimethamine IC_{50} and IC_{90} values for K_1 and $T_{9.96}$ strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Drug	Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
		Individual	Mean ± SD	Individual	Mean ± SD
Qinghaosu	K_1	12 25 35	24 ± 11.5	39 46 62	49 ± 12
	$T_{9.96}$	8 17 20	15 ± 6.2	42 44 44	43.3 ± 1.1
Pyrimethamine	K_1	>1000	-	>1000	-
	$T_{9.96}$	35 38	-	360 440	-

Table 3.2.2.2a — **Verapamil IC_{50} and IC_{90} values for K_1, FCR_3, $T_{9.96}$ and W2-mef strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	8000 6800 6000 6600 7000 7700	7017 ± 733.2	>10000	-
FCR_3	8000 11000	-	50000 90000	-
$T_{9.96}$	4000 4400 4500	4300 ± 264.5	52000 56000 60000	56000 ± 4000
W2-mef	5600 14000	-	43000 65000	-

Table 3.2.2.2b — **Chlorpromazine IC_{50} and IC_{90} values for K_1, FCR_3, $T_{9.96}$ and W2-mef strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	22000 26000	-	50000 60000	-
FCR_3	22000	-	49000	-
$T_{9.96}$	20000 22000	-	45000 48000	-
W2-mef	22000 23000	-	60000 62000	-

Table 3.2.2.2c **Fluoxetine IC_{50} and IC_{90} values for K_1, FCR_3, $T_{9.96}$ and W2-mef strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	10000 23000 32000	21667 ± 11060.4	71000 80000 76000	75667 ± 4509.2
FCR_3	17000	-	43000	-
$T_{9.96}$	17000 20000	-	40000 43000	-
W2-mef	5000 7000	-	19000 20000	-

Table 3.2.2.2d **Praziquantel and penfluridol IC_{50} and IC_{90} values for K_1, $T_{9.96}$ and W2-mef strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Drug	Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
		Individual	Mean ± SD	Individual	Mean ± SD
Praziquantel	K_1	96000 100000 130000	108667 ± 18583.1	620000 660000 680000	653333.3 ± 30550.5
	$T_{9.96}$	94000 97000	-	>100000	-
Penfluridol	K_1	330 410	-	3000 4000	-
	$T_{9.96}$	420 520	-	5200 7000	-
	W2-mef	1400 1800	-	5000 5400	-

Table 3.2.4a **Halofantrine IC_{50} and IC_{90} values for K_1 and $T_{9.96}$ strains of *P. falciparum* in the 72-hour [3H] hypoxanthine incorporation test (data from individual experiments)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	0.76 0.80	-	3.6 3.9	-
$T_{9.96}$	3.8 4.8	-	15 18	-

Table 3.2.4b **The ratios of IC_{50} (K_1, $T_{9.96}$) in 48-hour assay on IC_{50} (K_1, $T_{9.96}$) in 72-hour assay of halofantrine, using [3H] hypoxanthine incorporation**

Strain	IC_{50} (nmol/l) in 48hr		IC_{50} (nmol/l) in 72hr		IC_{50} in 48hr / IC_{50} in 72hr
	Individual	Mean ± SD	Individual	Mean ± SD	
K_1	1.7 2.2 2.3 2.3 2.6	2.2 ± 0.32	0.76 0.80	0.78	2.82
$T_{9.96}$	5.6 6.2 8	6.6 ± 1.2	3.8 4.8	4.3	1.53

Table 3.2.4c **Verapamil (VP), chlorpromazine (CZ) and fluoxetine (FX) IC_{50} and IC_{90} values for K_1 strain of *P. falciparum* in the 72-hour [3H] hypoxanthine incorporation assay (data from individual experiments)**

Drug	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
VP	3800	-	29000	-
CZ	12000 19000	-	64000 72000	-
FX	1500 1800	-	20000 30000	-

Table 3.2.5 **The ratios of IC_{50} (W2-mef)/IC_{50} (K_1) and IC_{50} (W2-mef)/IC_{50} ($T_{9.96}$) for the drugs evaluated in 48-hour assay, using [3H] hypoxanthine incorporation**

Strain Compound	K_1 Mean IC_{50} (nmol/l)	$T_{9.96}$ Mean IC_{50} (nmol/l)	W2-mef Mean IC_{50} (nmol/l)	IC_{50} (W2-mef) IC_{50} (K_1)	IC_{50} (W2-mef) IC_{50} ($T_{9.96}$)
Verapamil	7017	4300	9800	1.39	2.27
Chlorpromazine	24000	21000	22500	0.93	1.07
Fluoxetine	21667	18500	6000	0.27	0.32
Penfluridol	370	470	1600	4.32	3.40

Fig. 3.2.1 A comparison of the dose response curves for CQ and QN against K1(——) and T9.96(----) parasites.

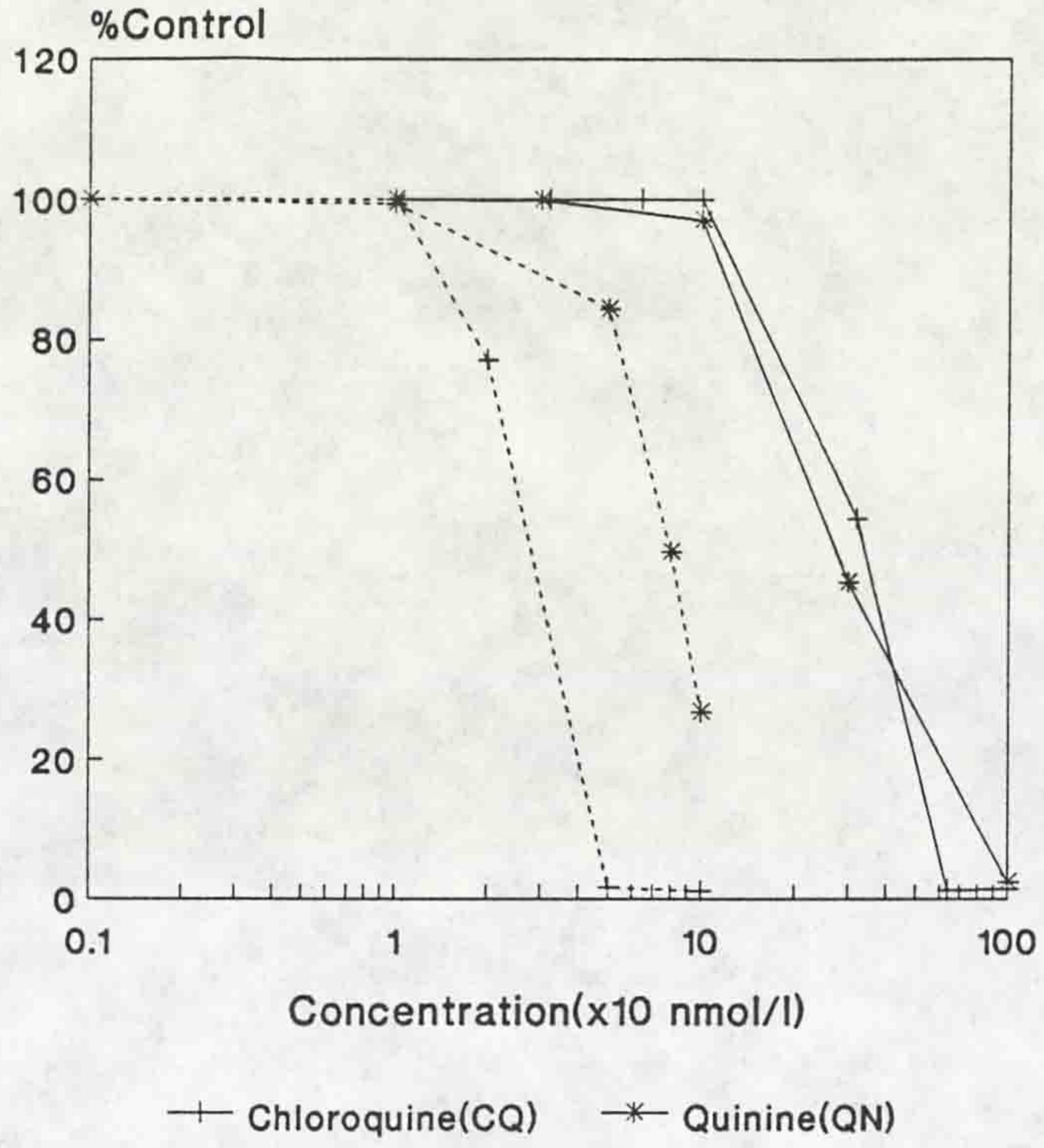


Fig. 3.2.2 A comparison of the dose response curves for MF,HF and QHS against K1(——) and T9.96(----) parasites.

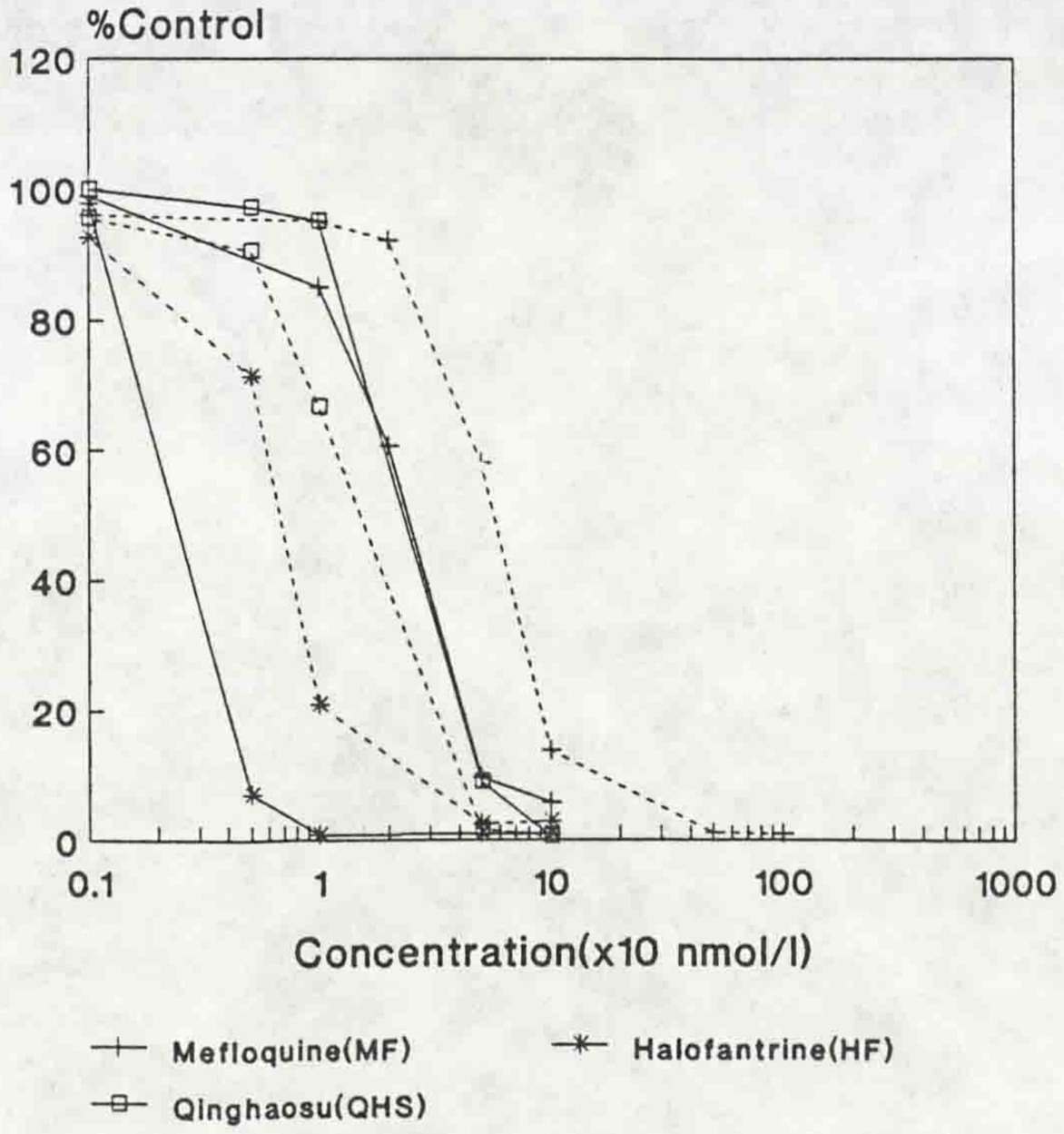


Fig.3.2.3 A comparison of the dose response curves for QN and QD against K1(——) and T9.96(----) parasites.

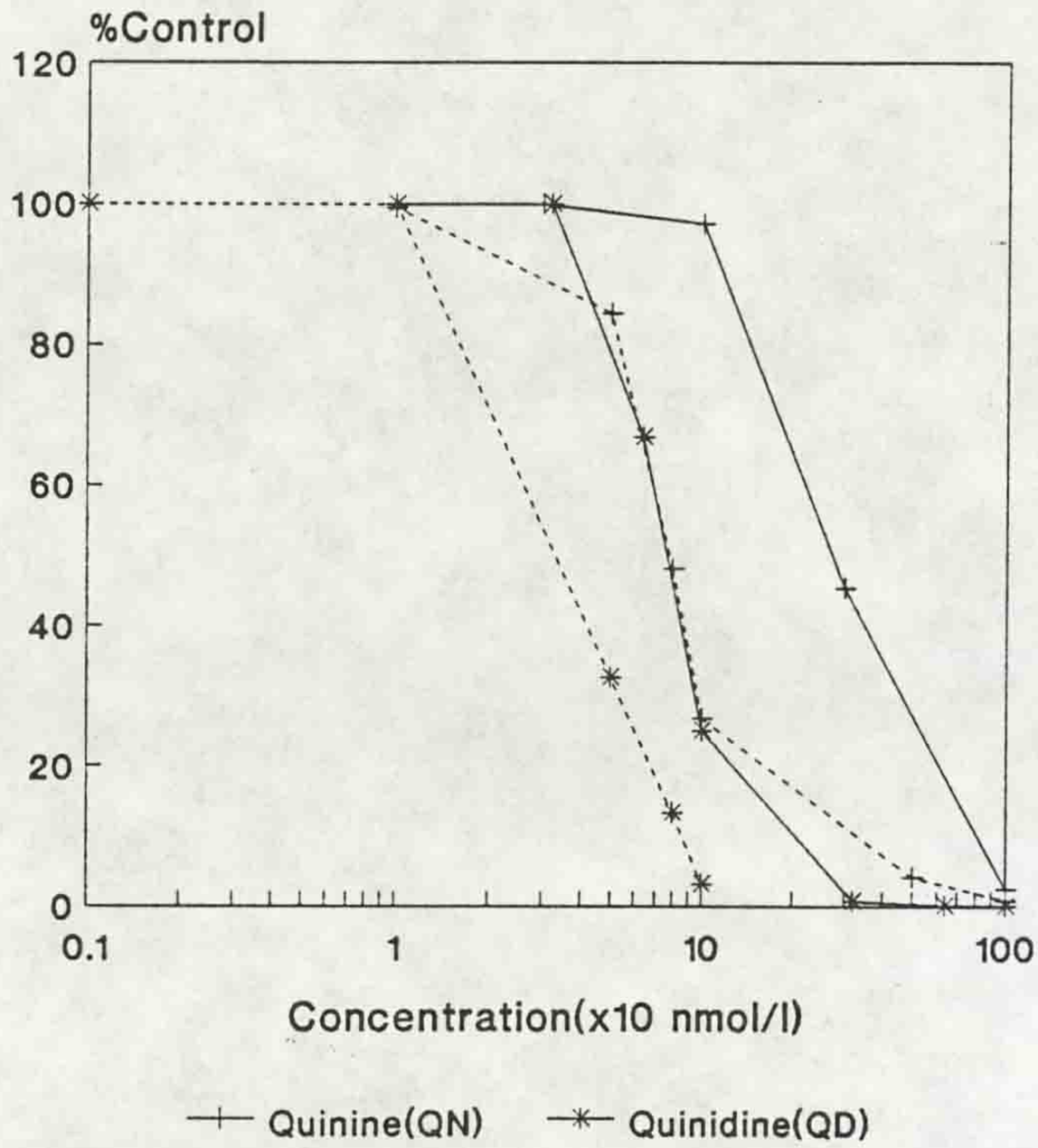


Fig.3.2.4 A comparison of the dose response curves for MP and AM against K1(——) and T9.96(----) parasites.

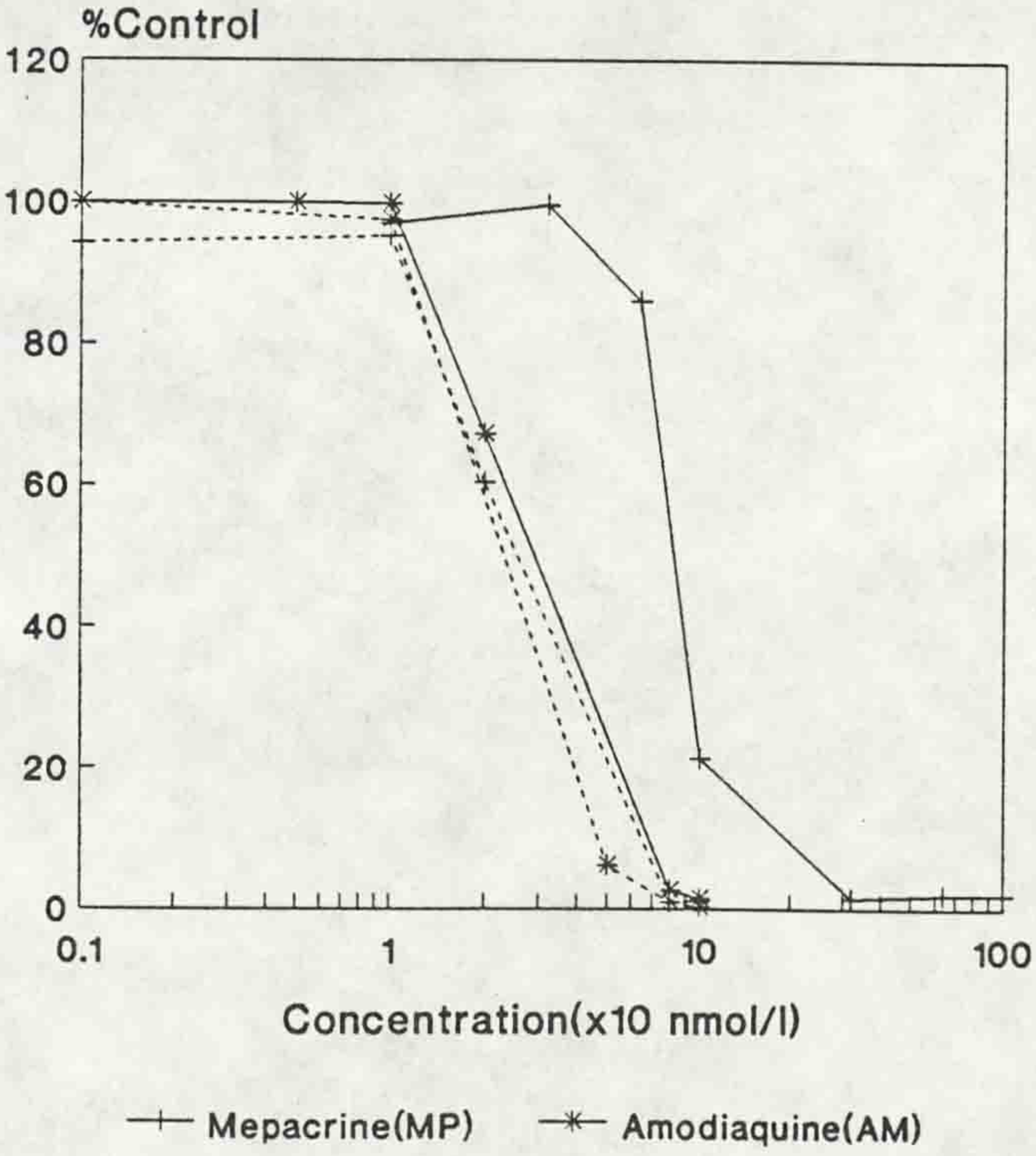


Fig.3.2.5 A comparison of the dose response curves for VP,CZ,FX,PQ and PF against K1 parasites.

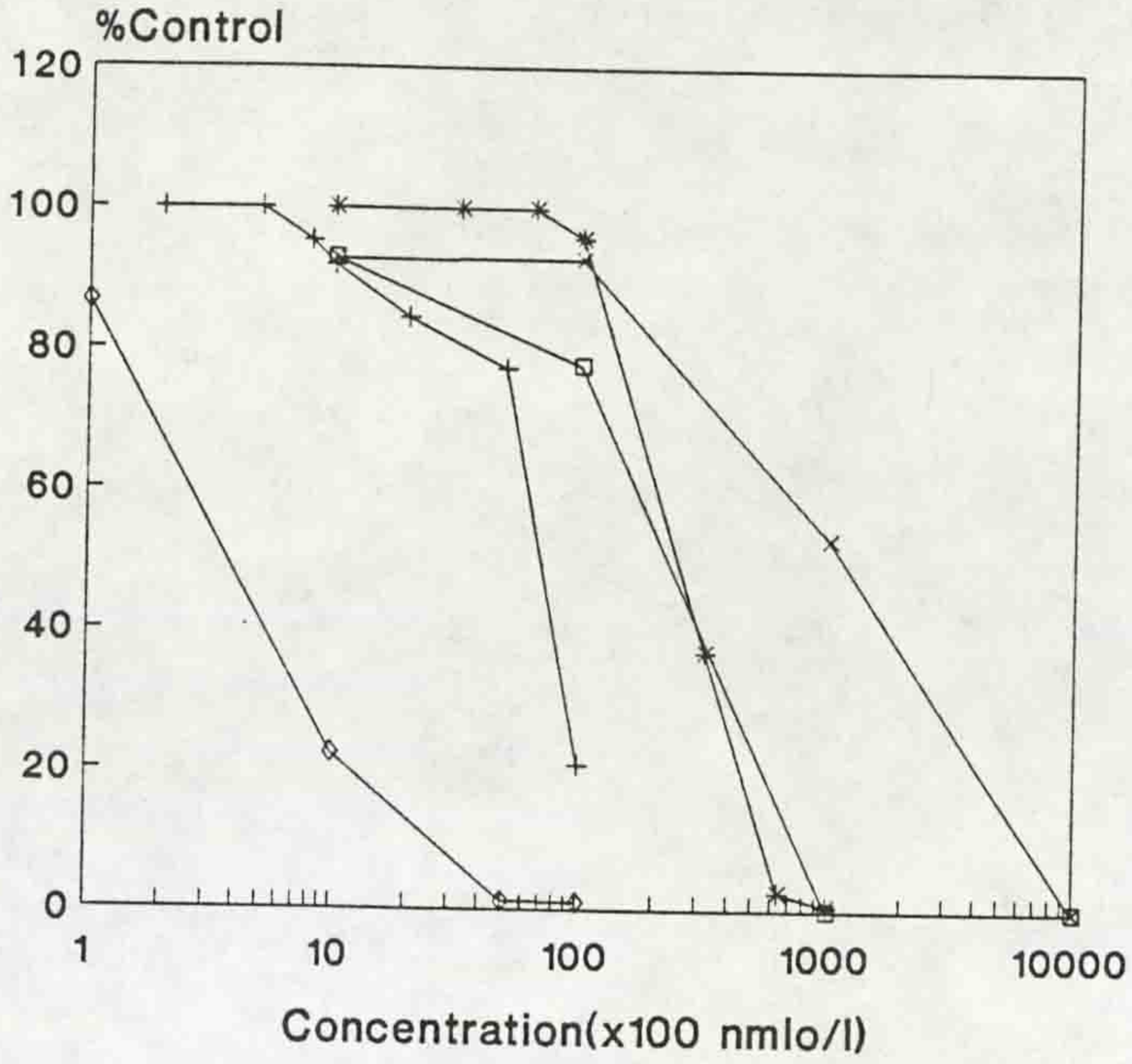


Fig.3.2.6 A comparison of the dose response curves for VP,CZ,FX,PQ and PF against T9.96 parasites.

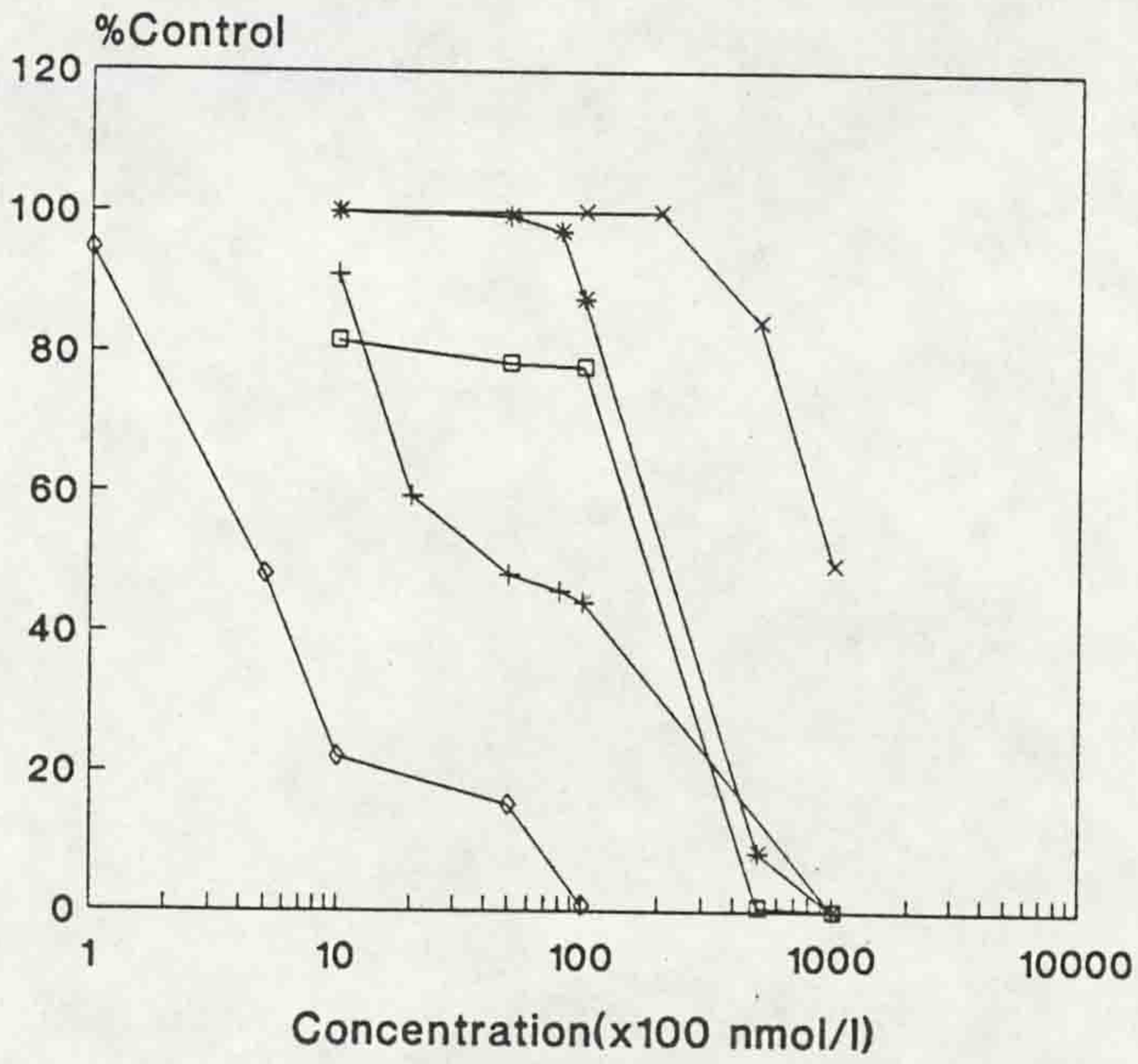


FIG.3.2.7 A comparison of the dose response curves for CQ,MF,HF and AM against FCR3 parasites.

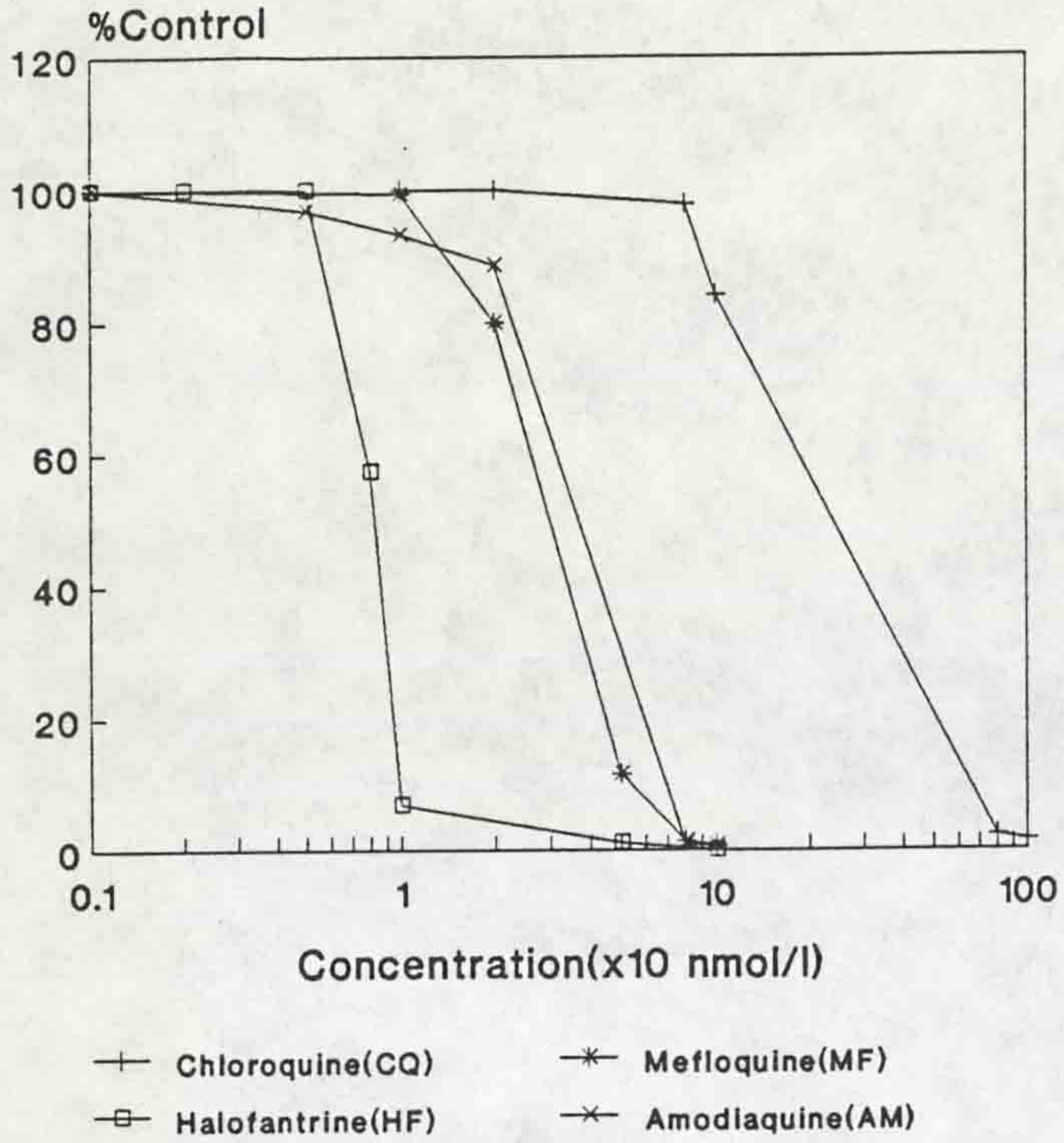


Fig.3.2.8 A comparison of the dose response curves for VP,CZ and FX against FCR3 parasites.

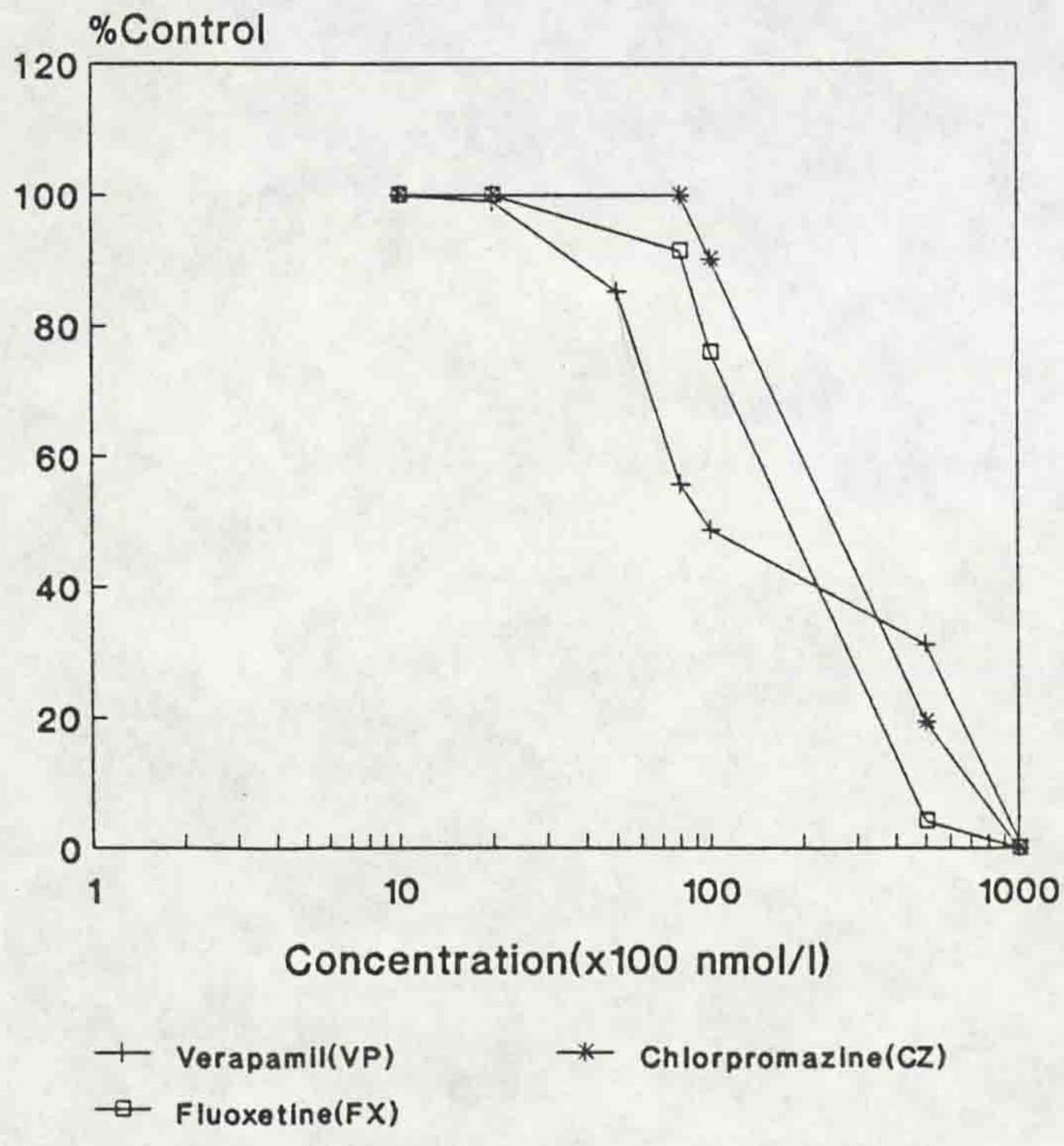


Fig.3.2.9 A comparison of the dose response curves for CQ and MF against W2-mef parasites.

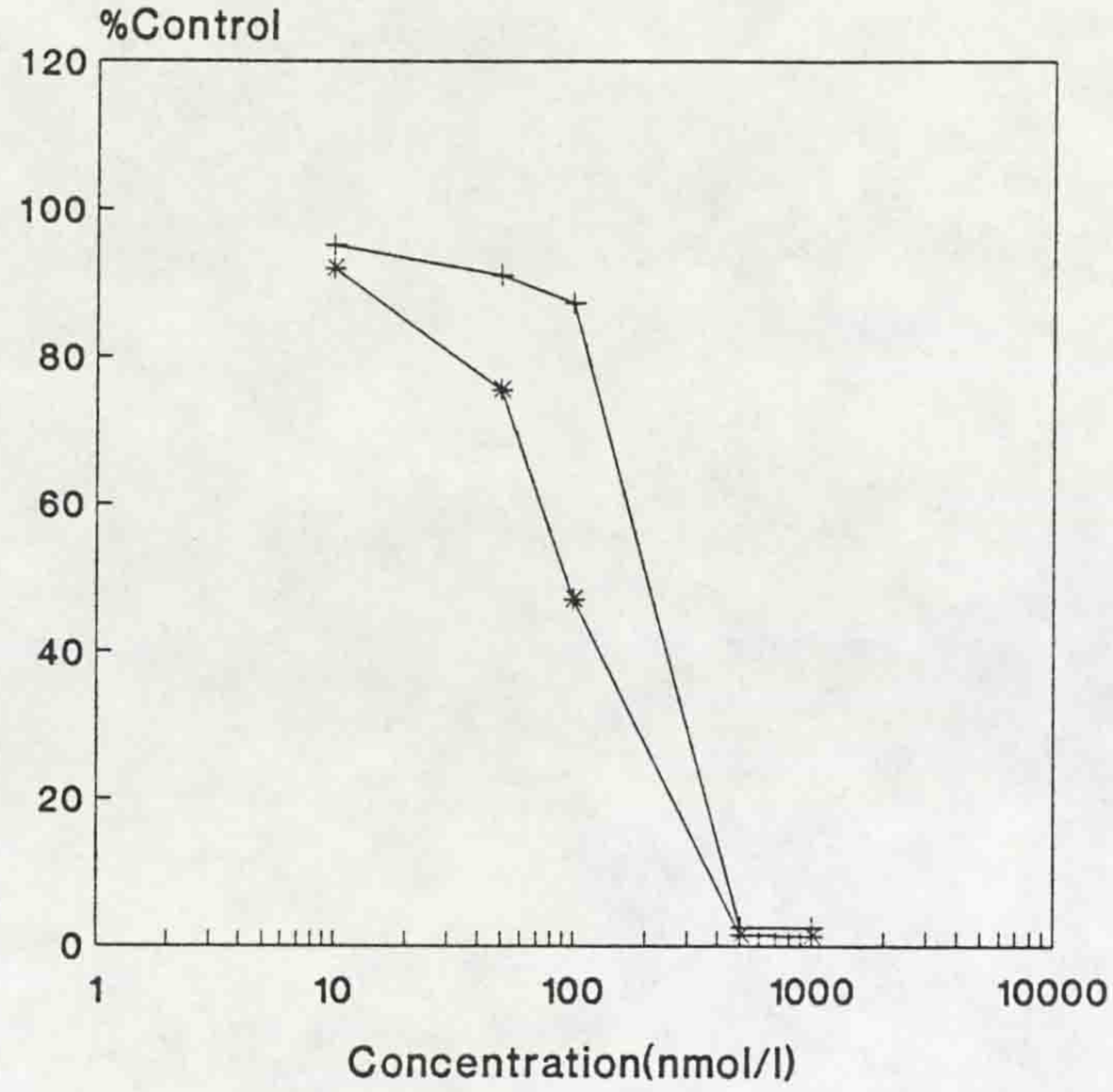


Fig.3.2.10 A comparison of the dose response curves for VP,CZ,FX and PF against W2-mef parasites.

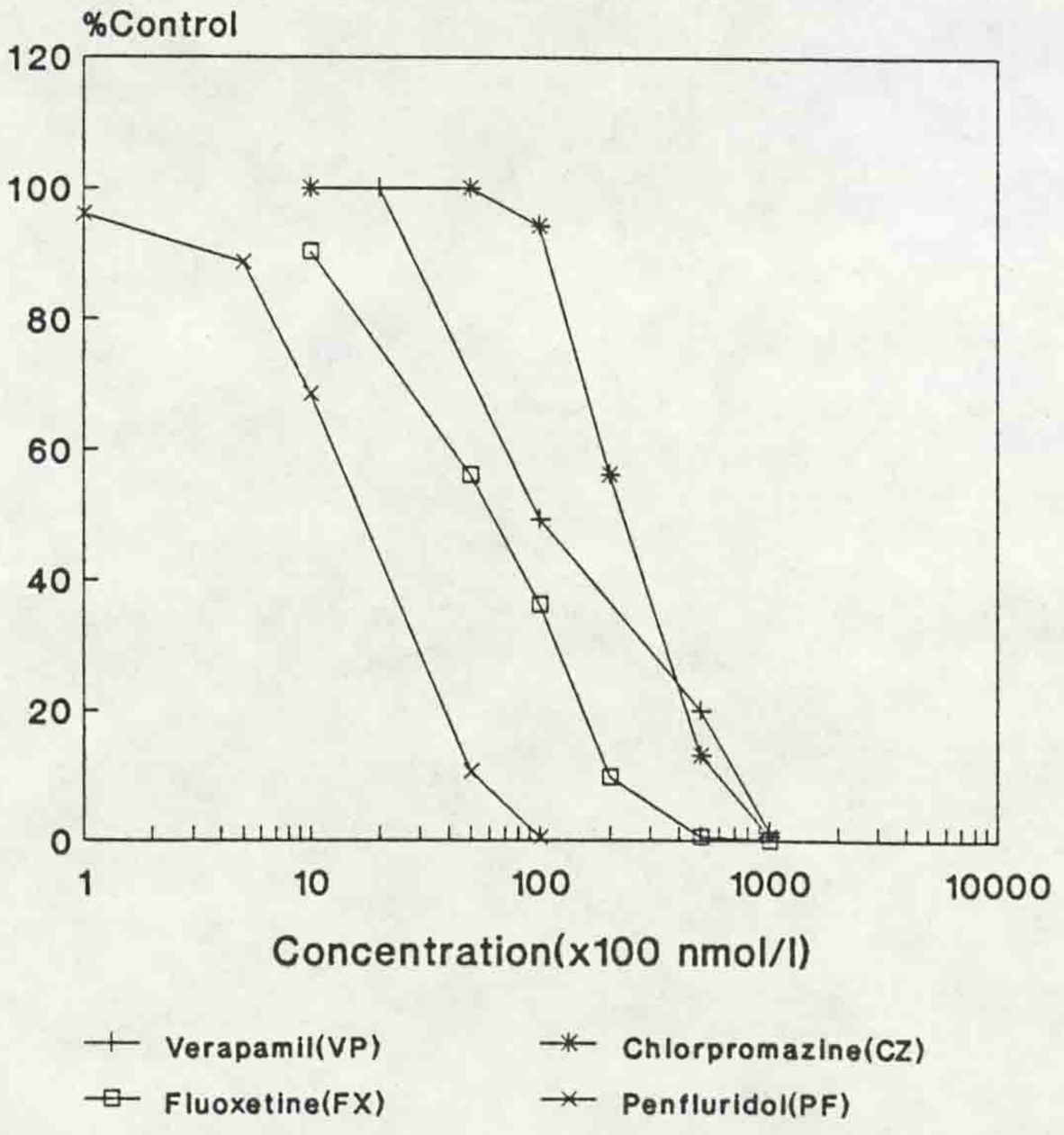


Fig.3.2.11 A comparison of the dose response curves for CQ,MF,VP,CZ,FX and PF against W2-mef parasites.

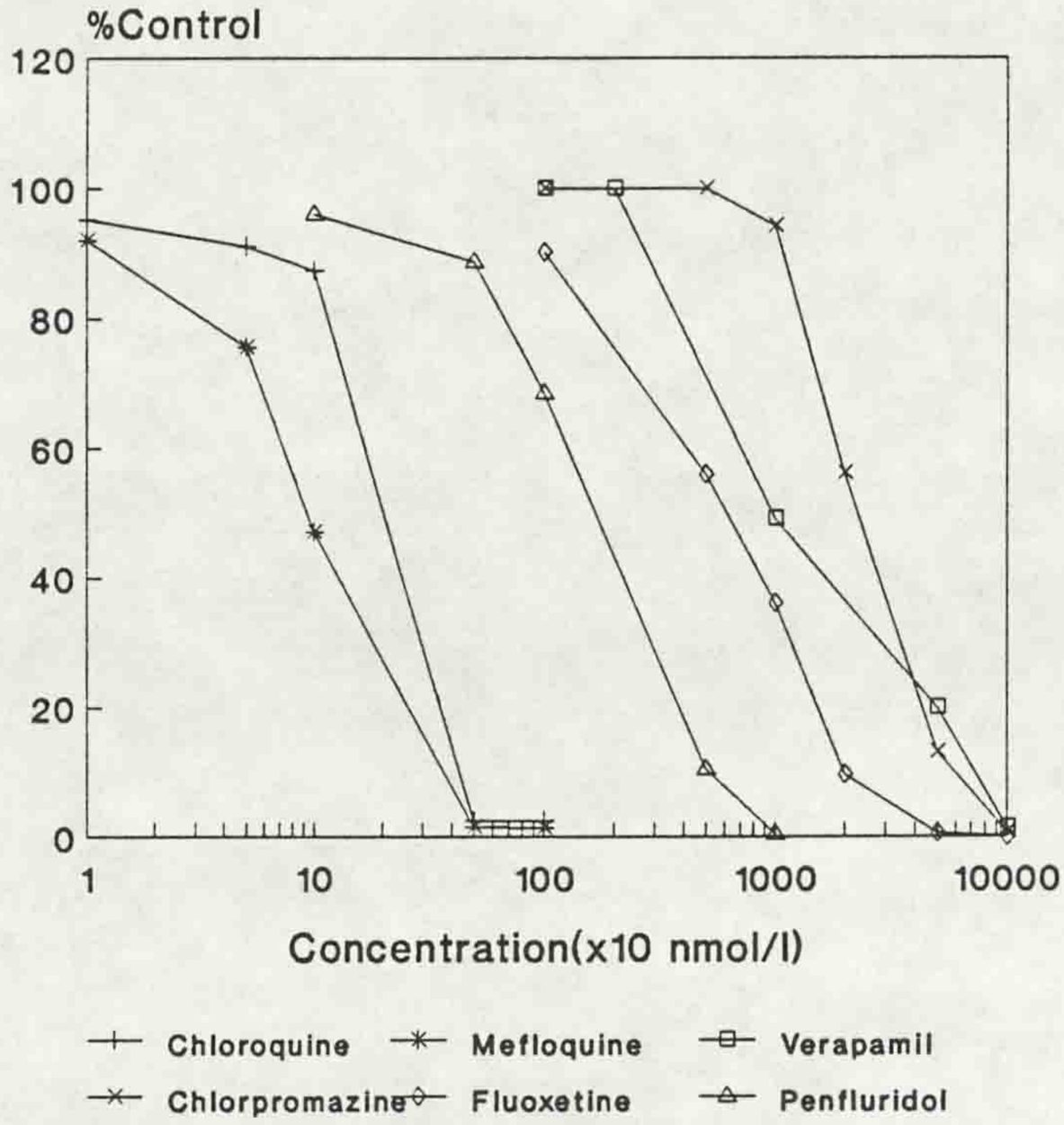


Fig.3.2.12 A comparison of the dose response curves for CQ and MF against CH150-R4 parasites.

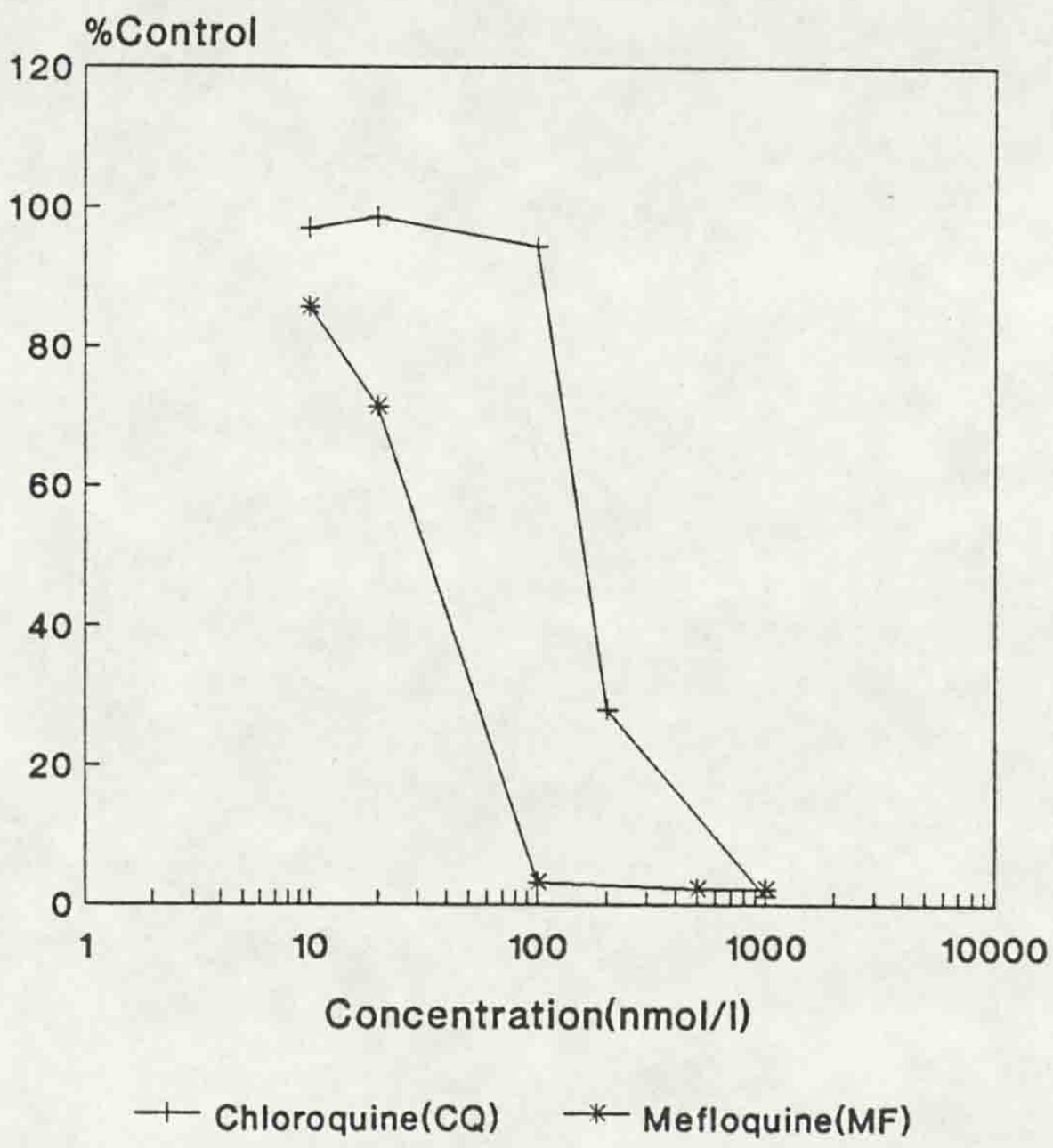


Fig.3.2.13 A comparison of the dose response curves for QN and PYR against T9.96 parasites.

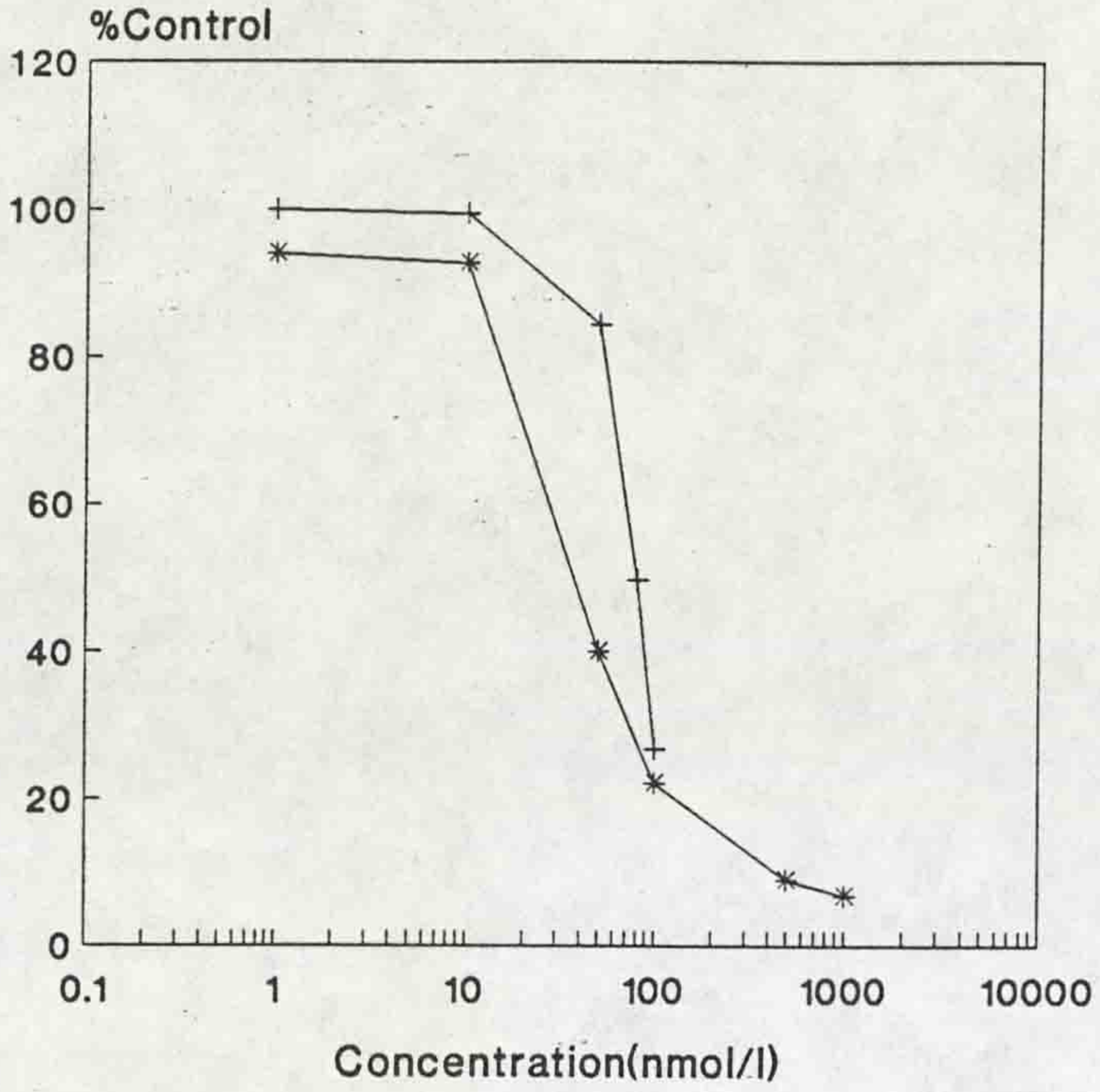


Fig.3.2.14 A comparison of the dose response curves for HF,VP,CZ and FX against K1 parasites(72-hr. assay).

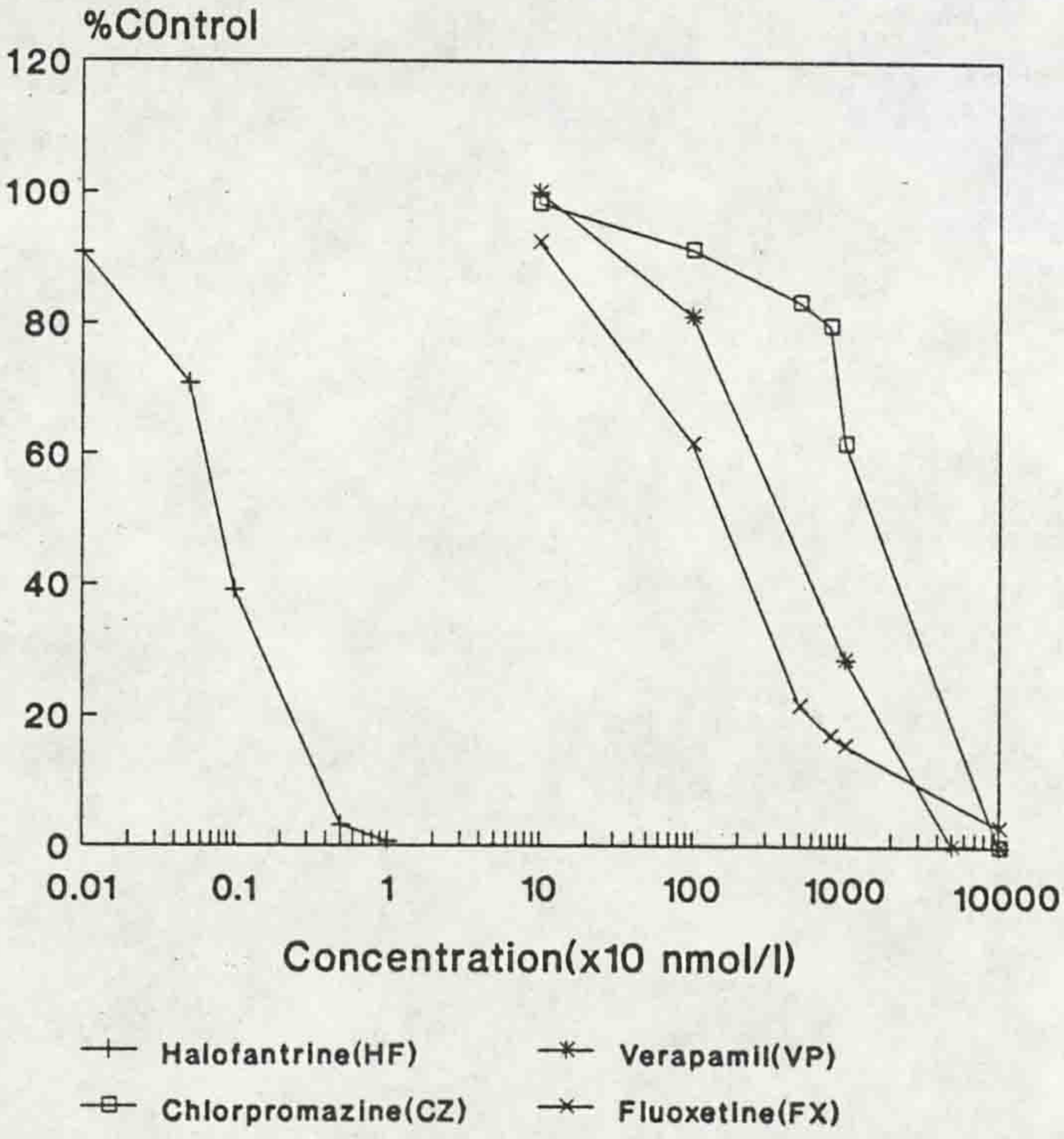
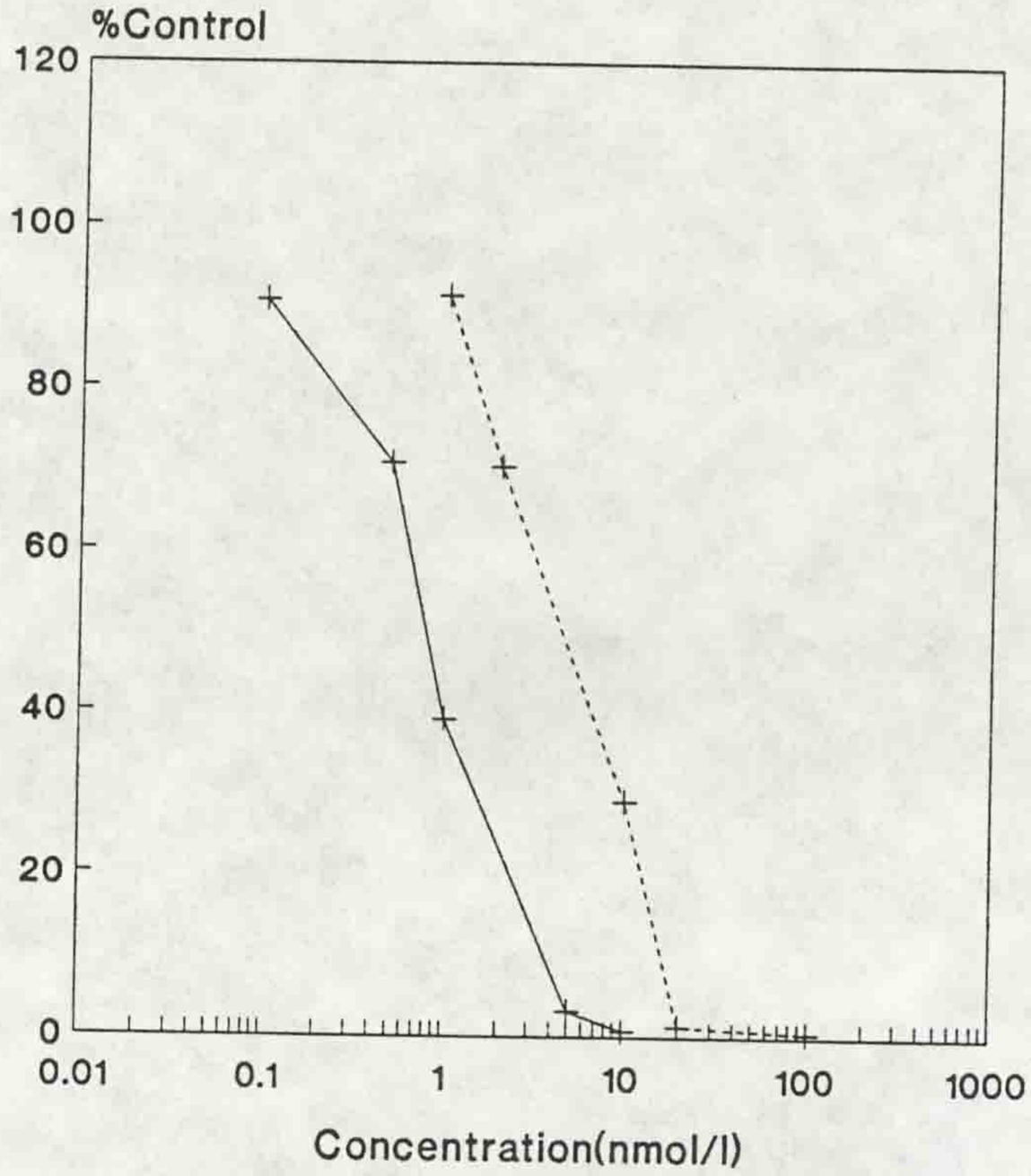


Fig.3.2.15 A comparison of the dose response curves for HF against K1(——) and T9.96(----) parasites(72-hr. assay).



CHAPTER 4 : THE EFFECT OF NOVEL DRUG COMBINATIONS AGAINST CHLOROQUINE-RESISTANT, CHLOROQUINE-SENSITIVE AND MEFLOQUINE-RESISTANT STRAINS

4.1 Introduction

Many reports suggest that the use of antimalarials in combination formulations will delay development of drug-resistance in *P. falciparum* (see Chapter 1.5) and this approach has been used with some success. More recently it has been shown that a number of drugs with no inherent antimalarial activity such as verapamil (Martin *et al*, 1987), desipramine (Bitonti *et al*, 1988) and cyproheptadine (Peters *et al*, 1989) are capable of reversing chloroquine resistance in *P. falciparum in vitro* and *in vivo*.

In this Chapter, we report the effect of the non-antimalarial agents verapamil, chlorpromazine, fluoxetine and praziquantel against isolates of *P. falciparum* when used in combination with chloroquine, mefloquine, halofantrine and qinghaosu. The non-antimalarial agents were selected on the basis of reports in the literature which were suggestive of a role in resistance reversal (Krogstad *et al*, 1987; Martin *et al*, 1987; Scheibel *et al*, 1987). The antimalarials represent the most promising of the agents currently available. The study is designed (a) to evaluate the potential of such combinations in reversing chloroquine resistance and (b) to determine any structural requirements essential for synergy or resistance reversal. In addition, the interaction between chloroquine and other antimalarial drugs (eg. quinine, quinidine and mepacrine) has also been screened with similar aims.

4.2 Materials and methods

Four strains of parasite K_1, $T_{9.96}$, FCR_3 and W2-mef, were employed during these studies. Cultivation of parasites was carried out as

described in Chapter 2.1. The interaction of chloroquine and mefloquine with verapamil, chlorpromazine, fluoxetine and praziquantel was assessed by means of two different methods. In the first method an appropriate range of concentrations of chloroquine and mefloquine was combined with the concentrations of 1000nmol/l verapamil, 3200nmol/l chlorpromazine and 10000nmol/l fluoxetine and praziquantel. The determination of IC_{50} values was as described in Chapter 2 (2.2.3.1). The second method was based on a modification of the test described by Chawira & Warhurst (1987). This method relies on the use of the IC_{50} values for each drug to be used in the combination. Parasites are exposed to the drug combination such that if the effects were additive 50% of the parasites would be killed, ie. fixed ratios of the IC_{50}s of the two drugs in question are used. In simple terms it involves the combination of x% of the IC_{50} of one drug with y% of the IC_{50} of other such that x + y = 100%, ie. 90:10, 70:30, 50:50, 30:70 and 10:90μl respectively.

The assay procedures were as described in Chapter 2 (2.2.3.1). The IC_{50} concentrations of the individual (including percentage inhibition values) drugs were plotted on two ordinates and these values joined with a straight line. The results of the fixed ratios were plotted in between the ordinates. If the two drugs had no effect on each other's action, then the resulting fixed ratio points would lie on or about the line, indicating an additive effect. If the points lay above the line, potentiation was indicated, namely the two drugs influenced positively each other's activity. In contrast, if the points dropped below the line, antagonism was indicated, namely the drugs interfered with one another's action.

4.3 Results

4.3.1 The effect of fixed concentrations of non-antimalarial drugs in combination with chloroquine or mefloquine against *P. falciparum*

All experiments in this study were performed at least in duplicate. The results are given in Tables 4.3.1 to 4.3.4. The results indicate that the sensitivity of the chloroquine resistant K_1 parasites to chloroquine was increased 4.42, 7.75 and 11.07 times when combined with verapamil, chlorpromazine and fluoxetine respectively, with no alteration in sensitivity in combination with praziquantel (Table 4.3.3). Table 4.3.4 illustrates that the susceptibility of the K_1 parasites to mefloquine was reduced in combination with chlorpromazine with the ratio of 0.75. Relatively small increases in mefloquine sensitivity were seen in combination with verapamil and fluoxetine with the ratios of 1.90 and 2.12 respectively. The results are graphically presented in Fig. 4.3.1 and 4.3.2.

4.3.2 The interaction between standard antimalarial drugs and non-antimalarial drugs, using the fixed ratios method

4.3.2.1 The interaction between chloroquine and non-antimalarial drugs against K_1, FCR_3 and $T_{9.96}$ strains

The effects of combinations of chloroquine with verapamil, chlorpromazine, fluoxetine and praziquantel against K_1 are plotted in Figs. 4.3.3-4.3.6. All the non-antimalarial compounds excluding praziquantel increased, synergistically, the activity of chloroquine against the K_1 strain. The ratios of 70:30 and 10:90 for chloroquine + verapamil with 96.05% and 77.96% growth inhibition showed the highest and lowest effect of the combination on the strain (Fig. 4.3.3). The interaction between chloroquine and chlorpromazine again

demonstrated a high level of potentiation against the K_1 strain, particularly in the ratios of 90:10, 70:30, 50:50 and 30:70 with 98.06, 99.02, 98.91 and 90.49 per cent growth inhibition, respectively (Fig. 4.3.4). The greatest potentiation occurred with the combination of chloroquine with fluoxetine against K_1. This was most marked with the ratios of 50:50 and 30:70 with 99.38 and 99.43 per cent growth inhibition respectively (Fig. 4.3.5). The interaction between chloroquine and praziquantel indicated antagonism against K_1 strain (Fig. 4.3.6).

Verapamil and chloroquine were seen to act synergistically against the chloroquine resistant FCR_3 strain of *P. falciparum* in an analogous fashion to their effects against K_1 (Fig. 4.3.7).

The interaction between chloroquine and verapamil, chlorpromazine or fluoxetine against the $T_{9.96}$, chloroquine-sensitive, strain are illustrated in Figs. 4.3.8-4.3.10. All three compounds antagonised the effect of chloroquine against this strain. The greatest antagonism occurred when chloroquine was combined with either verapamil or chlorpromazine. The ratio of 70:30 with 0.91% inhibition for chloroquine + verapamil and the same ratio with 0.62% inhibition for chloroquine + chlorpromazine produced the greatest degree of antagonism.

4.3.2.2 The interaction between mefloquine and non-antimalarial drugs against the K_1 and W2-mef strains

The combination of mefloquine with verapamil, chlorpromazine, fluoxetine and praziquantel was tested against the K_1 strain. The results indicated that an additive effect was obtained for combination of mefloquine with verapamil against the K_1 parasites (Fig. 4.3.11). Fluoxetine, chlorpromazine and praziquantel all antagonised the effect of mefloquine against the parasites (Figs.

4.3.12 to 4.3.14).

The W2-mef, mefloquine-resistant, strain was treated by mefloquine + verapamil, mefloquine + chlorpromazine and mefloquine + fluoxetine combinations. The results are illustrated in Figs. 4.3.15-4.3.17. The combination of mefloquine with fluoxetine resulted in an additive effect against the W2-mef strain, whereas the combinations of mefloquine + verapamil and mefloquine + chlorpromazine demonstrated a degree of antagonism.

4.3.2.3 The interaction between halofantrine and non-antimalarial drugs against the K_1 and $T_{9.96}$ strains

In the next group of experiments, combinations of halofantrine with verapamil, chlorpromazine and fluoxetine were tested against the K_1 and $T_{9.96}$ strains. The results are illustrated in Figs. 4.3.18-4.3.23. All three halofantrine combinations resulted in antagonism of effect against K_1 parasites with the greatest degree of antagonism being seen with the combination of halofantrine and chlorpromazine (Fig. 4.3.19), and the weakest antagonism with the combination of halofantrine and fluoxetine against the strain. Antagonism was again demonstrated with halofantrine and the three non-antimalarial agents when tested against the $T_{9.96}$ strain (Figs. 4.3.21 to 4.3.23).

4.3.2.4 The interaction between qinghaosu and non-antimalarial compounds against the K_1 strain

Qinghaosu was tested in combination with verapamil, chlorpromazine, fluoxetine and praziquantel against the K_1 strain. The results indicated that all the four non-antimalarials antagonised the effect of qinghaosu against this strain (Figs. 4.3.24-4.3.27). The combination of qinghaosu with

praziquantel showed the greatest antagonistic effect on the K_1 strain especially in the ratios of 90:10, 70:30 and 50:50 producing no inhibition in parasite growth (Fig. 4.3.27).

4.3.3 The interaction between chloroquine and quinine, quinidine and mepacrine against the K_1 strain

Combinations of chloroquine with quinine, quinidine and mepacrine were tested against the K_1 strain. The results indicated that the effect of chloroquine against these parasites was antagonised by all of the other antimalarials tested. Antagonism was greatest between chloroquine and quinidine especially in the ratio of 50:50 with 0.49% inhibition. The results are graphically presented in Figs. 4.3.28-4.3.30.

A summary of the results from these drug combination experiments tested against the K_1, $T_{9.96}$, W2-mef and FCR_3 strains of *P. falciparum* are tabulated in Tables 4.3.5 and 4.3.6.

4.4 Discussion

The traditional method for evaluating potential drug interactions involves constructions of isoblogram using data obtained from many separate sensitivity assays (Chequerboard technique) (Hewlett & Plackett, 1979; Gershon & Howells, 1984). This method is obviously very time consuming, so for the purpose of our studies the fixed ratios method (Chawira & Warhurst, 1987) was employed as the same results can be achieved in a much shorter period, without the requirement for multiple complex sensitivity tests.

Comparative results between the IC_{50} values for chloroquine alone

and in combination with verapamil against the K_1 strain indicated that sensitivity to chloroquine increased about four times when used in combination form (Table 4.3.3). Similar results had previously been reported by Martin and co-workers (1987) using the chloroquine-resistant W-2 strain. Using the fixed ratios method, combination of chloroquine with verapamil showed a marked potentiating effect against both K_1 and FCR_3 strains. The ratio of 70:30 (70% x IC_{50} CQ : 30% x IC_{50} VP) producing 96 and 95 per cent inhibition. Therefore, verapamil reversed chloroquine resistance in these strains. The same results were observed by Martin *et al* (1987) via the chequerboard technique, employing chloroquine-resistant W-2 and IEC-306 strains. In addition, Kyle and colleagues (1990) reported that verapamil potentiated the activity of a series of quinolines (chloroquine, desethylchloroquine, quinine and quinidine) against a W-2 clone. Marked potentiation has also been reported between chloroquine and verapamil against a chloroquine-resistant strain of *Plasmodium berghei* in infected mice, with an additive effect produced against the sensitive parasites of *P. berghei* (Peters *et al*, 1989).

Verapamil belongs to a group of drugs collectively referred to as calcium antagonists or calcium-channel blockers. This drug was initially developed as a cardiovascular agent (Snyder & Reynolds, 1985). Fleckenstein (1971) elucidated that the activity of verapamil and other calcium antagonists could be reversed competitively by calcium ions. This observation stimulated a number of investigations looking at the effect of these calcium channel blockers against voltage-dependent calcium channels. Further investigations described at least two distinct receptors for these drugs, both of which were associated with the

voltage-dependent calcium channels (Snyder & Reynolds, 1985). In 1975, Devis and colleagues reported that verapamil inhibited glucose induced secretion of insulin. They observed that the inhibitory effect of verapamil was associated inversely with calcium concentration. Reversal of drug-resistance in carcinoma cells by verapamil and other calcium-channel blockers prompted investigations into the mechanism of this interaction (Tsuruo *et al*, 1982a, 1982b, 1983; Tsuruo, 1983; Rogan *et al*, 1984; Fojo *et al*, 1985; Safa *et al*, 1987). It was established very early on that resistance reversal was independent of calcium channel blocking activity as only one isomeric form of verapamil has calcium channel blocking activity, but both isomers can reverse resistance (Dr S A Ward, pers. comm.). Tsuruo *et al* (1982a, 1982b) and Tsuruo (1983) showed that verapamil greatly enhanced the intracellular levels of vincristine and adriamycin in vincristine- and adriamycin-resistant P388 leukaemia cells. In addition, Fojo *et al* (1985) stated that the resistance of human KB carcinoma cells to colchicine was partially reversed by verapamil. However, the reversal of anticancer drug-resistance in the drug-resistant carcinoma cells by verapamil was attributed to the enhancement of cellular accumulation of the anticancer agents (Tsuruo *et al*, 1983). The mechanism of this reversal is not entirely understood, but speculations have been made based on the ability of verapamil to bind to a 150K to 170K membrane-associated glycoprotein (Cornwell *et al*, 1986). Additionally, Safa and his associates (1987) suggested that P-glycoprotein plays a direct role in the establishment and maintenance of drug-resistance in carcinoma cells and is also involved in the reversal of resistance by calcium antagonists. In other investigations Fojo and colleagues (1985) concluded that more than one

mechanism may be responsible for the reversal of drug-resistance in the drug-resistant carcinoma cells. In 1987, Martin and co-workers demonstrated that verapamil reversed chloroquine resistance in Indochina (W-2) and Brazil (IEC-306), chloroquine resistant, strains of *P. falciparum*. They speculated that the mechanism of this resistance reversal by verapamil may be analogous to the situation in neoplastic cells. The efflux of chloroquine from chloroquine-resistant strains of *P. falciparum* was investigated by Krogstad *et al* (1987). They also showed that the release of chloroquine from the chloroquine-resistant Indochina I/CDC strains was inhibited by verapamil. These workers were able to demonstrate that chloroquine-sensitive parasites release pre-accumulated chloroquine with a half-time which was substantially longer than in chloroquine-resistant parasites. They concluded that failure to accumulate drug in resistant parasites may result from enhanced active efflux. These workers were able to show that agents such as verapamil which are capable of reversing resistance also reduced drug efflux in resistant parasites. However, there are a number of assumptions which have been made in drawing analogies between cancer cells and malaria parasites which do not necessarily hold true (Ginsburg & Stein, 1991). Additionally, recent molecular studies designed to show the importance of the multi-drug efflux pump gene in *P. falciparum* have proved inconclusive. Ginsburg (1988) suggested: "verapamil or diltiazem exert their effect on Ca^{2+}-Na+ exchange in the parasites reducing proton leak and resulting in a more acidic vacuolar pH". On the other hand, Warhurst (1988) stated that verapamil might affect the lysosomal membrane or internal compartments, thus reducing the efflux of chloroquine from the resistant parasites. In an ultrastructural study, Jacobs and

co-workers (1988) demonstrated that the use of chloroquine against the chloroquine-sensitive D-6 strain produced considerable alterations within the parasites food vacuole which was not apparent in the chloroquine-resistant W-2 parasites. When a small amount of verapamil was combined with chloroquine, similar perturbations were seen within the food vacuole of drug-resistant parasites, indicating an association between resistance reversal and loss of vacuolar integrity.

We have demonstrated that the sensitivity of chloroquine-resistant K_1 parasites to chloroquine increased markedly when exposed to a chloroquine + chlorpromazine combination using either the fixed concentration or ratios method. The results obtained in this study also were comparable to those obtained by Kyle *et al* (1990). They looked at combinations of chlorpromazine with a number of quinoline antimalarials and invariably observed potentiation against chloroquine-resistant strains and antagonism against chloroquine-sensitive strains, observations which are consistent with our own results. It has been suggested that resistance reversal by chlorpromazine may be related to one of its minor properties, ie. inhibition of calmodulin. Scheibel and colleagues (1987) looked at combinations of chloroquine with three other calmodulin antagonists (cyclosporin A, R24571 and W-7), separately against chloroquine-resistant FCB_{K+} and chloroquine-sensitive 7G8 strains of *P. falciparum*. They observed a low level of potentiation between chloroquine and W-7 against the FCB_{K+} strain, but not between chloroquine and the two other calmodulin antagonists. The combination of chloroquine with the three calmodulin antagonists showed an antagonistic effect against the 7G8 strain.

Calmodulin, a calcium-dependent protein, has been extensively

studied in a variety of vertebrate species (Klee *et al*, 1980; Kanno & Sasaki, 1982; Reynolds & Claxton, 1982; Tomlinson *et al*, 1984), segmented worms (Waisman *et al*, 1978) and plants (Anderson & Cormier, 1978). Recently, calmodulin was isolated from the obliquely striated skeletal muscle of *Ascaris suum* by Masaracchia and co-workers (1986). Calmodulin is very broadly distributed, and probably present in all eukaryotic cells. The protein is a heat-stable, acidic polypeptide of 148 amino acids and also contains four calcium binding sites. The binding of four calcium ions (as a result of movement of calcium through channels in the plasma membrane or its release from intracellular membranes to calmodulin) changes the conformation of the protein. Consequently, calmodulin is able to activate a wide variety of enzyme systems, such as cyclic nucleotide phosphodiesterase, adenylate cyclase and protein kinases (Tomlinson *et al*, 1984). Calmodulin antagonists bind to and inhibit the activity of calmodulin. The mechanism of this inhibition is not clear, but presumably, this inhibition appears to be related to the binding of drugs to the hydrophobic domains of calmodulin which are exposed by a Ca^{2+} induced conformational changes (La Porte *et al*, 1980; Tomlinson *et al*, 1984). However, Tsuruo *et al* (1982, 1983) and Slater *et al* (1986) reported that some calmodulin antagonists enhanced the intracellular levels of two anticancer (vincristine and adriamycin) drugs in vincristine- and adriamycin-resistant P388 leukaemia cells by inhibiting the efflux of the drugs.

Scheibel and colleagues (1987) investigated the effects of calmodulin antagonists, alone and in combination with chloroquine, quinine and mepacrine, against *P. falciparum in vitro*. By using a calmodulin radioimmunoassay and electron microscopy, they demonstrated that calmodulin is diffusely scattered in

the cytoplasm of trophozoites and early schizonts, and in mature schizonts the protein is concentrated in the apical (rhoptry) area of the merozoite which is most important in the penetration of the host erythrocyte. Nickell and co-workers (1982) suggested that some calmodulin inhibitors such as cyclosporin A may inhibit the calcium-calmodulin activity at the apical of the merozoite, and thereby prevent invasion of the erythrocyte. This suggestion was later implicitly corroborated by Scheibel *et al* (1987). As mentioned previously, the results of interaction between chloroquine and chlorpromazine on the chloroquine-resistant strain are comparable with those obtained by Kyle and colleagues (1990), but not with the results of combination of chloroquine with other calmodulin antagonists, ie. cyclosporin A, W-7 and R24571, obtained by Scheibel and co-workers (1987). The discrepancy may indicate that the resistance reversal potential of chlorpromazine is totally unrelated to any anti-calmodulin activity or due to differences in the binding of antagonists to the calmodulin molecule. Some calmodulin inhibitors interact with three or more calcium-dependent sites, for instance, chlorpromazine interacts strongly with three and weakly with seventeen calcium-dependent sites (Levin & Weiss, 1979). The quantity and variety of the sites may play an important role for the sensitivity of parasites to calmodulin inhibitors. Asano & Hidaka (1984) and Loffler *et al* (1985) reported that chloroquine also possesses anti-calmodulin activity. Therefore, antagonism between calmodulin antagonists and chloroquine in chloroquine-sensitive parasites may reflect competition between these agents for calmodulin which may implicate this protein as a potential site of chloroquine action.

The non-antimalarial agents fluoxetine and praziquantel were

selected in part due to their reputed interference with calcium homeostasis. Fluoxetine potentiated the actions of chloroquine against chloroquine-resistant parasites whereas praziquantel did not.

Although, both fluoxetine and praziquantel interfere with the Ca^{2+} movement in the cells, each possesses independent properties. Fluoxetine is a potent inhibitor of serotonin (5-hydroxytryptamine) (5-HT) uptake into synaptosomes of rat brain (Wong *et al*, 1974) and in schistosomules (Catto & Ottensen, 1978). Seeman (1972) demonstrated that fluoxetine like other amphiphilic cationic drugs (chlorpromazine, imipramine) fluidises acidic phospholipid membranes and displaces calcium from membranes. Pax and colleagues (1979) in their studies with *Schistosoma mansoni* suggested that fluoxetine might directly or indirectly interfere with schistosome calcium transport mechanisms. In other investigations Allison & Young (1964) and also de Duve *et al* (1974) elucidated that cationic, amphiphilic agents such as chloroquine bind to lysosomes. Furthermore, although both chloroquine and fluoxetine interfere with Ca^{2+} transport systems (Pax *et al*, 1979; Tanabe *et al*, 1982; Ginsburg, 1988), it seems unlikely that this is related to their actions in *P. falciparum*.

Although the precise mechanism of action of praziquantel is unknown, Pax *et al* (1979) and Ruenwangsa *et al* (1983) demonstrated that the drug increases the influx of Ca^{2+} into *Schistosoma mansoni* and *Opisthorchis viverrini* (a liver fluke), respectively. Pax and colleagues (1979) showed that fluoxetine readily reduced praziquantel-induced uptake of $45Ca^{2+}$ by schistosomes. Ruenwongsa and co-workers (1983) also observed a similar phenomenon employing verapamil and the liver fluke instead of fluoxetine and

schistosomes, respectively. Harder *et al* (1987) suggested that although praziquantel, like fluoxetine, may interfere with serotonin (5HT), there are some important discrepancies between the drugs such as; fluoxetine displaces Ca^{2+} from membranes which culminates in a reduction in membrane bound Ca^{2+} but praziquantel enhances the membrane Ca^{2+} permeability. Fluoxetine unlike praziquantel is able to fluidise a synthetic phosphatidylglycerol membrane. According to the results obtained in this study praziquantel, unlike verapamil, chlorpromazine and fluoxetine, antagonised the activity of chloroquine against the K_1 strain. Although the precise reason for such discrepancy is unclear, one may speculate that praziquantel interferes with calcium cycling via enhancement of the influx of calcium into the parasites. If calcium homeostasis is important praziquantel may cause changes which operate in the opposite direction to the effects of the other compounds tested.

Despite efforts to rationalise how resistance reversal is achieved by the compounds tested, no single property of a drug can be invoked. Although alterations in calcium homeostasis is a common feature the directions of the changes produced and the distinct biochemical pathways involved are varied. It may well be that the mechanism of resistance reversal is a much more general feature of these agents perhaps relating to the PKa's and lipophilicity (Kanno & Sasaki, 1982). In terms of drug transport (efflux) resistance reversal can be explained to some extent. However, our consistent findings that these agents antagonise the effects of chloroquine in sensitive parasites would not conform with an effect on a drug exporting glycoprotein.

The antagonism produced by combinations of chloroquine with

quinine against the chloroquine-resistant K_1 strain in this study is in agreement with work of Stahel *et al* (1988). In a similar study Geary and colleagues (1986a) observed an additive effect between the drugs. Furthermore, chloroquine in combination with quinidine and mepacrine also resulted in reduced sensitivity in K_1 strain (Chapter 4.3.3). Geary *et al* (1986) and Stahel *et al* (1988) demonstrated that chloroquine + mefloquine and chloroquine + amodiaquine combinations resulted in antagonism against chloroquine-resistant and sensitive strains of *P. falciparum*.

The foregoing results altogether indicate that most of the quinoline-containing antimalarial drugs antagonised the activity of chloroquine against chloroquine-resistant and chloroquine-sensitive strains. Although the precise mechanism of action of the combinations on the parasites is unknown, the results may support the hypothesis that quinoline-derivative drugs share a common mechanism of action, albeit that they contain some differences in structure which could affect their interactions with infected erythrocytes (Fitch *et al*, 1979; Geary *et al*, 1986a). However, this idea opposes some of the conclusions drawn from the observations of cross-resistance patterns in *P. falciparum*. Fitch and colleagues (1979) also elucidated that chloroquine and mefloquine competitively inhibit each others accumulation in the parasites.

Evaluation of the effects of mefloquine, halofantrine or qinghaosu in combination with verapamil, chlorpromazine, fluoxetine or praziquantel failed to show any synergistic potential against K_1, $T_{9.96}$ or W2-mef strains of *P. falciparum* (ie. additive or antagonistic responses).

Table 4.3.1 **Comparative response of the K_1 strain to chloroquine alone and in combination with verapamil, chlorpromazine, fluoxetine or praziquantel**

Drug	IC_{50} (nmol/l)	
	Individual	Mean $\pm$ SD
Chloroquine	230	
	290	310 $\pm$ 91.65
	410	
Cholorquine + Verapamil*	65	-
	75	-
Chloroquine + Chlorpromazine*	37	-
	43	-
Chloroquine + Fluoxetine*	23	
	32	28 $\pm$ 4.5
	29	
Chloroquine + Praziquantel*	120	
	180	210 $\pm$ 108.16
	330	

* Concentration of non-antimalarial components were 1000, 3200, 10000 and 10000nmol/l for verapamil, chlorpromazine, fluoxetine and praziquantel respectively

Table 4.3.2 **Comparative response of the K_1 strain to mefloquine alone and in combination with verapamil, chlorpromazine or fluoxetine**

Drug	IC_{50} (nmol/l)	
	Individual	Mean ± SD
Mefloquine	15	
	18	21 ± 7.9
	30	
Mefloquine + Verapamil*	9.3	
	9.7	11 ± 2.6
	14	
Mefloquine + Chlorpromazine	21	
	*26	28 ± 8.1
	37	
Mefloquine + Fluoxetine*	7	
	8.7	9.9 ± 3.6
	14	

* Concentration of non-antimalarial compounds were 1000, 3200 and 10000nmol/l for verapamil, chlorpromazine and fluoxetine respectively

Table 4.3.3 **The ratios of the IC_{50} value for chloroquine on the Ic_{50} values for the drug in combination with verapamil, chlorpromazine, fluoxetine and praziquantel against the K_1 strain**

Drug	Individual IC_{50} (nmol/l)	IC_{50} of chloroquine / IC_{50} of combination
Chloroquine	310	-
Chloroquine + Verapamil*	70	4.42
Chloroquine + Chlorpromazine*	40	7.75
Chloroquine + Fluoxetine*	28	11.07
Chloroquine + Praziquantel*	210	1.47

* Concentration of non-antimalarial compounds were 1000, 3200, 10000 and 10000nmol/l for verapamil, chlorpromazine, fluoxetine and praziquantel respectively

Table 4.3.4 **The ratios of the IC_{50} value for mefloquine on the IC_{50} values for the drug in combination with verapamil, chlorpromazine and fluoxetine against the K_1 strain**

Drug	Individual IC_{50} (nmol/l)	IC_{50} of mefloquine / IC_{50} of combination
Mefloquine	21	-
Mefloquine + Verapamil*	11	1.90
Mefloquine + Chlorpromazine*	28	0.75
Mefloquine + Fluoxetine*	9.9	2.12

* Concentration of non-antimalarial compounds were 1000, 3200 and 10000nmol/l for verapamil, chlorpromazine and fluoxetine respectively

Table 4.3.5 **Summary of results of drug combinations against the K_1 strain of *P. falciparum*, using [3H] hypoxanthine in 48-hour assays.**

An: Antagonism; Po: Potentiation; Ad: Additive effect

Drug	Chloroquine	Mefloquine	Halofantrine	Qinghaosu
Quinine	An.	-	-	-
Quinidine	An.	-	-	-
Mepacrine	An.	-	-	-
Verapamil	Po.	Ad.	An.	An.
Chlorpromazine	Po.	An.	An.	An.
Fluoxetine	Po.	An.	An.	An.
Praziquantel	An.	An.	-	An.

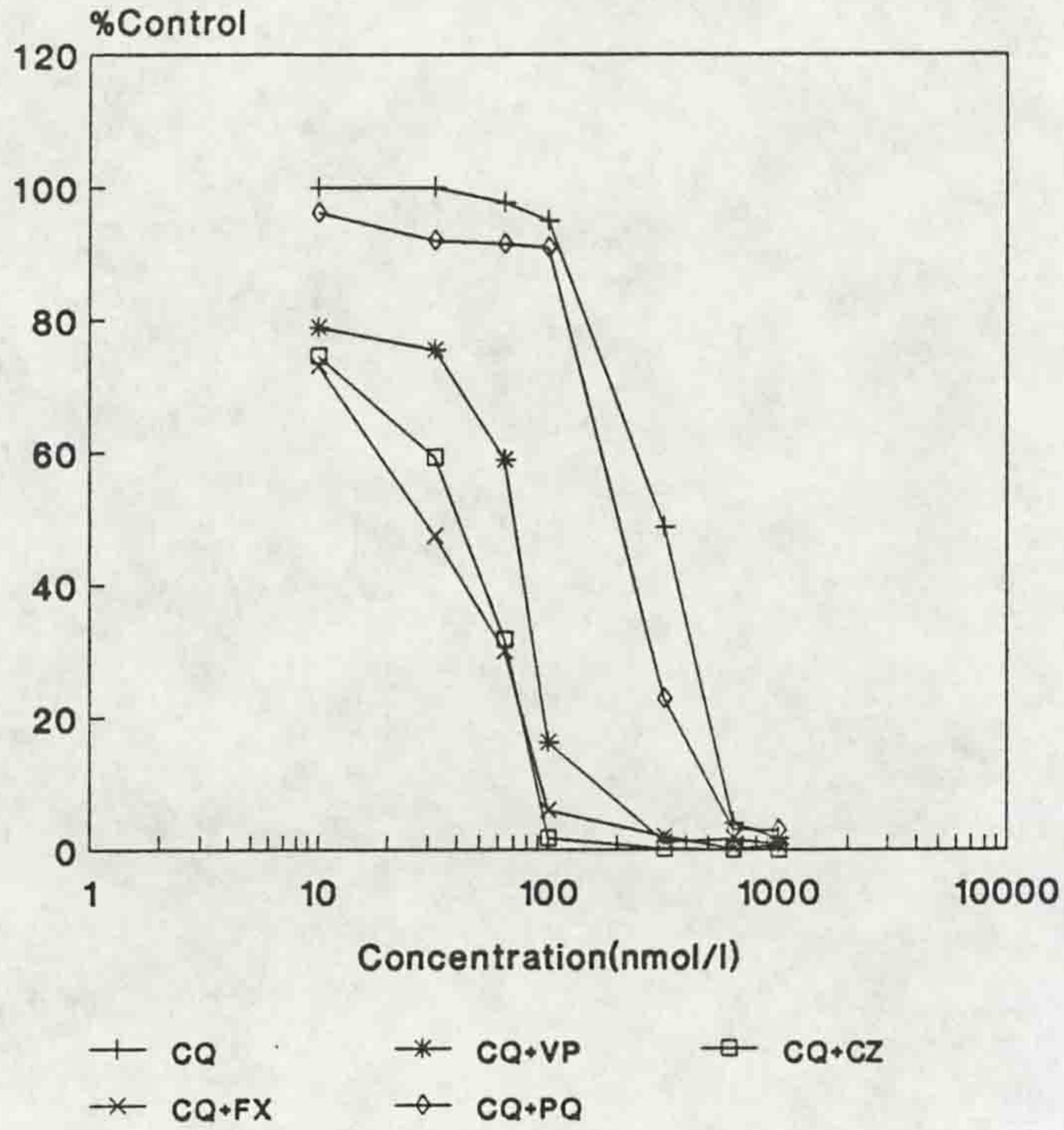
Table 4.3.6 **Summary of results of drug combinations against the $T_{9.96}$, W2-mef and FCR_3 strains of *P. falciparum*, employing [3H] hypoxanthine in 48-hour assays**

An: Antagonism; Po: Potentiation; Ad: Additive effect

Drug	Chloroquine	Mefloquine	Halofantrine
Verapamil	Po. X; An. *	An. +	An. *
Chlropromazine	An. *	An. +	An. *
Fluoxetine	An. *	Ad. +	An. *

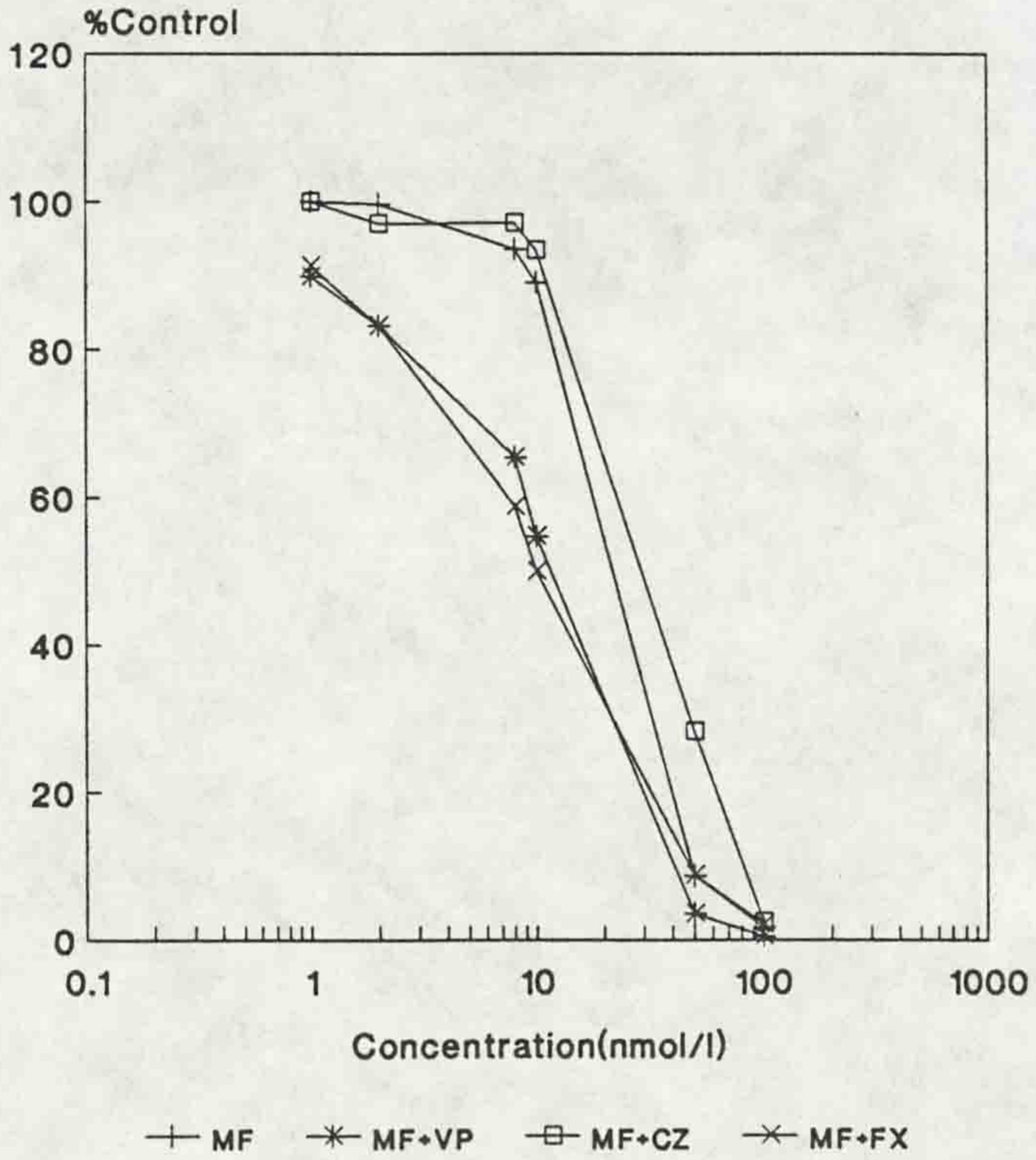
*: $T_{9.96}$; +: W2-mef; X: FCR_3

Fig.4.3.1 Comparative dose response of K1 to CQ, CQ+VP,CQ+CZ,CQ+FX and CQ+PQ.



CQ:chloroquine;VP:verapamil;CZ:chlorpro-mazine;FX:fluoxetine;PQ:praziquantel.

Fig.4.3.2 Comparative dose response of K1 to MF,MF+VP,MF+CZ,and MF+FX.



MF:mefloquine

Fig.4.3.3 Interaction between chloroquine(CQ) and verapamil (VP) on K1 strain.

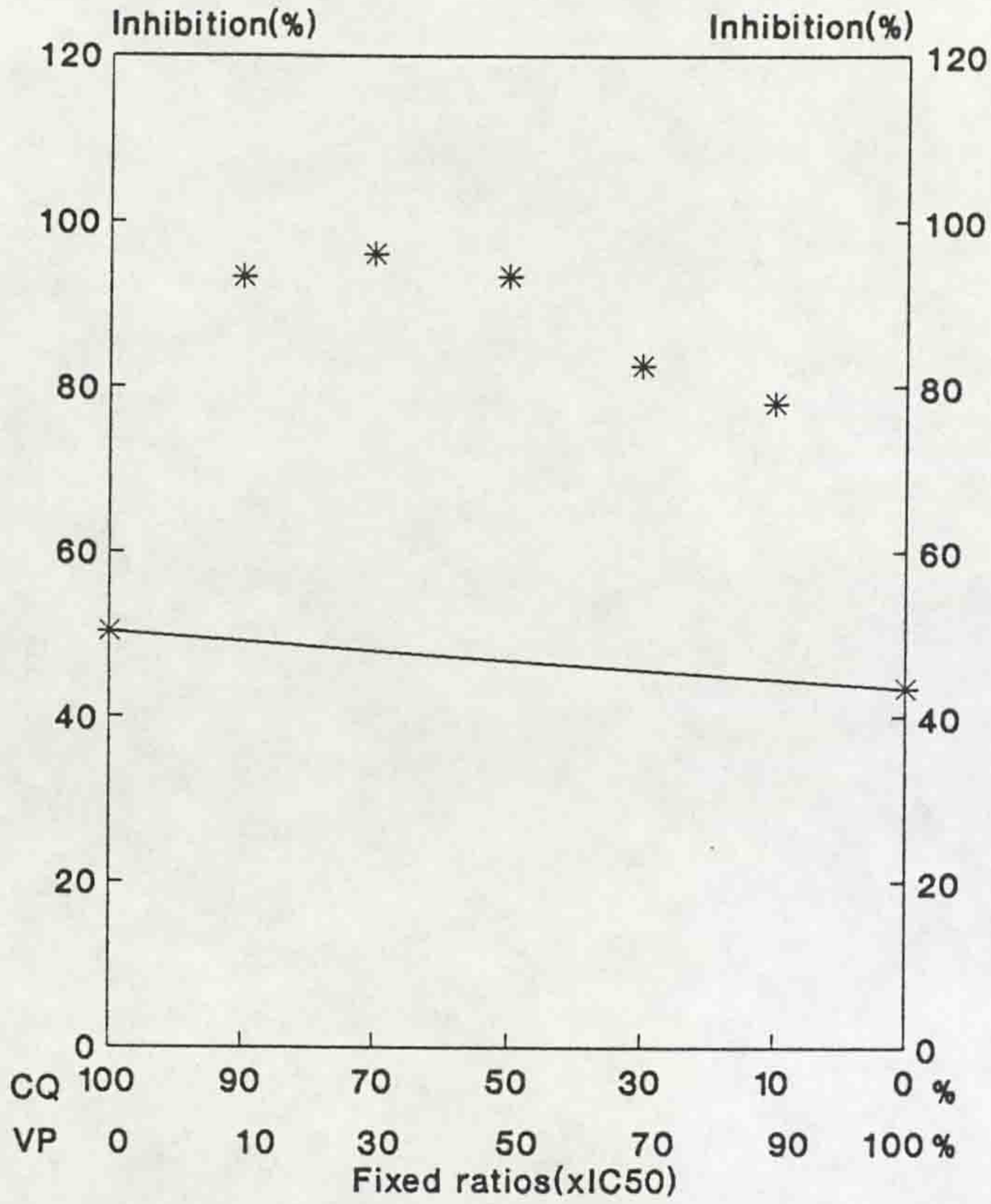


Fig.4.3.4 Interaction between chloroquine(CQ) and chlorpromazine (CZ) on K1 strain.

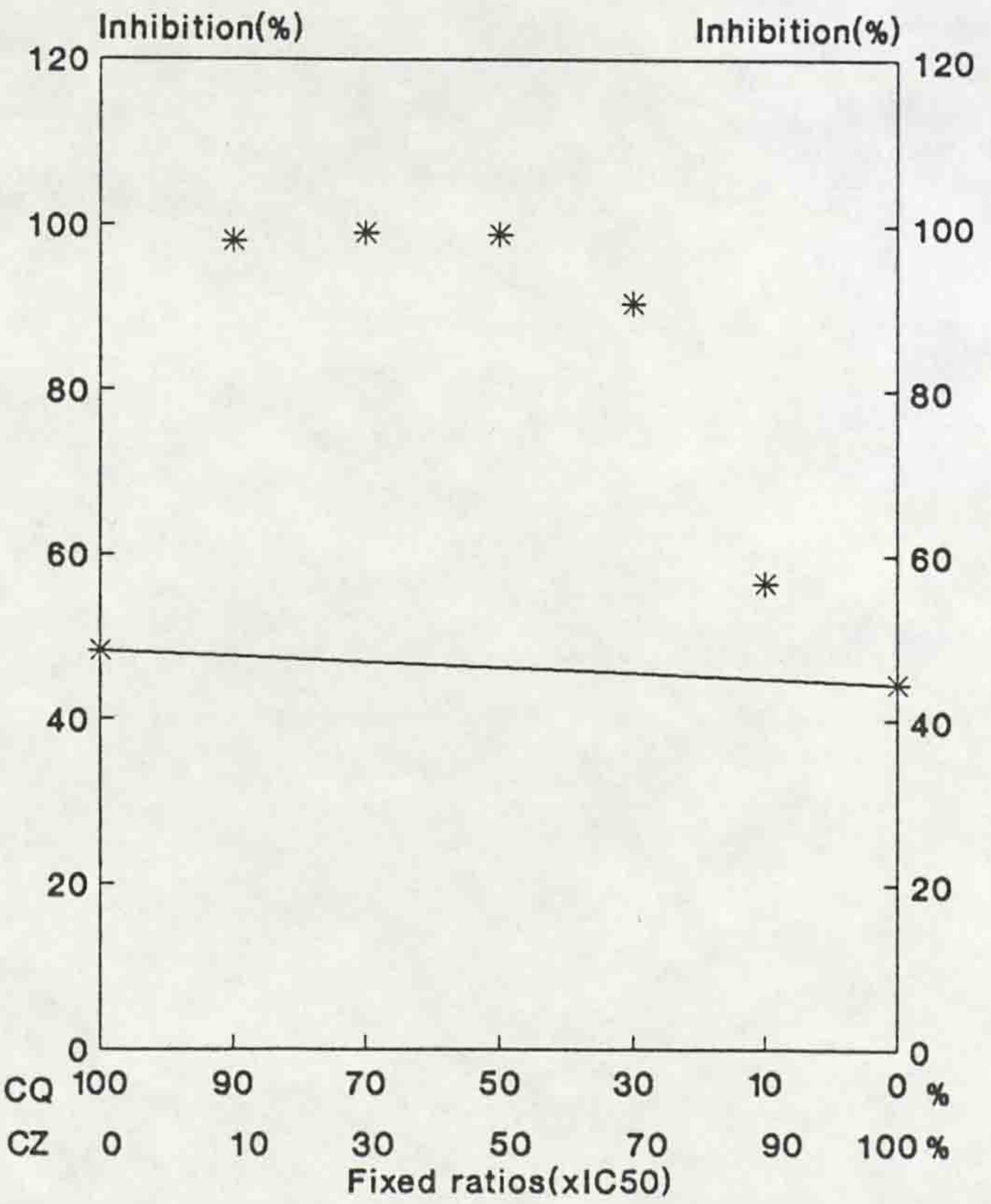


Fig.4.3.5 Interaction between chloroquine(CQ) and fluoxetine (FX) on K1 strain.

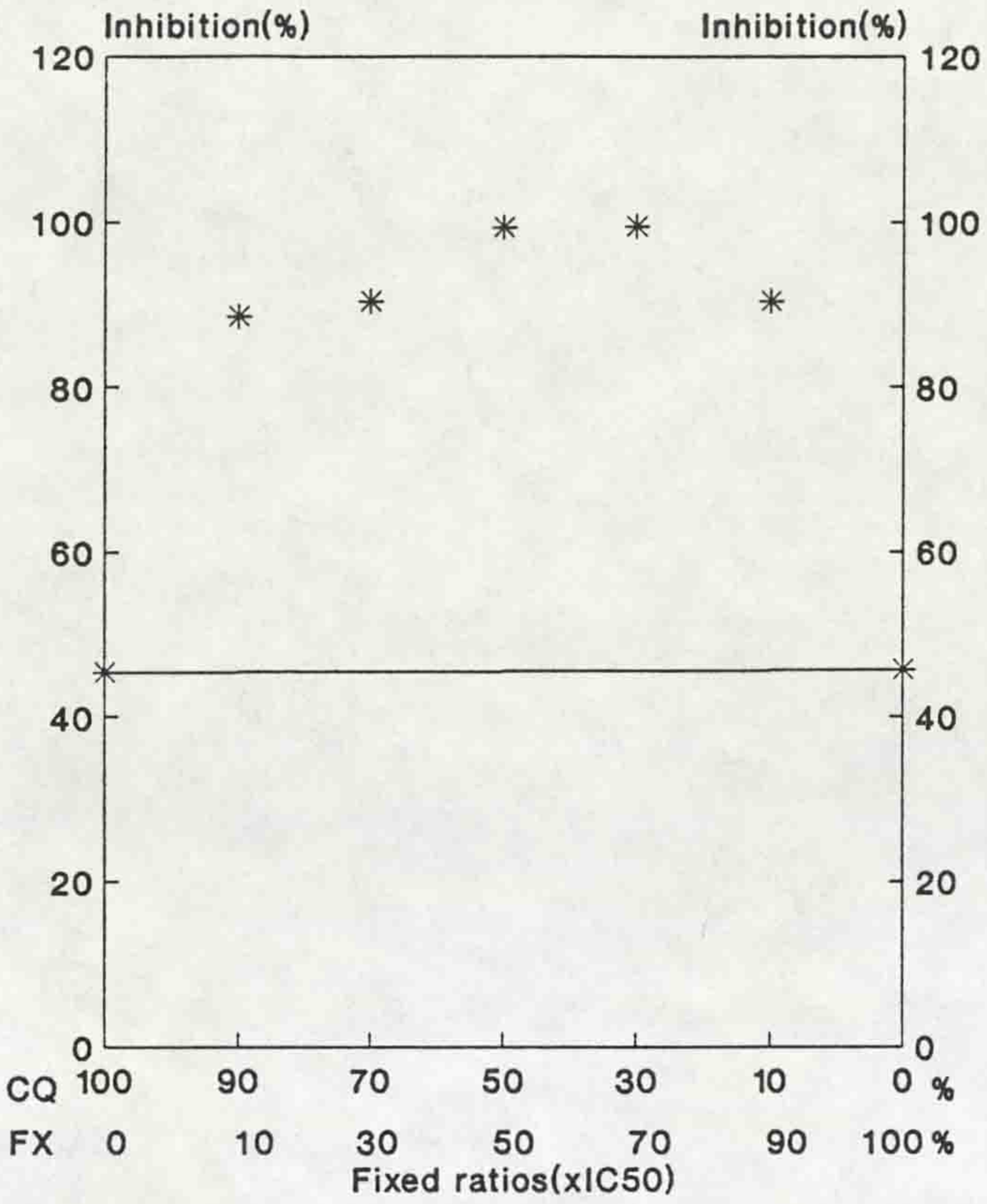


Fig.4.3.6 Interaction between chloroquine(CQ) and praziquantel (PQ) on K1 strain.

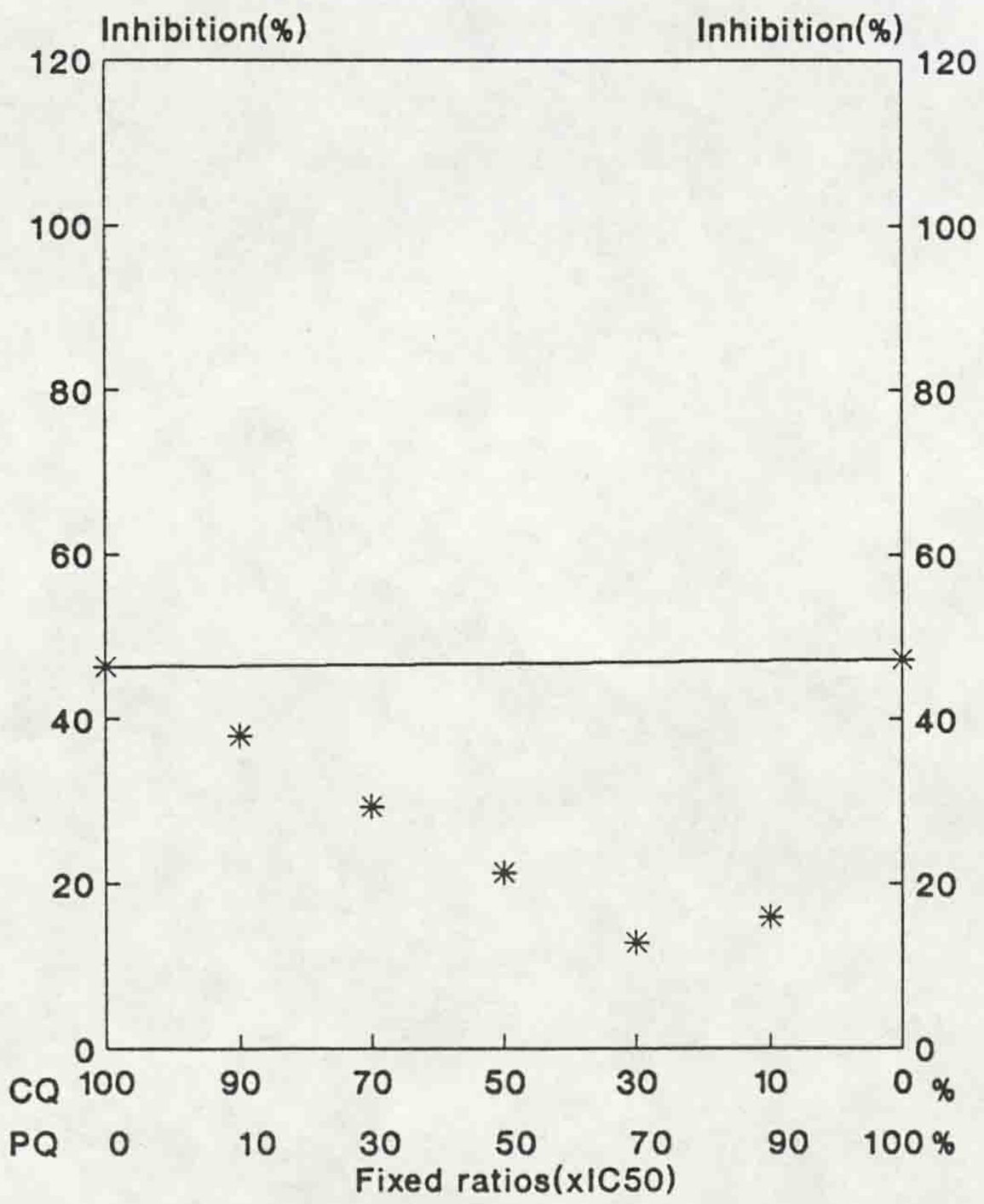


Fig.4.3.7 Interaction between chloroquine(CQ) and verapamil (VP) on FCR3 strain.

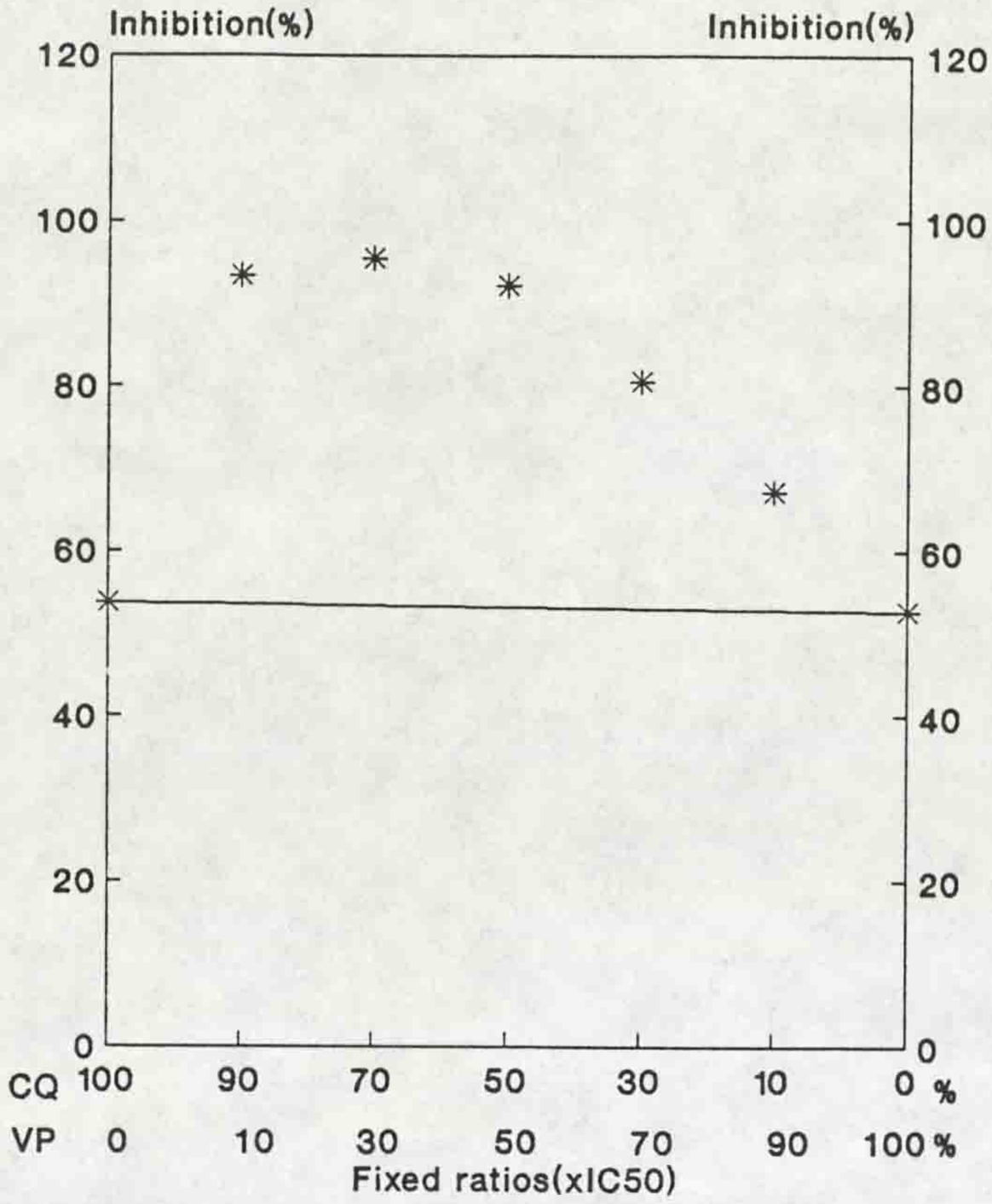


Fig.4.3.8 Interaction between chloroquine(CQ) and verapamil (VP) on T9.96 strain.

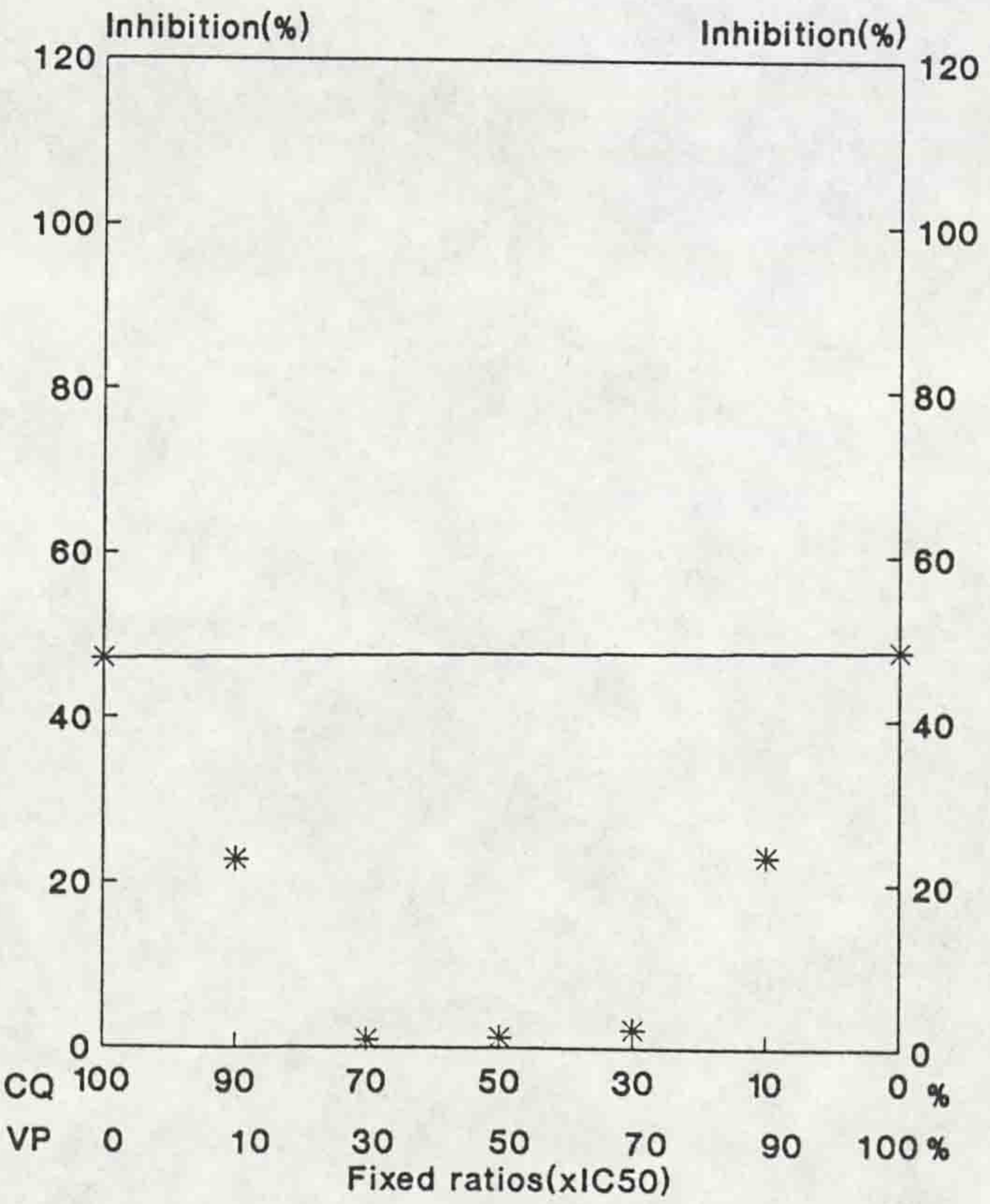


Fig.4.3.9 Interaction between chloroquine(CQ) and chlopromazinen (CZ) on T9.96 strain.

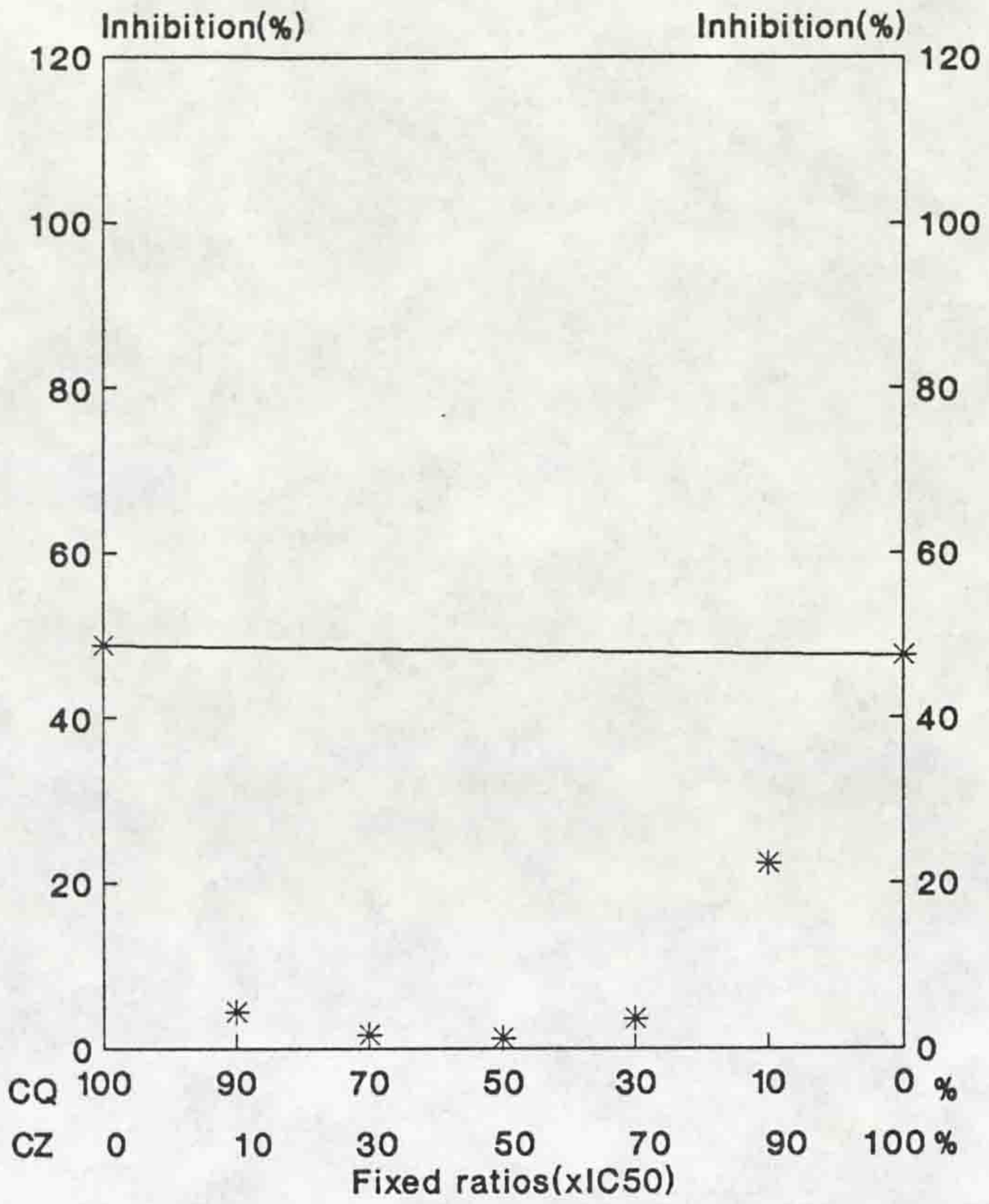


Fig.4.3.10 Interaction between chloroquine(CQ) and fluoxetine (FX) on T9.96 strain.

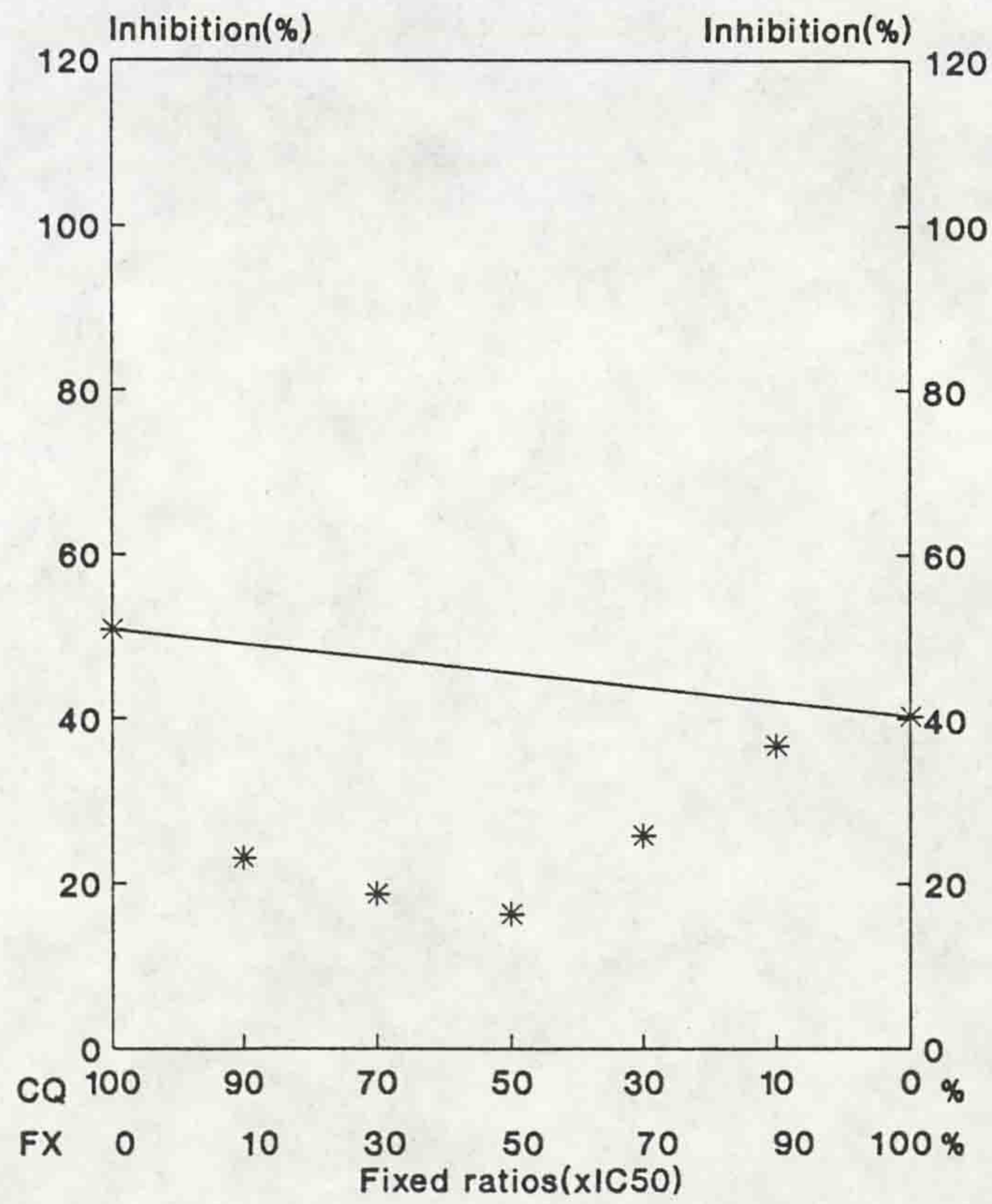


Fig.4.3.11 Interaction between mefloquine(MF) and verapamil (VP) on K1 strain.

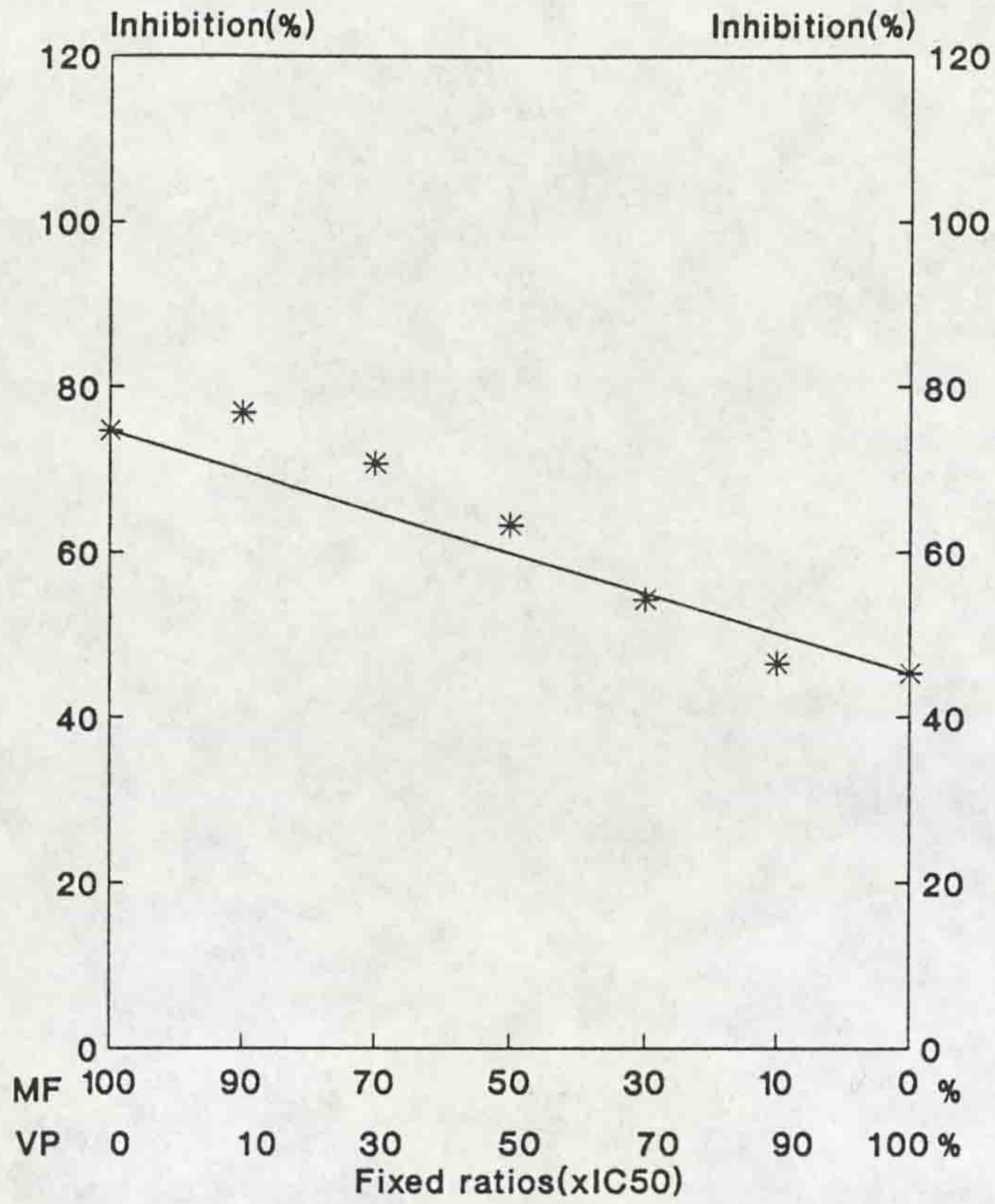


Fig.4.3.12 Interaction between mefloquine(MF) and chlorpromazine (CZ) on K1 strain.

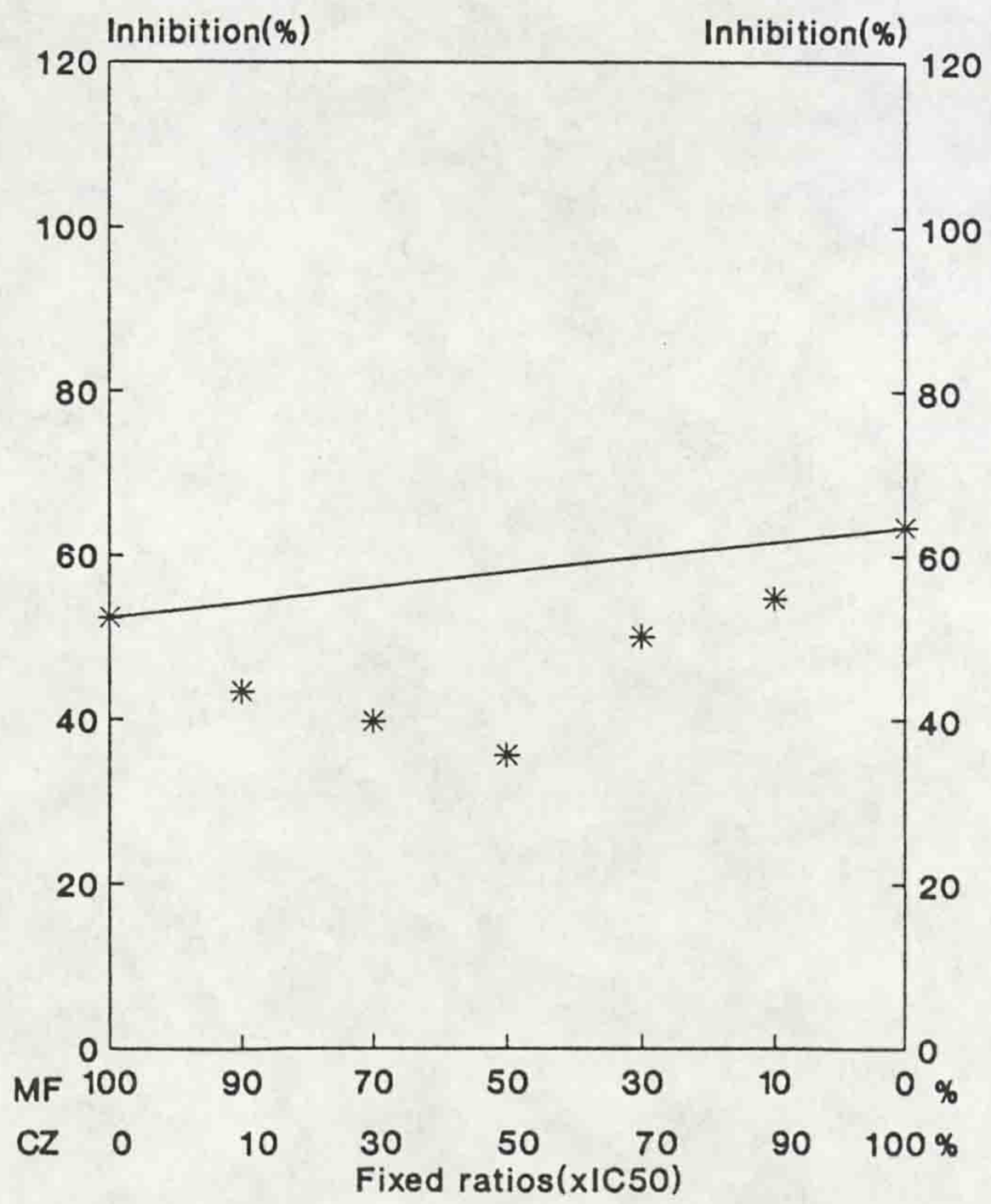


Fig.4.3.13 Interaction between mefloquine(MF) and fluoxetine (FX) on K1 strain.

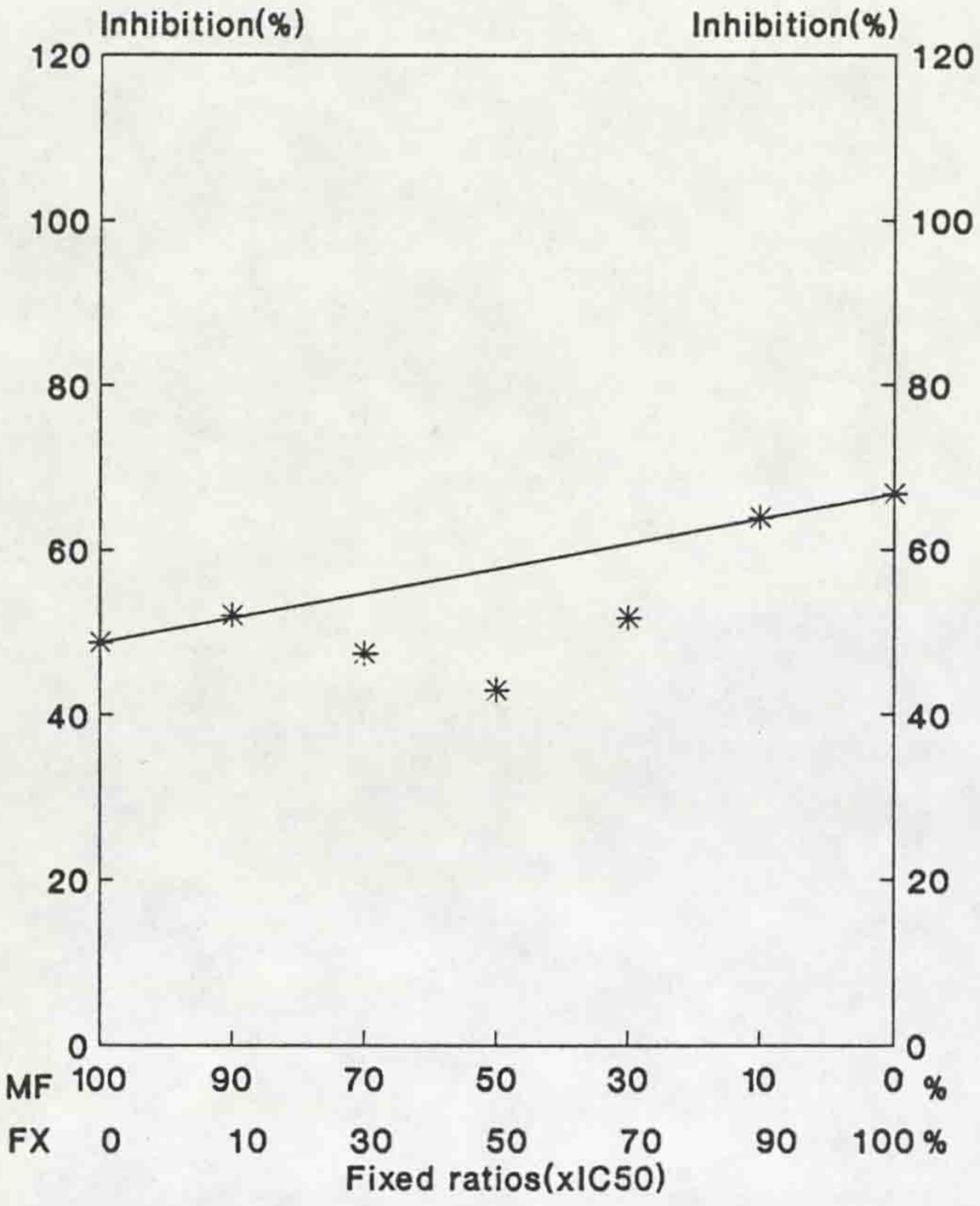


Fig.4.3.14 Interaction between mefloquine(MF) and praziquantel (PQ) on K1 strain.

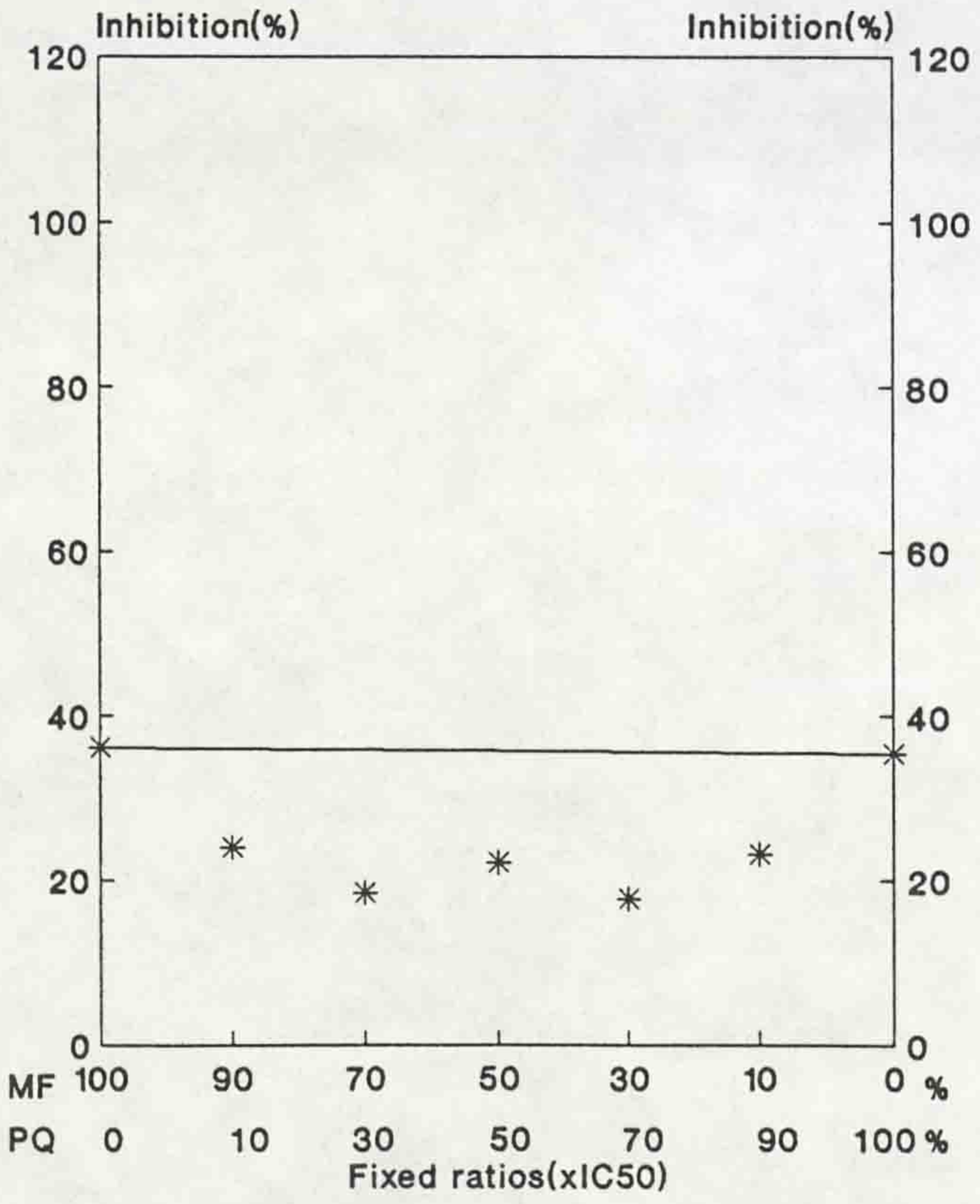


Fig.4.3.15 Interaction between mefloquine(MF) and verapamil(VP) on W2-mef strain.

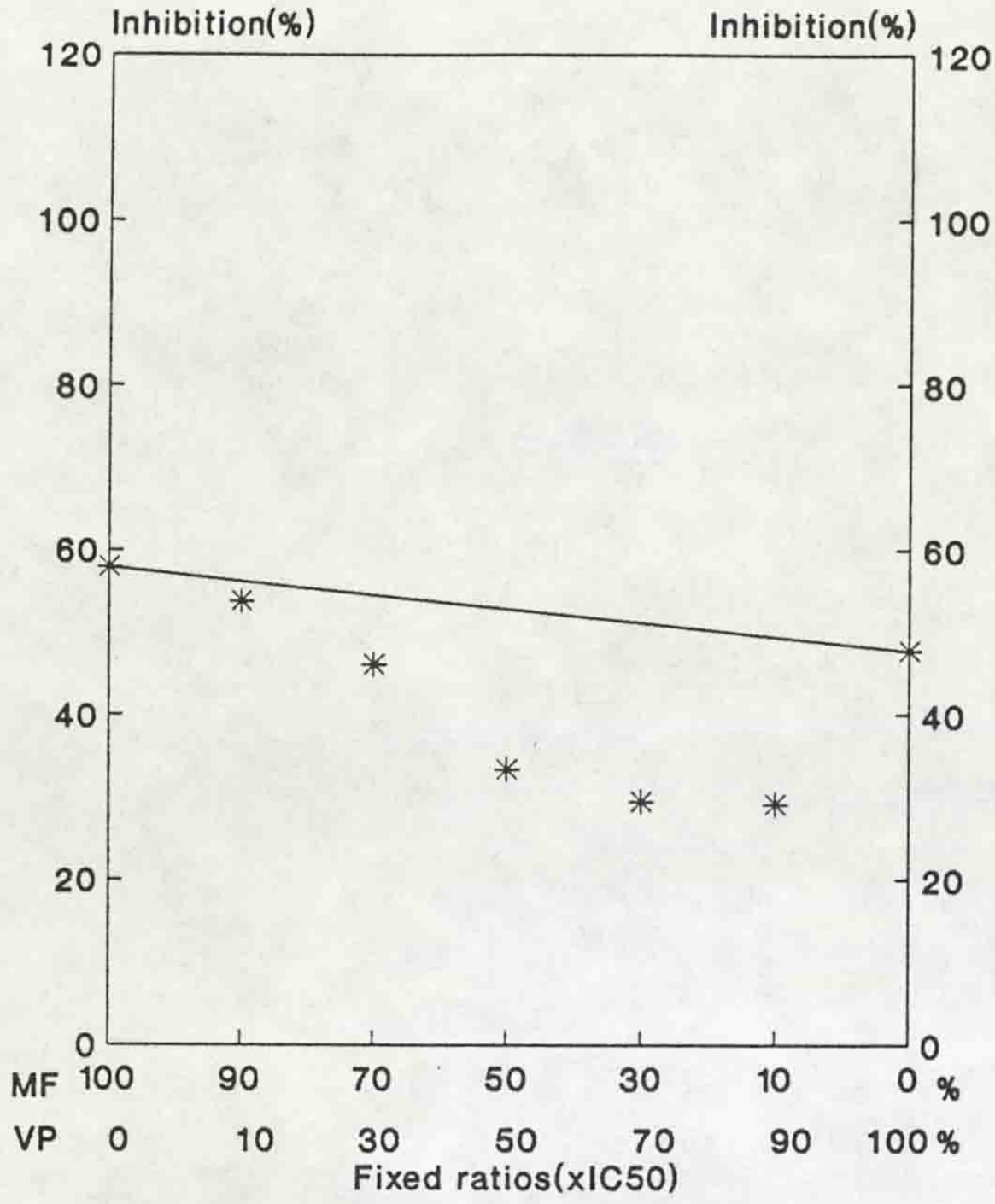


Fig.4.3.16 Interaction between mefloquine(MF) and chlorpromazine (CZ) on W2-mef strain.

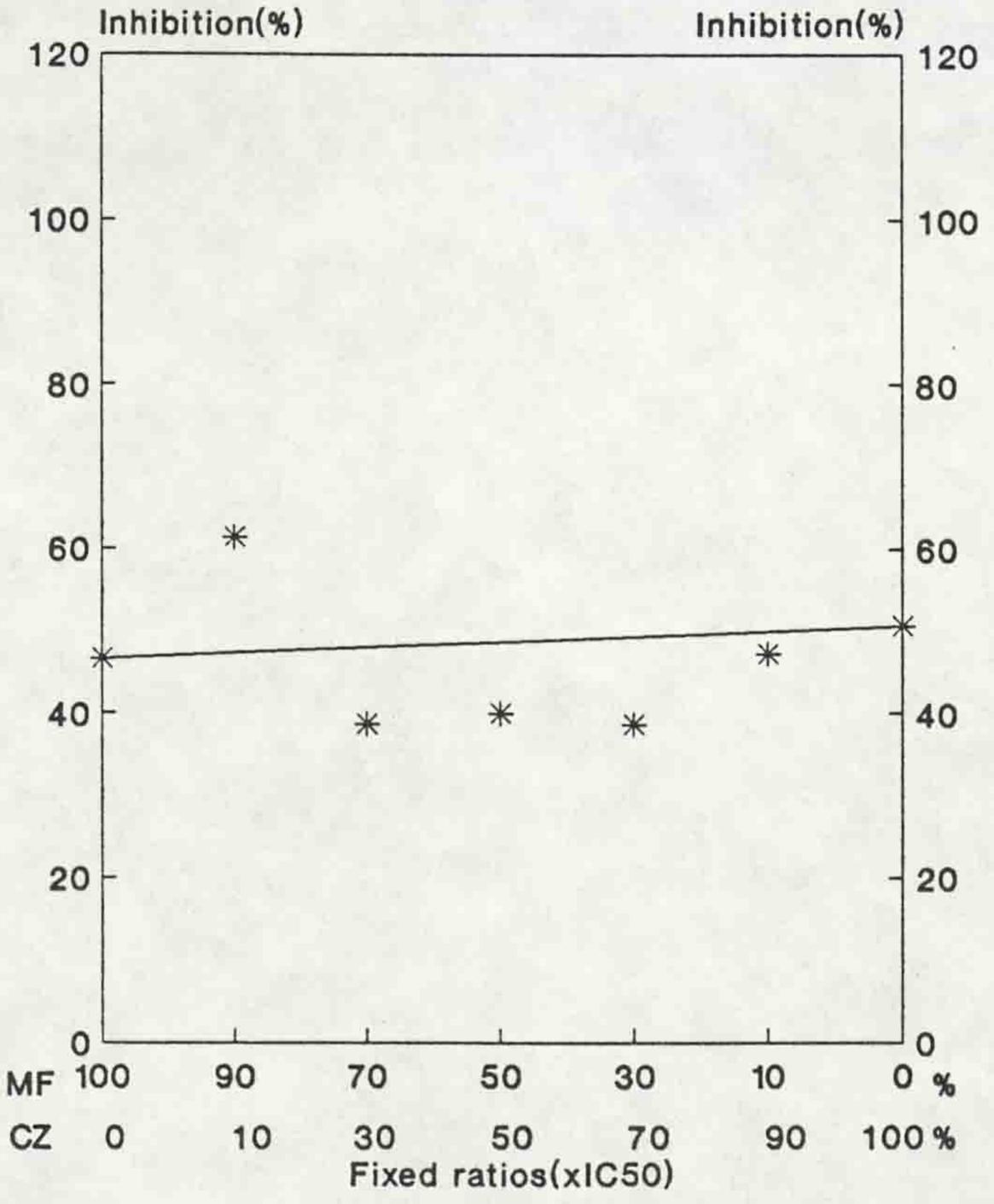


Fig.4.3.17 Interaction between mefloquine(MF) and fluoxetine (FX) on W2-mef strain.

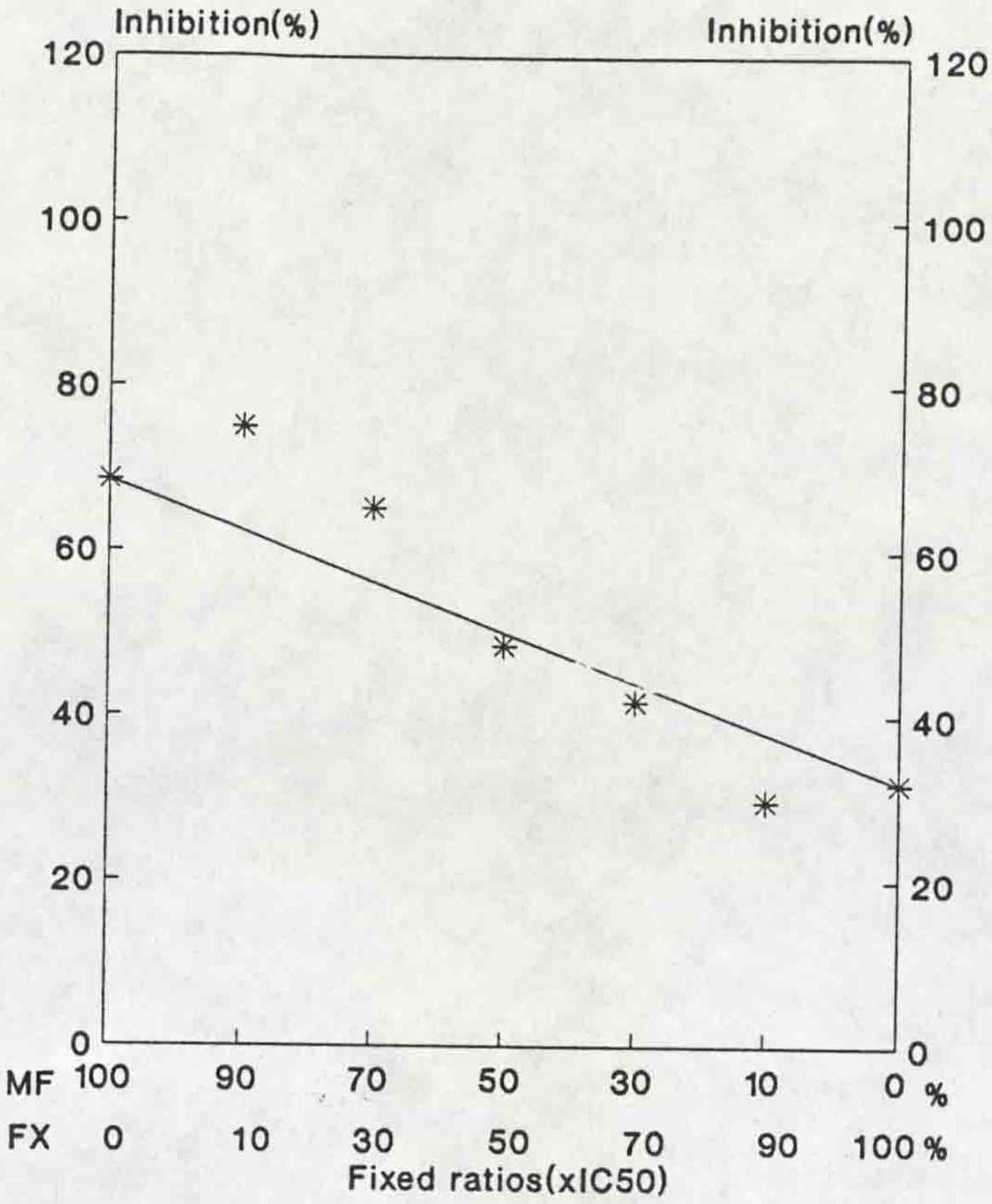


Fig.4.3.18 Interaction between halofantrine(HF) and verapamil (VP) on K1 strain.

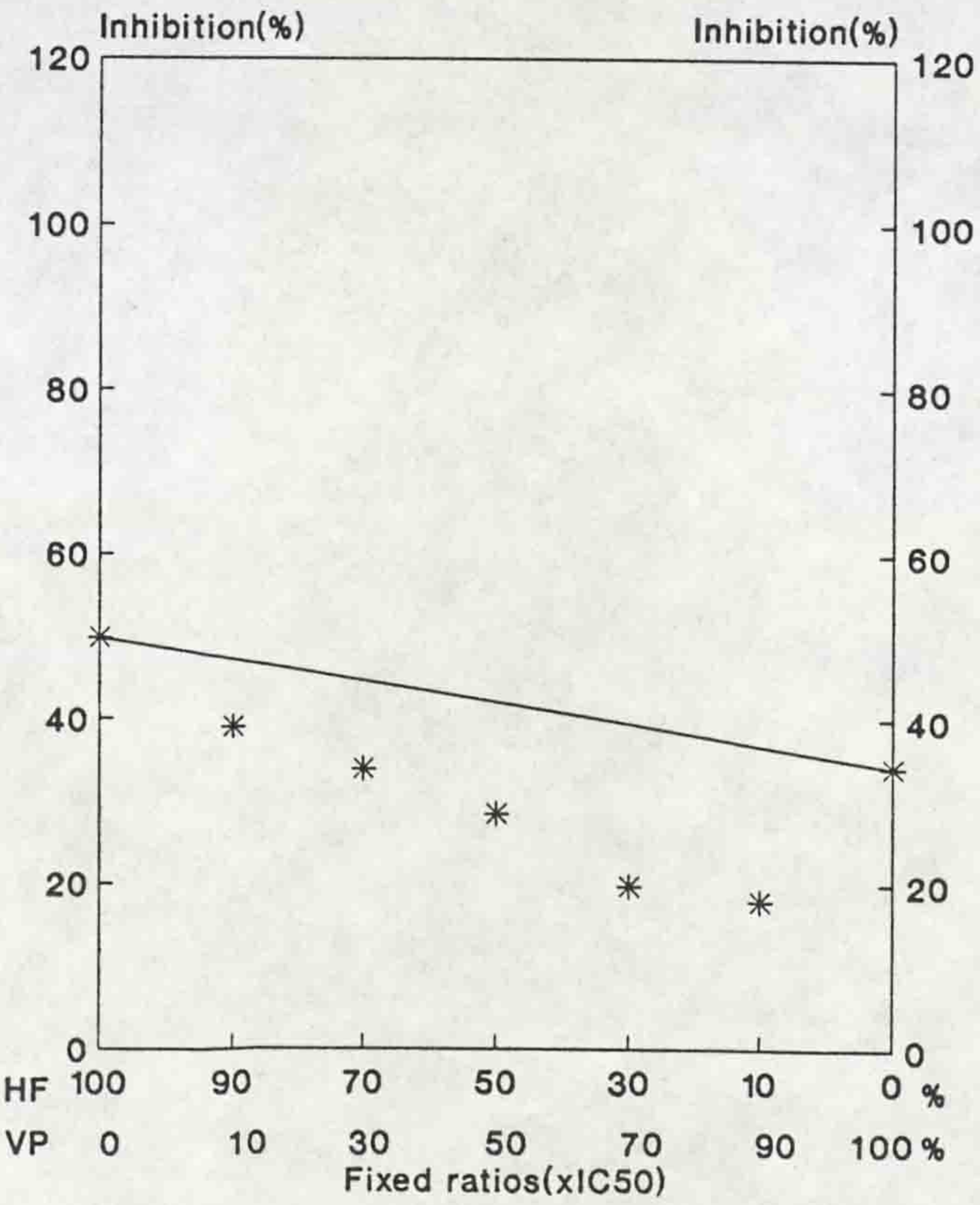


Fig.4.3.19 Interaction between halofantrine(HF) and chlorpromazine (CZ) on K1 strain.

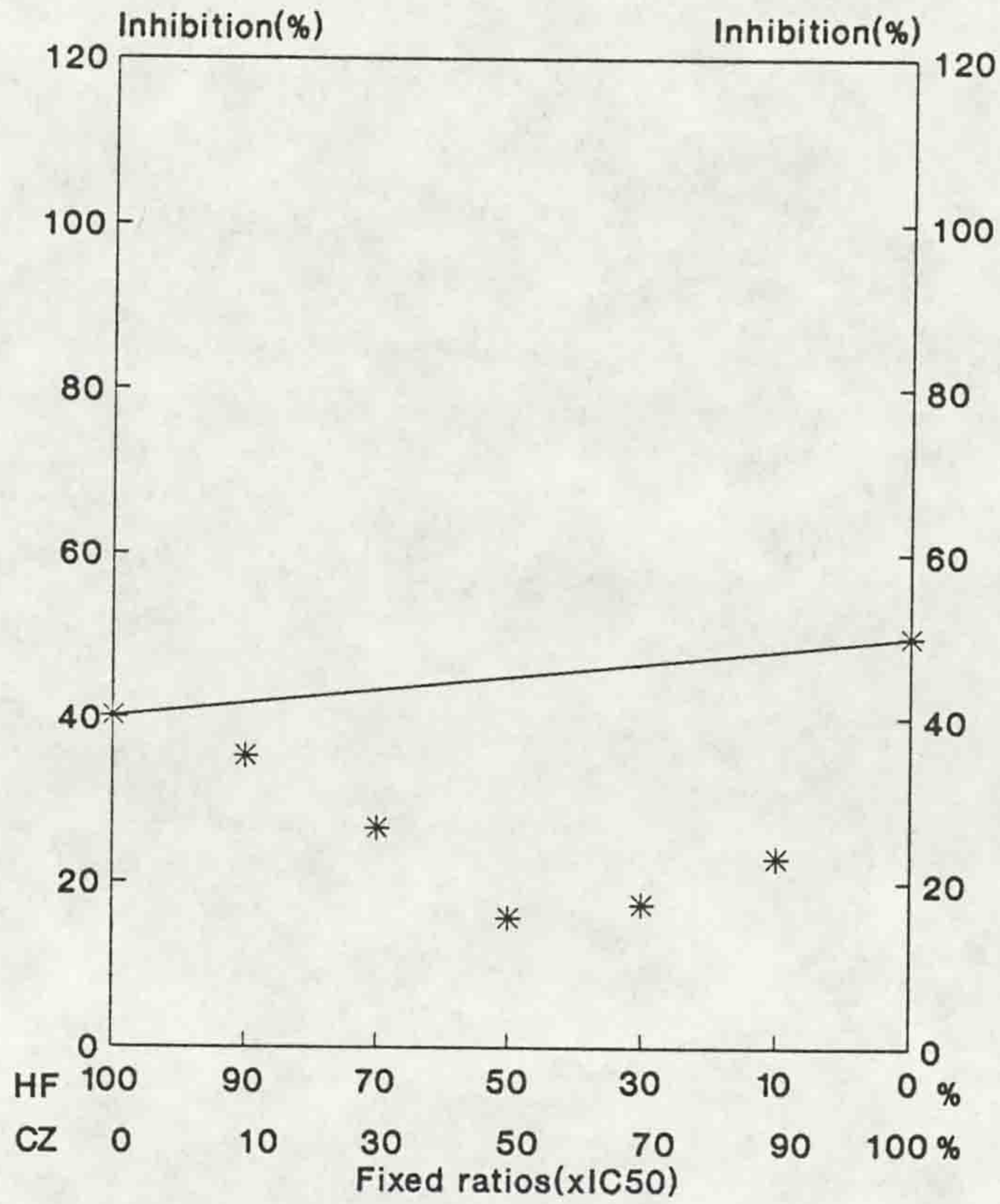


Fig.4.3.20 Interaction between halofantrine(HF) and fluoxetine (FX) on K1 strain.

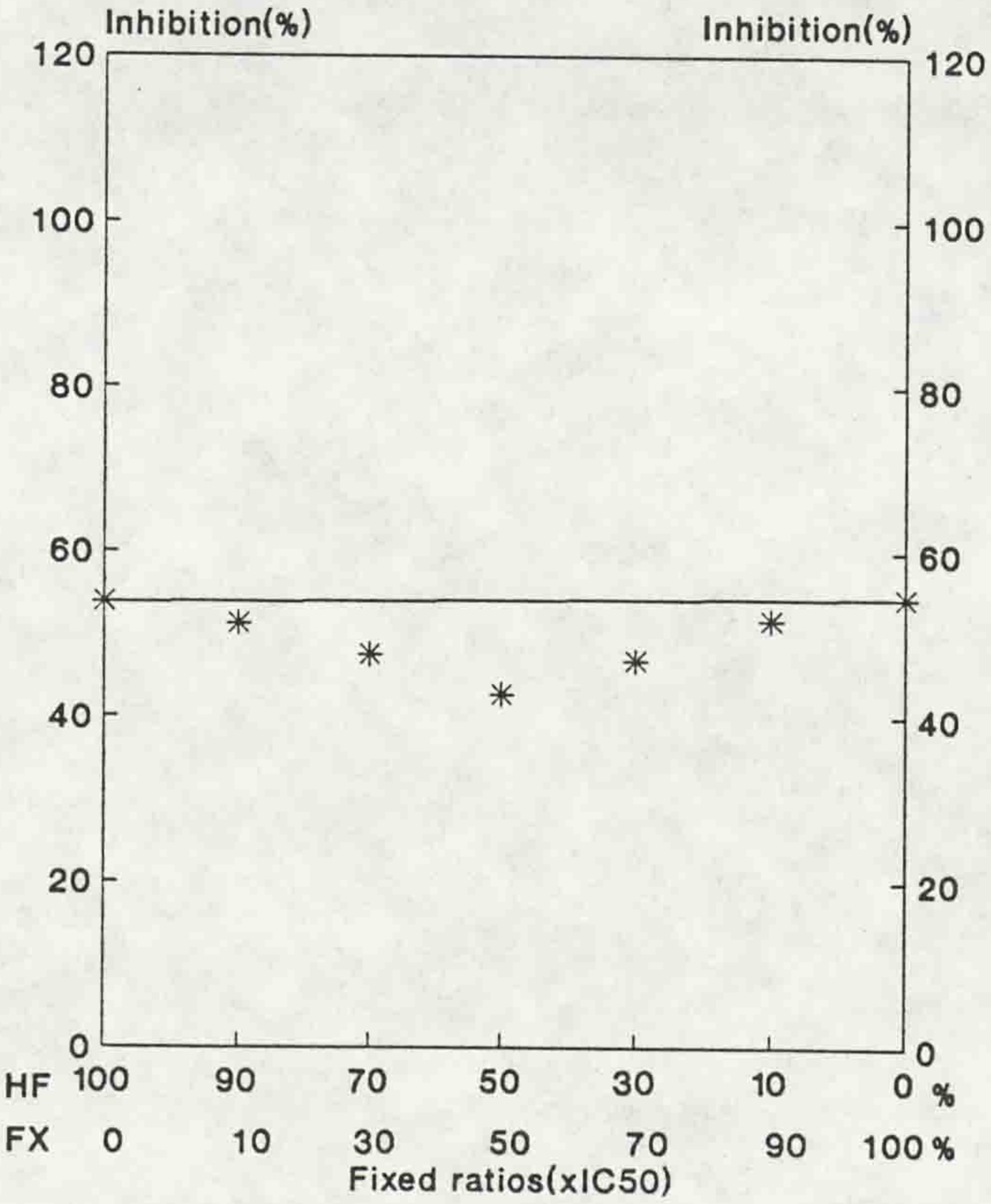


Fig.4.3.21 Interaction between halofantrine(HF) and verapamil (VP) on T9.96 strain.

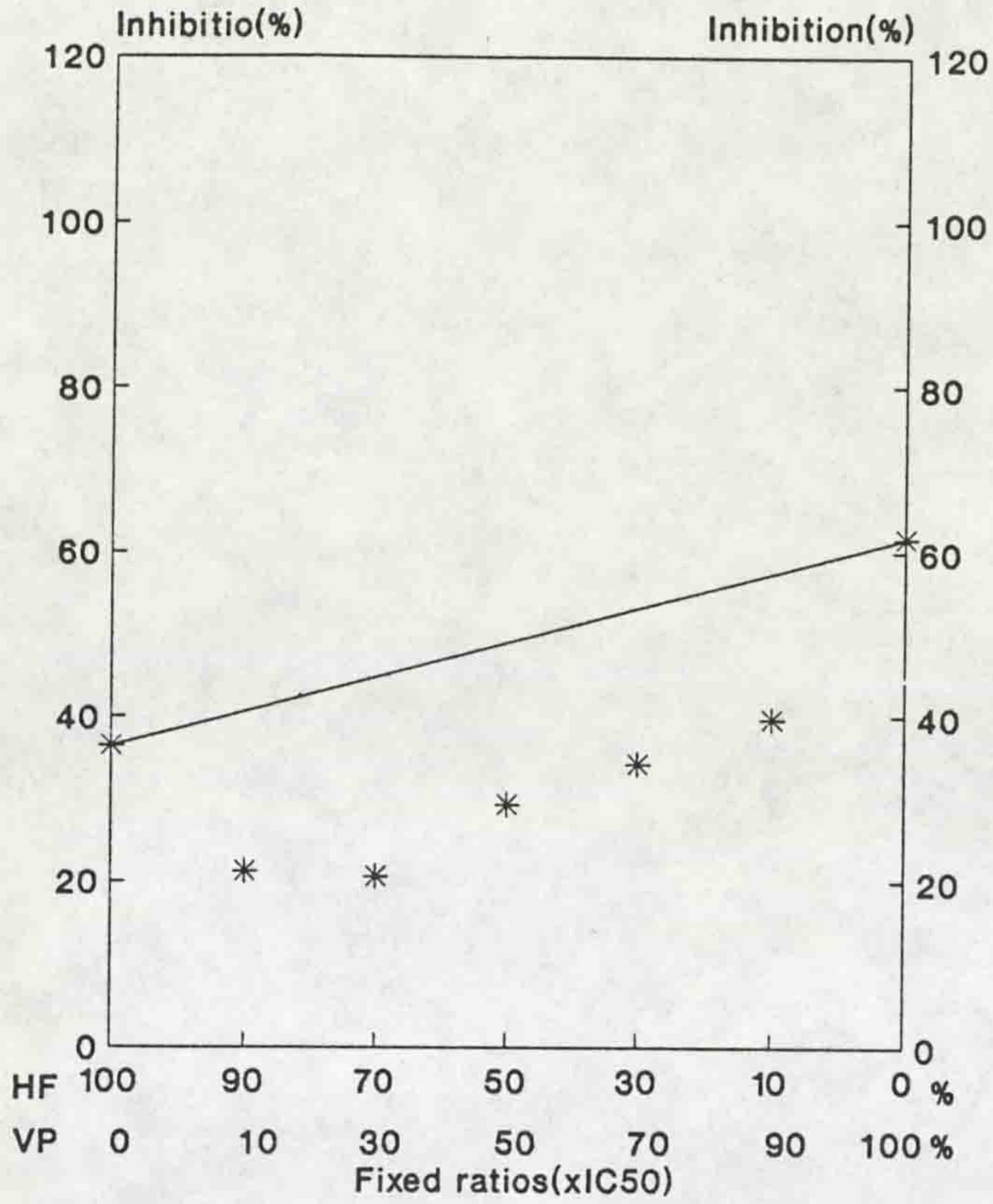


Fig.4.3.22 Interaction between halofantrine(HF) and chlorpromazine (CZ) on T9.96 strain.

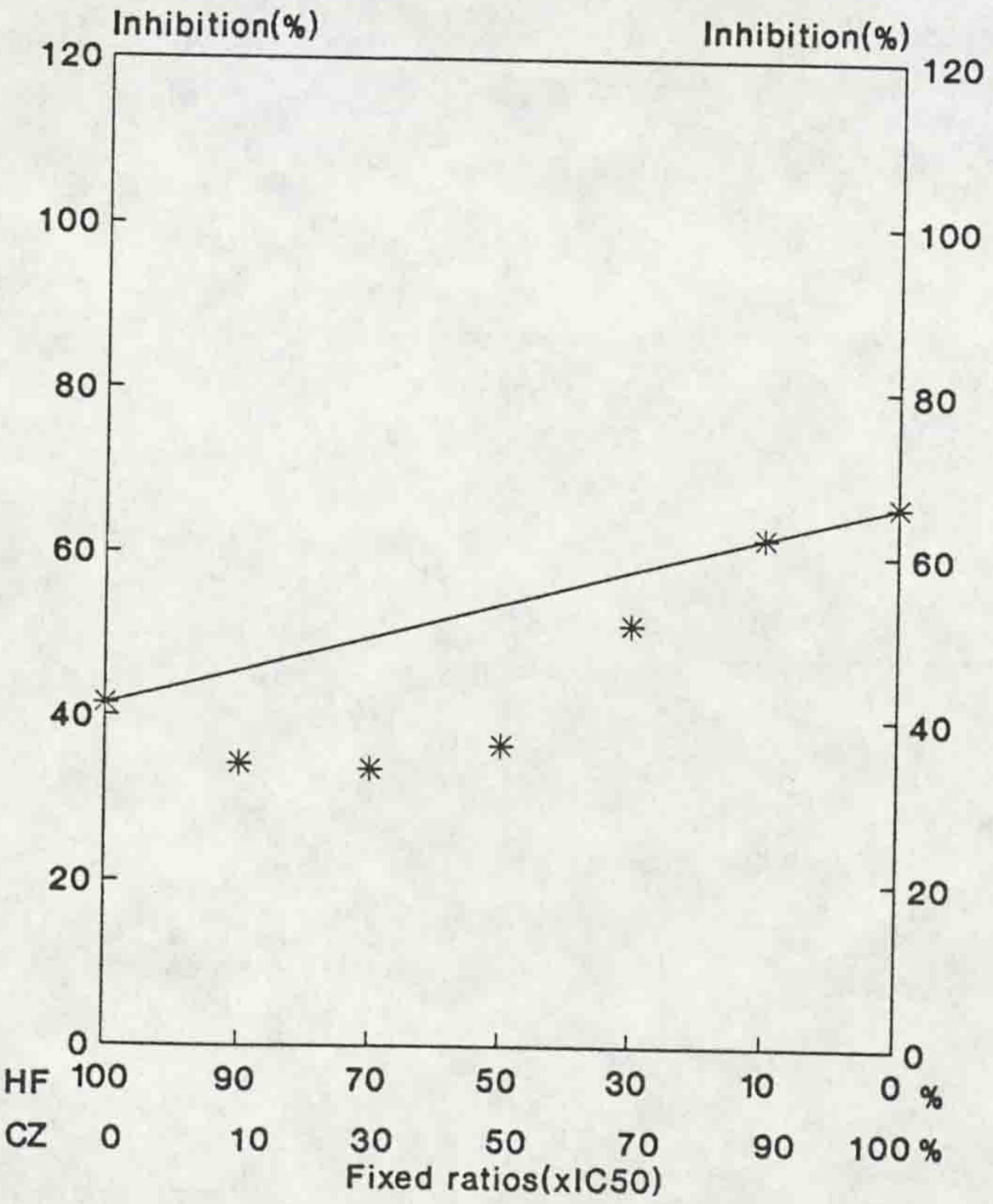


Fig.4.3.23 Interaction between halofantrine(HF) and fluoxetine (FX) on T9.96 strain.

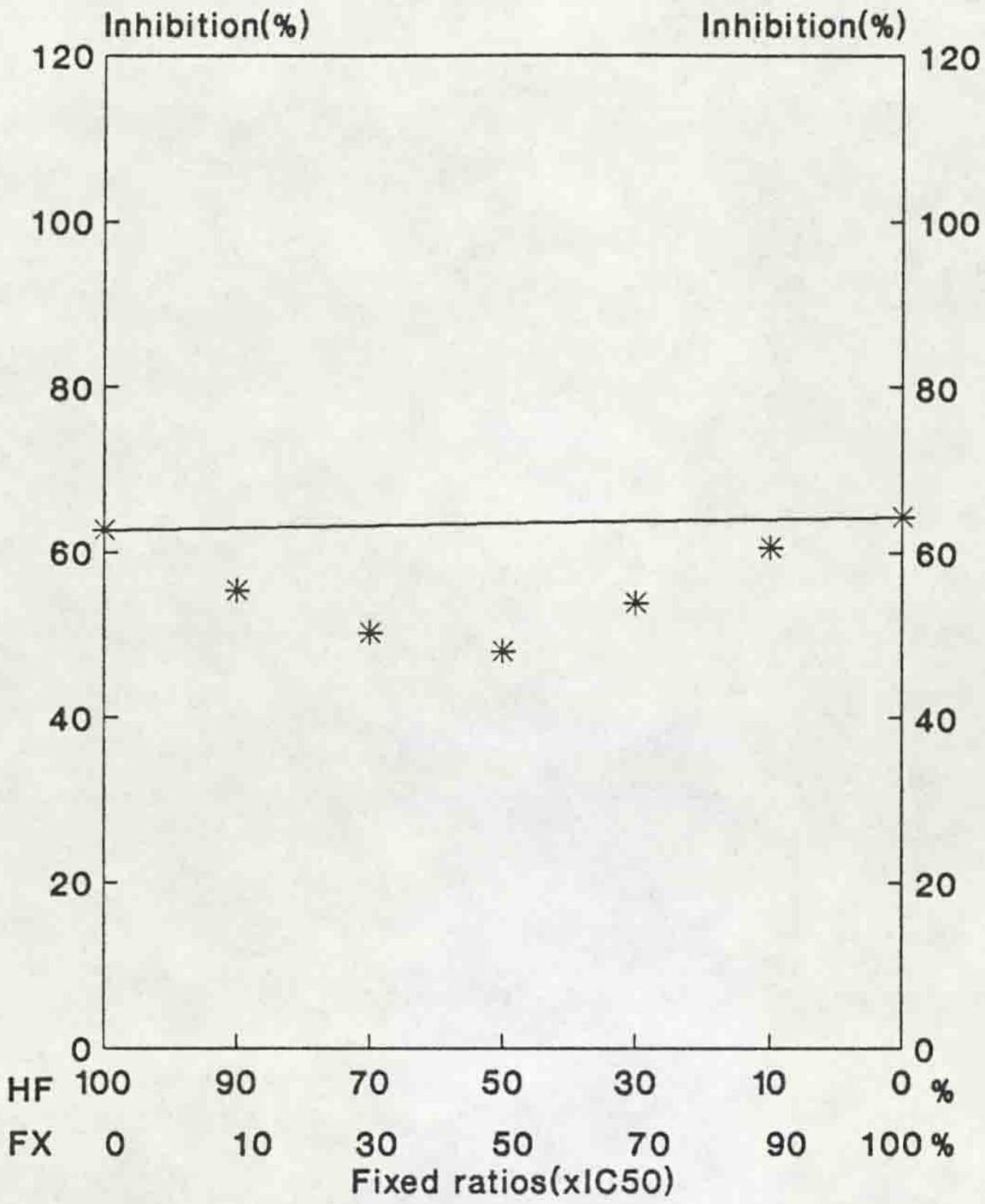


Fig.4.3.24 Interaction between qinghaosu(QHS) and verapamil (VP) on K1 strain.

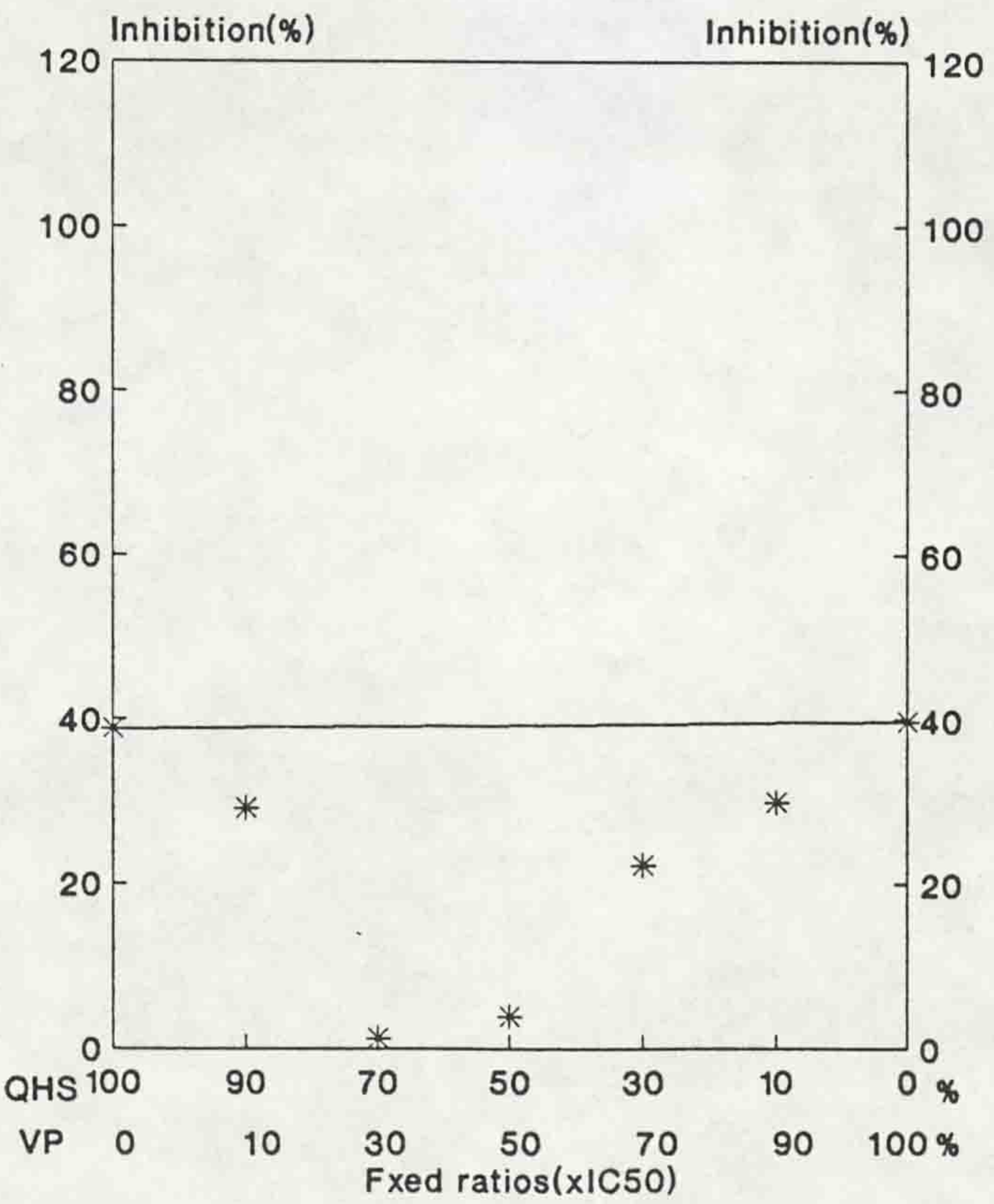


Fig.4.3.25 Interaction between qinghaosu(QHS) and chlorpromazine (CZ) on K1 strain.

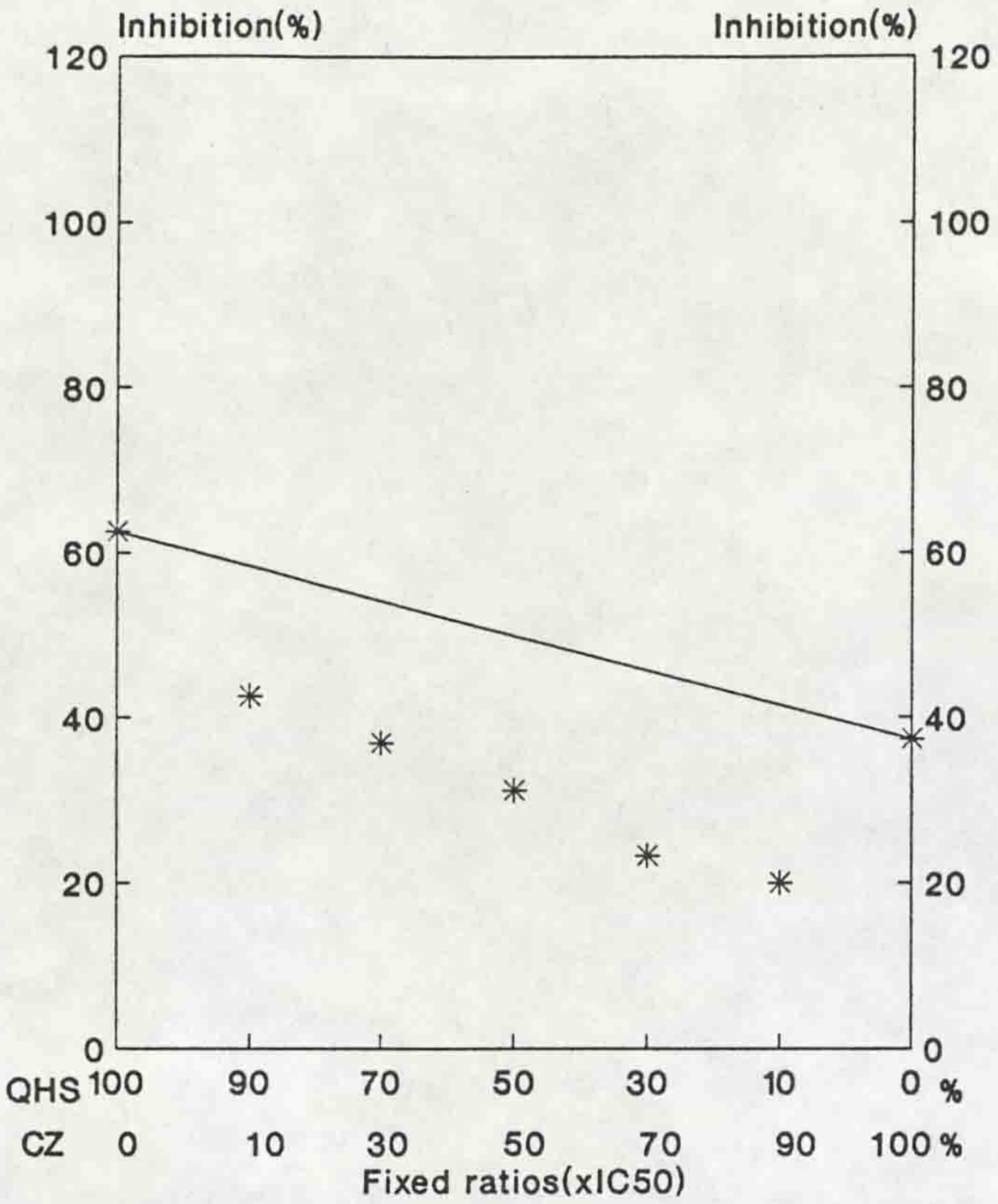


Fig.4.3.26 Interaction between qinghoasu(QHS) and fluoxetine (FX) on K1 strain.

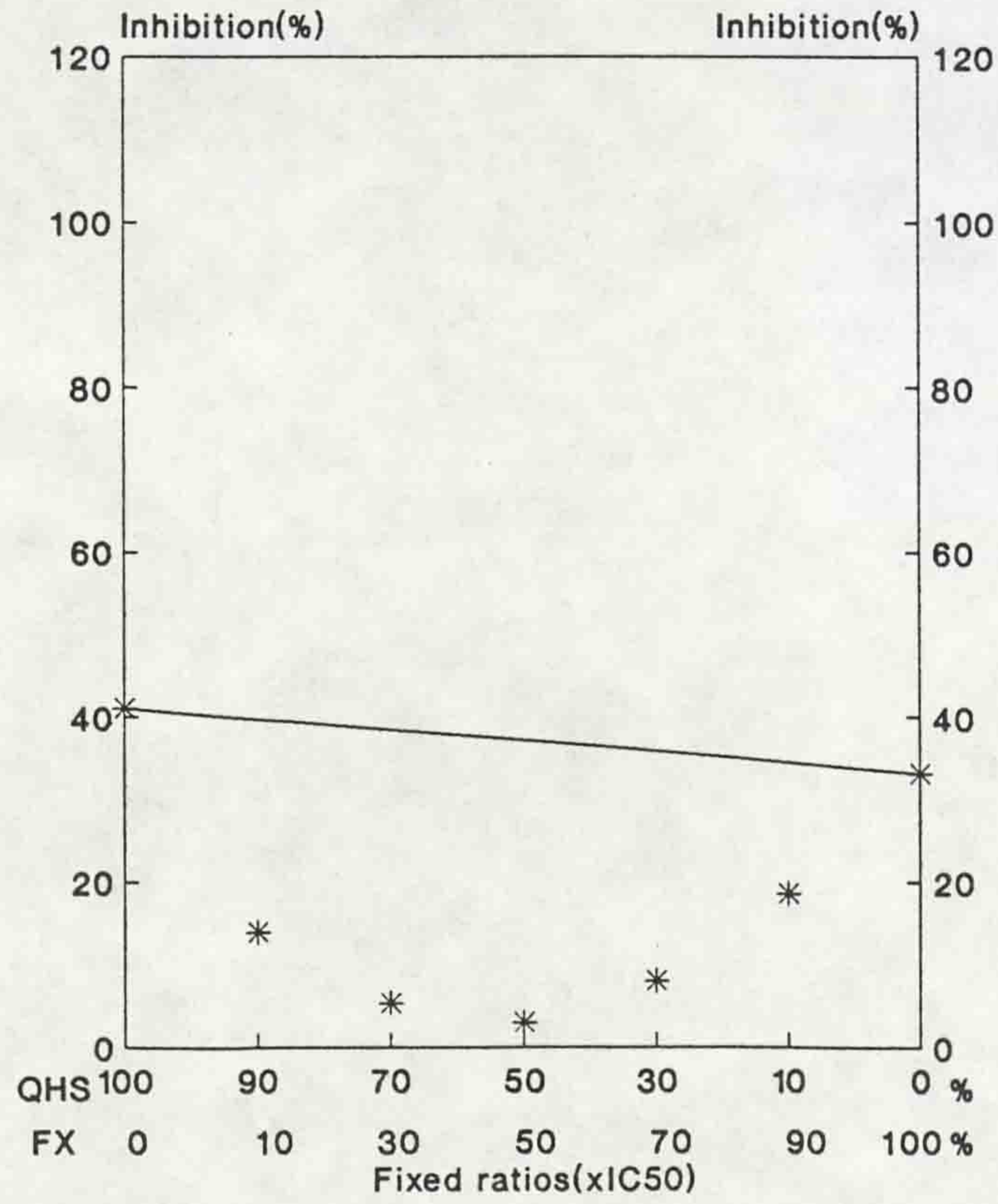


Fig.4.3.27 Interaction between qinghaosu(QHS) and praziquantel (PQ) on K1 strain.

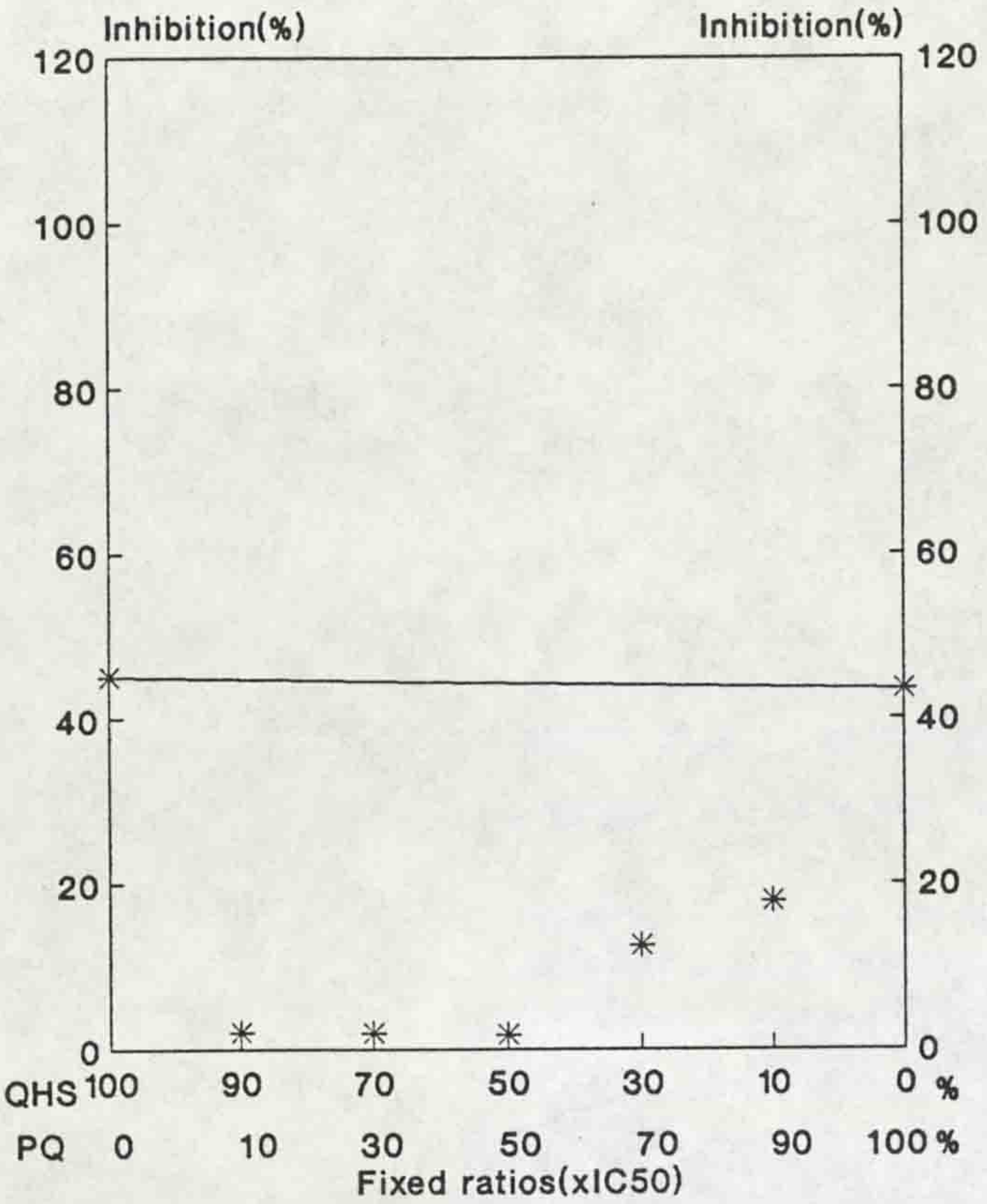


Fig.4.3.28 Interaction between chloroquine(CQ) and quinine(QN) on K1 strain.

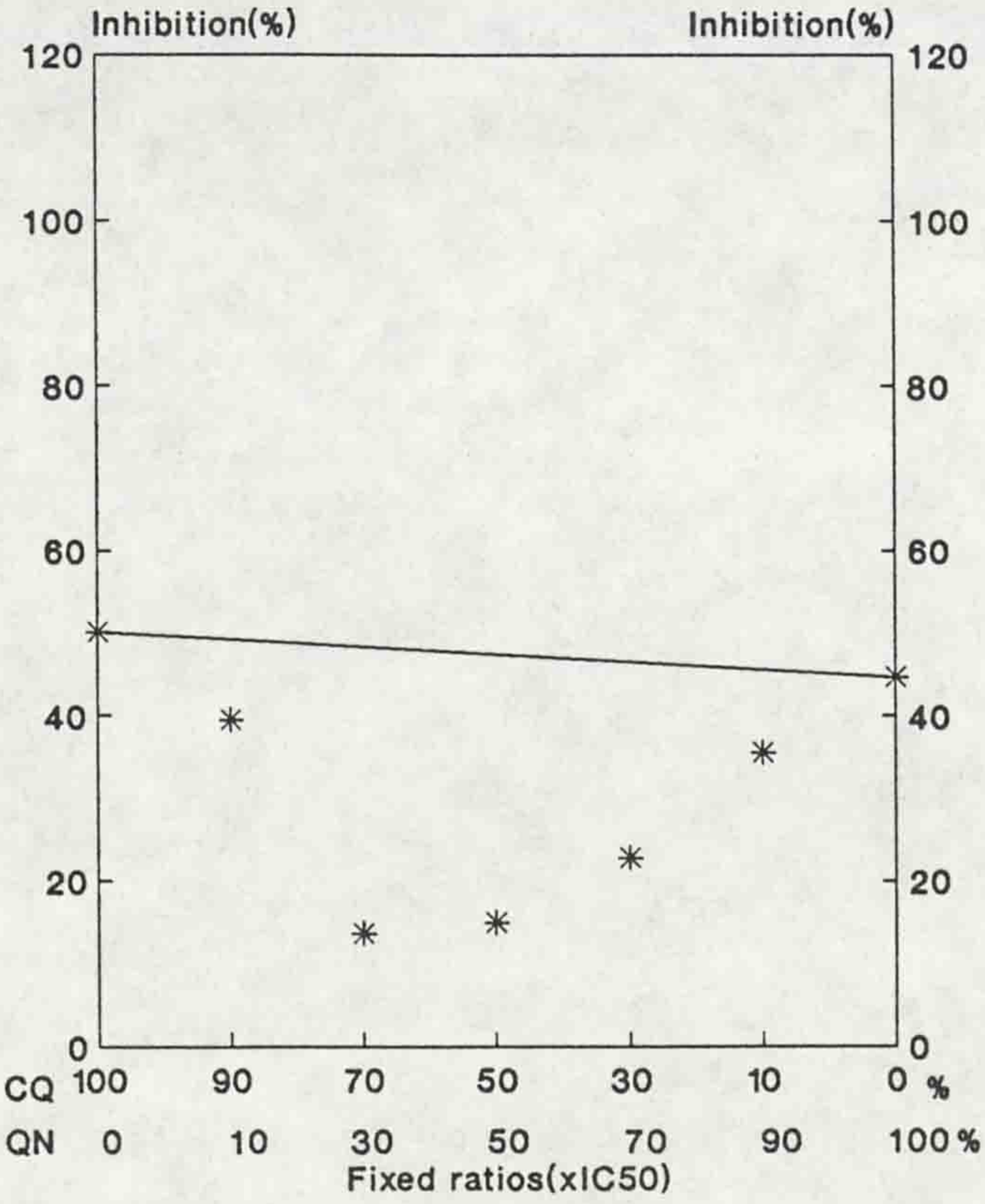


Fig.4.3.29 Interaction between chloroquine(CQ) and quinidine (QD) on K1 strain.

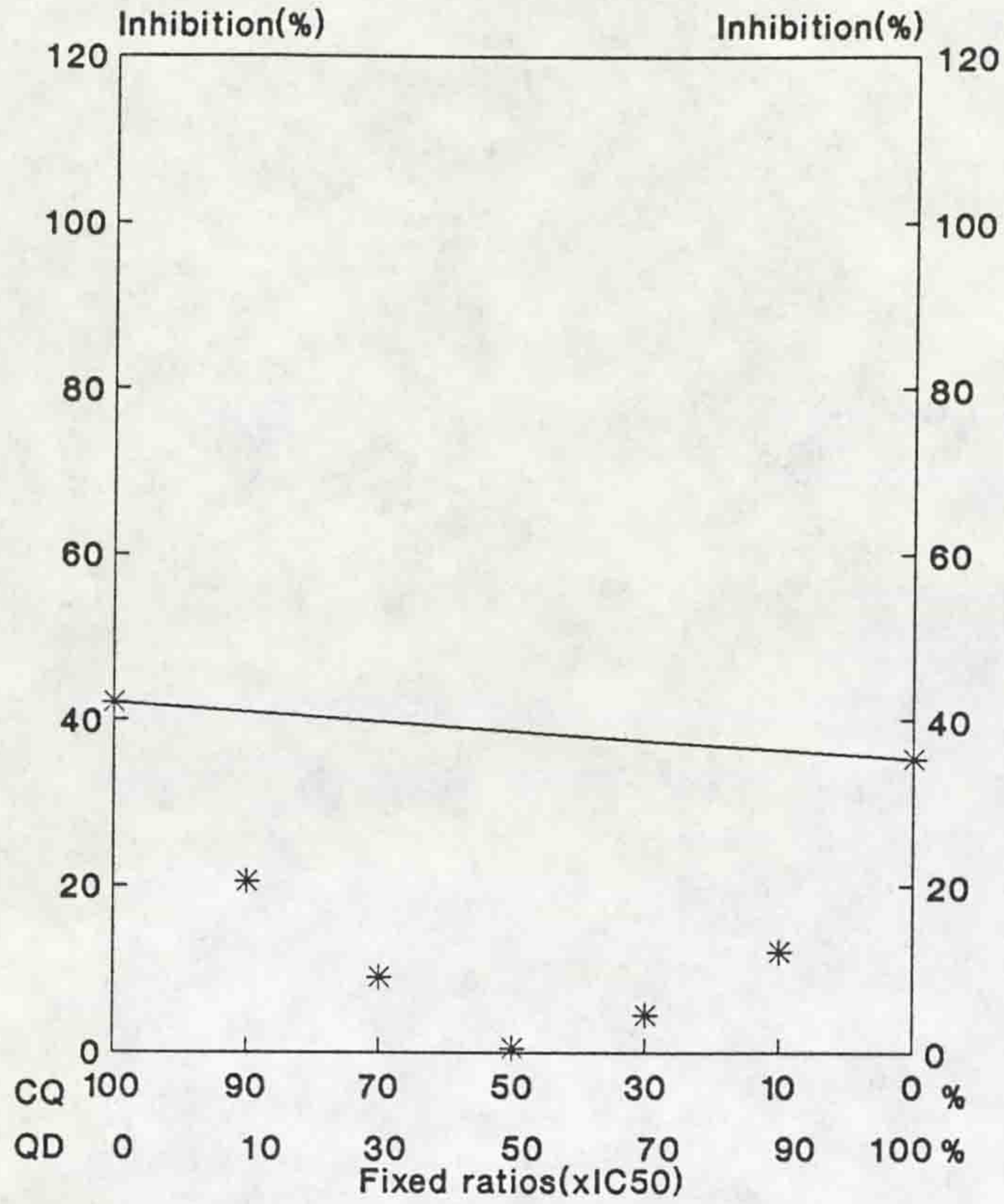
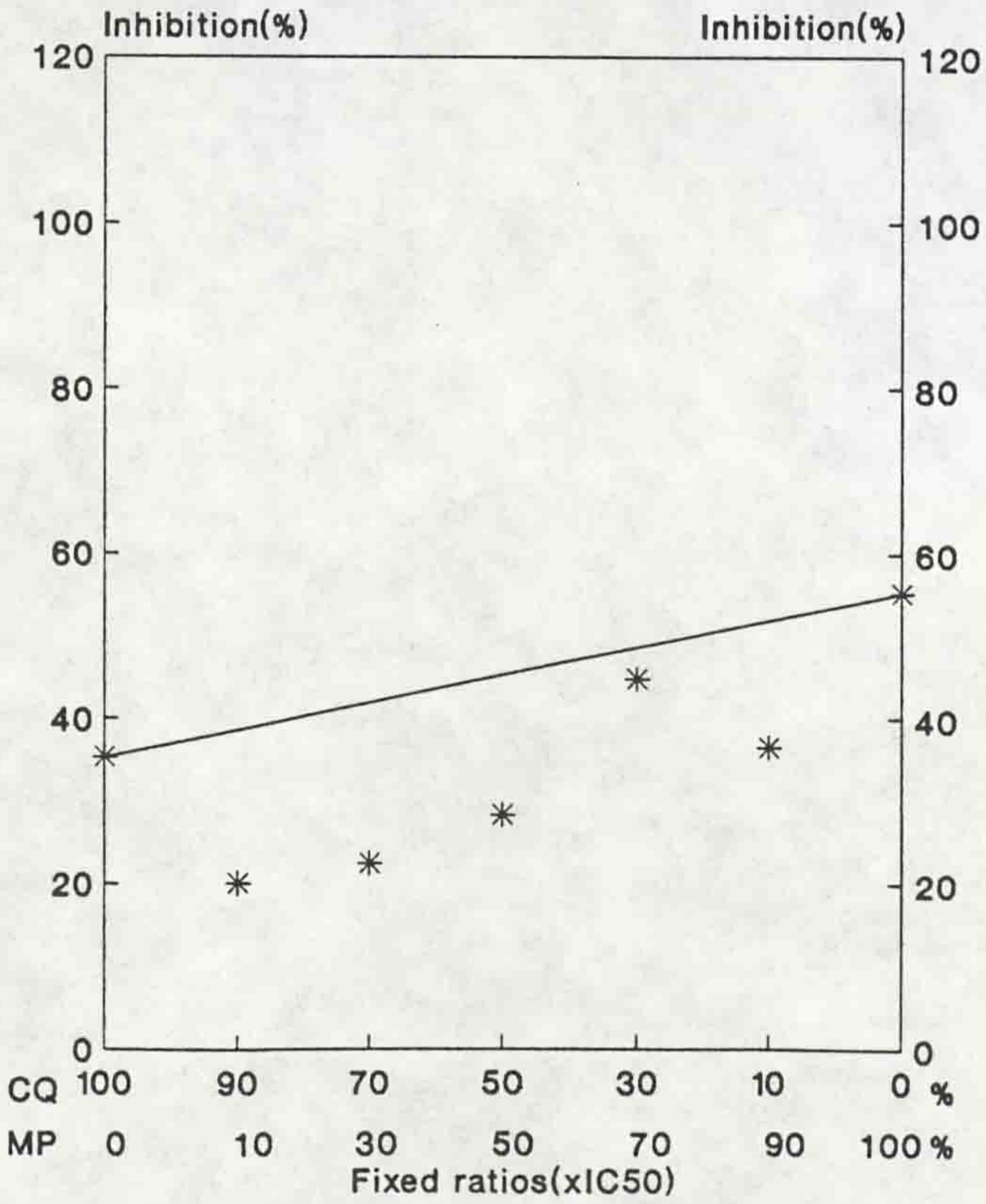


Fig.4.3.30 Interaction between chloroquine(CQ) and mepacrine (MP) on K1 strain.



CHAPTER 5 : THE INDUCTION OF HALOFANTRINE RESISTANCE IN *PLASMODIUM FALCIPARUM IN VITRO*

CHAPTER 5 Page

The induction of halofantrine resistance in *Plasmodium falciparum* *in vitro*

5.1 Introduction

The development of drug resistance in malaria parasites depends on a variety of factors, which have been summarised by Peters (1984d) (Table 5.1). He stated: "The only clearly defined ways in which malaria parasites have been shown to become resistant to drugs are: (a) the selection under drug pressure of pre-existing mutants, eg. Bishop (1958), who selected for metachloridine resistance in *Plasmodium gallinaceum* ..., (b) simple Mendelian inheritance, as shown for several antimalarials by the group headed by Beale in Edinburgh, and (c) phenotypic adaptation, as is apparent in the highly chloroquine-resistant Rc-type lines of *P. berghei*".

The standard procedures for the selection of drug-resistant strains of *P. falciparum* from established *in vitro* cultures of sensitive parasites have involved the continuous exposure of cultures to increasing sublethal drug concentrations (Nguyen-Dinh & Trager, 1978; Golenser *et al*, 1981; Lambros & Notsch, 1984; Oduola *et al*, 1988b) or the exposure of cultures to mutagenic agents followed by drug pressure to select resistant mutants (Inselburg, 1984, 1985; Banyal & Inselburg, 1986), although the relevance of resistance mechanisms developed as a result of mutagenesis to resistance mechanisms developed in the field is unclear. Brockelman *et al* (1981) produced a mefloquine-tolerant line of *P. falciparum* by exposing cultures to progressive doses of mefloquine (0.04, 8, 16, 32, 64 and 128nmol/l) for 48-hour periods separated by several cycles of culture in non-drug treated medium.

In this study we have compared the ability of two different methods to select resistance to the 9-phenanthrenemethanol halofantrine in a chloroquine-

sensitive ($T_{9.96}$) and chloroquine-resistant (K_1) strain of *P. falciparum*, namely intermittent -vs- continuous drug exposure.

5.2 Materials and methods

5.2.1 Culture maintenance

The maintenance of parasites was conducted as described in Chapter 2.1.2. Drug and non-drug treated medium were changed daily and replaced with fresh medium. The cultures were diluted to a parasitaemia of 1% every two to three days. Parasitaemia was monitored daily using Giemsa-stained thin blood smears. The multiplication rates were determined compared to control as follows:

$$c = \frac{100\ pi}{pc}$$

where: c = multiplication compared to control (%)

pi = parasitaemia of drug or non-drug treated cultures

pc = parasitaemia of control cultures

Halofantrine hydrochloride was obtained in powder form (Smith Kline and French Laboratories Limited). The stock solution contained halofantrine dissolved in 70% ethanol (agitated for fifteen minutes by ultrasonicator < Decon FS100 >) to a $10^{-2}\underline{M}$ final concentration. The stock solution was stored at 4°C and fresh solutions were prepared every two to three weeks.

5.2.2 Induction of resistance

5.2.2.1 Continuous drug exposure

The chloroquine-resistant K_1 and chloroquine-sensitive$T_{9.96}$ strains were initially exposed to 1.7 and 2.5nmol/l halofantrine respectively. These concentrations correspond to the IC_{40} and IC_{25} (40% and 25% inhibitory concentrations) values for halofantrine against each strain. Drug concentrations were increased to 2.5nmol/l (day 29) for the K_1 and 3.2nmol/l (day 30) for the $T_{9.96}$ strain, when both strains had become resistant to the initial drug concentrations. One drug free culture was set up to act as a control for each treated culture.

5.2.2.2 Intermittent drug exposure

This method was employed to induce resistance at halofantrine concentrations of 3.2, 4.5 and 8nmol/l for K_1 parasites and 4.5, 8 and 10nmol/l for $T_{9.96}$ (the K_1 and $T_{9.96}$ parasites used had exhibited resistance to halofantrine at a concentration of 2.5 and 3.2nmol/l, respectively, in the continuous exposure studies). Parasite cultures were exposed to halofantrine until the parasitaemia fell below 40% of control values. Medium containing halofantrine was then replaced with drug free media and the parasites were cultured in the absence of drug until parasite multiplication rate returned to control values. At this time the medium was removed and replaced with medium containing drug. This procedure was continued until equivalent parasite growth was achieved in the halofantrine treated parasites compared to control for at least 10 days at which point the drug concentration was increased.

5.2.3 Freezing

At the end of each phase of resistance development some of the resistant parasites were preserved in liquid nitrogen (see Chapter 2.1.2.8). This provided reference material in the development of resistance.

5.2.4 Drug susceptibility tests

The drug susceptibility tests were accomplished as described in Chapter 2.2.3. The sensitivity of the resistant parasites developed to amodiaquine, chloroquine, halofantrine, mefloquine, qinghaosu, quinine and pyrimethamine was also carried out to determine cross-resistance patterns.

5.2.5 Parasite nomenclature

The original parasites used were $T_{9.96}$ and K_1. When a halofantrine resistant parasite line was achieved the abbreviated name was modified to include this, eg. K_1HF or $T_{9.96}$HF. The numbers used after each name, eg. K_1HF1 or K_1HF3 represent the first and third resistant K_1 lines developed sequentially to increasing drug concentrations.

5.3 Results

5.3.1 The effect of continuous drug pressure

The development of halofantrine resistance in K_1 chloroquine-resistant parasites at concentrations of 1.7 and 2.5nmol/l halofantrine and in $T_{9.96}$ chloroquine-sensitive parasites at concentrations of 2.5 and 3.2nmol/l halofantrine are shown in Fig. 5.3.1 and 5.3.2. Exposure of K_1 parasites to 1.7nmol/l halofantrine (Fig. 5.3.1) culminated in an initial decrease in parasite growth to 6% of control values at day 6, thereafter the growth of parasites increased attaining

control levels by day 20 which was maintained for 10 days in the presence of the drug (these parasites were termed K_1HF1). Further exposure of these parasites to halofantrine 2.5nmol/l resulted in the development of resistance 10 days after exposure. Unfortunately, these parasites became contaminated by day 18 and had to be replaced with previously cryopreserved parasites (Fig. 5.3.1). Repeated attempts to induce resistance at 3.5nmol/l halofantrine failed resulting in complete death of parasites within a few days (Fig. 5.3.3).

The development of halofantrine resistance in $T_{9.96}$ parasites to a concentration of 2.5nmol/l halofantrine was achieved within 15 days (parasites termed $T_{9.96}$HF1). This level of resistance was maintained for 10 days in the presence of the drug. Further exposure of the $T_{9.96}$HF1 parasites to a 3.2nmol/l concentration of halofantrine culminated in the development of resistance 16 days after exposure (Fig. 5.3.2). Although these parasites exhibited stable resistance for over 10 days continuous exposure, repeated attempts to induce resistance to 4.5nmol/l halofantrine failed again, resulting in complete death of all parasites within a few days (Fig. 5.3.4).

Parasites resistant to halofantrine 2.5 and 3.2nmol/l are referred to as K_1HF2 and $T_{9.96}$HF2, respectively which were used subsequently in the intermittent exposure experiments.

5.3.2 The effect of intermittent drug exposure

5.3.2.1 Chloroquine resistant K_1 parasites

Figures 5.3.5 to 5.3.7 illustrate the time course for the development of halofantrine resistance at drug concentrations of 3.2, 4.5 and 8nmol/l. Parasite exposure at a halofantrine concentration of 3.2nmol/l had little effect on parasite

growth and multiplication until day 8 of exposure. Thereafter parasite growth compared to control decreased to 30% by day 16. At this time the parasite culture was used to prepare two sub-cultures, one containing 3.2nmol/l halofantrine and the other was cultured in the absence of drug until control growth rates were restored. The parasitaemia in the drug treated sub-culture continued to decline to zero by day 18. The untreated sub-culture exhibited normal growth and multiplication four days after the removal of drug pressure. The untreated parasites were treated again with the same concentration of halofantrine on day 20. The reintroduction of drug pressure on day 20 resulted in an initial decrease in growth (30% by day 23) but growth rate returned to normal values by day 25 and remained at this level for 10 days despite the continued presence of 3.2nmol/l halofantrine (Fig. 5.3.5). Parasites resistant to 3.2nmol/l are referred to as K_1HF3 strain. Figure 5.3.6 illustrates the time course for the development of halofantrine resistance at concentration of 4.5nmol/l halofantrine in the K_1HF3 parasites. The pattern is similar to that observed using drug concentration of 3.2nmol/l with complete resistance to 4.5nmol/l halofantrine (stable for 10 days) being achieved by day 20 (Fig. 5.3.6). The resulting parasites are referred to as K_1HF4. Induction of halofantrine resistance at 8nmol/l halofantrine in the K_1HF4 strain proved more difficult to achieve requiring four periods of drug exposure separated by intervals of up to 8 days growth in the absence of the drug. Halofantrine resistance at concentration of 8nmol/l was achieved by day 50. These parasites exhibiting halofantrine resistance to the latter concentration (8nmol/l) are referred to as K_1HF strain, which was formally appointed as a halofantrine-resistant strain, derived from

chloroquine-resistant parasites, in this study.

5.3.2.2 Chloroquine sensitive $T_{9.96}$ parasites

Figures 5.3.8 to 5.3.10 illustrate the time course of resistance development to halofantrine at concentrations of 4.5, 8, 10 and 20nmol/l in $T_{9.96}$ parasites. The initial parasites used were obtained from the continuous exposure experiment which exhibited resistance to 3.2nmol/l ($T_{9.96}$HF2). Within three days exposure at a concentration of 4.5nmol/l parasite growth decreased to 30% of control values. At this time two sub-cultures were prepared from these parasites, one in the presence of 4.5nmol/l halofantrine and the other in the absence of the drug. The parasitaemia in the drug treated sub-culture continued to decline to zero by day 8. The parasite growth in the untreated sub-cultures returned to control values by day 7 when they were re-exposed to the drug. Again parasite growth decreased to 40% of control values by day 18. Sub-cultures were again prepared as described above. Parasite growth continued to decline in those cultures exposed to halofantrine, while the untreated sub-cultures returned to 80% control values by day 22. Re-exposure of these parasites (4.5nmol/l) culminated in an initial decline in parasite growth but by day 30, parasite growth returned to control levels and remained at this level for 12 days despite the continued presence of 4.5nmol/l halofantrine (Fig. 5.3.8). The resulting parasites are referred to as $T_{9.96}$HF3. Resistance to 8nmol/l was obtained after a single cycle of exposure, washout and re-exposure of the $T_{9.96}$HF3 parasites (Fig. 5.3.9). These parasites are referred to as $T_{9.96}$HF4. Initial attempts to produce halofantrine resistance at 20nmol/l halofantrine proved unsuccessful despite three exposure cycles (Fig. 5.3.10). At day 23 these parasites were exposed to 10nmol/l

halofantrine and parasite resistance to this concentration was achieved within one exposure cycle (Fig. 5.3.10). These parasites are referred to as $T_{9.96}$HF strain, which was formally appointed as a halofantrine-resistant strain, derived from chloroquine-sensitive parasites, in this study.

5.3.3 Drug sensitivity tests

The sensitivity of K_1HF and $T_{9.96}$HF to halofantrine were significantly reduced compared to the original parasite strains K_1 and $T_{9.96}$ (Figs. 5.3.11-5.3.14). These differences were apparent from both the microscopic examination method (Fig. 5.3.11 and 5.3.12) or from the radiolabelled hypoxanthine incorporation method (Figs. 5.3.13 and 5.3.14). Nine and three fold increases in the IC_{50} values for halofantrine were observed for K_1HF and $T_{9.96}$HF parasites respectively compared to the parent strains when assessed by the radiolabelled hypoxanthine incorporation method. A similar change in sensitivity of the K_1HF was determined by microscopic examination, however using this method $T_{9.96}$HF showed a five fold decrease in sensitivity to halofantrine (Table 5.3.1).

The response of the parent strains, K_1HF and $T_{9.96}$HF to chloroquine, quinine, mefloquine, amodiaquine, qinghaosu and pyrimethamine and the apparent cross-resistance patterns are presented in Tables 5.3.1 to 5.3.5. The development of halofantrine resistance in the chloroquine-resistant K_1 parasites was associated with increased resistance to mefloquine, quinine, qinghaosu, and an increase in sensitivity to the 4-aminoquinolines most notably chloroquine (Figs. 5.3.15 to 5.3.19). Halofantrine resistance developed in the chloroquine-sensitive $T_{9.96}$ parasites was also associated with increased resistance

to mefloquine, quinine, qinghaosu and pyrimethamine with little effect on the 4-aminoquinolines (Figs. 5.3.20 to 5.3.25). The resistance developed in the K_1HF and $T_{9.96}$HF parasites was stable after freezing in liquid nitrogen and after six weeks continuous culture in complete drug free medium (Figs. 5.3.26 and 5.3.28).

5.4 Discussion

Halofantrine {WR171,669; 1,3-dichloro-α-[2-(dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol} is a 9-phenanthrenemethanol member of the aminoalcohols (Schmidt *et al*, 1978b; Childs *et al*, 1984; Schuster & Canfield, 1989). As such it possesses greater structural similarities to mefloquine and quinine than it does to the 4-aminoquinoline antimalarials. A number of workers have shown halofantrine to be highly active against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (Schmidt *et al*, 1978b; Cosgriff *et al*, 1982; Childs *et al*, 1984; Webster *et al*, 1985; Boudreau *et al*, 1988; Salako *et al*, 1990). This is in agreement with the present findings, halofantrine being effective against both strains, with greater antimalarial activity against K_1 chloroquine-resistant strain than the $T_{9.96}$ chloroquine-sensitive strain.

Human pharmacokinetics of halofantrine and its N-desbutyl metabolite have been investigated by Broom (1989). The results show that the extent of absorption of halofantrine hydrochloride is low and variable, hence the occasional recrudescence of falciparum infection in malarious patients following halofantrine treatment may be attributed to an inadequate drug level in the body (Boudreau *et al*, 1988). Halofantrine has an elimination half-life of about 1-2.6 days (Boudreau *et al*, 1988; Broom, 1989).

Clinically, halofantrine is well tolerated. Minor gastrointestinal symptoms are the most common side-effects of halofantrine treatment (Horton & Parr, 1989; Salako *et al*, 1990). At present halofantrine appears to be a useful antimalarial drug for the treatment of acute malaria, although some recrudescences have recently been reported by a number of investigators (Boudreau *et al*, 1988; Rab *et al*, 1989; Salako *et al*, 1990).

Drug resistance in *P. falciparum* is a major problem which is not restricted to the 4-aminoquinoline chloroquine. Notably resistance to mefloquine prior to its clinical release (Boudreau *et al*, 1982; Hoffman *et al*, 1985), amodiaquine (see Peters, 1987), the antifolates (Field & Edeson, 1949; Chaudhuri & Chaudhuri, 1949; Wilson, 1952) and an apparent reduction in the sensitivity of parasites to the action of quinine (Jaroonvesama *et al*, 1974; Bastien, 1987) raises the concern that parasite drug resistance is an inevitable consequence of drug usage. In the present study we have attempted to devise experimental procedures, based on drug pressure, to develop strains of *P. falciparum* with stable drug resistance. We believe that resistance developed in this way will mimic the resistance likely to be encountered in the field. As such this parasite material is ideal for studying the mechanism of resistance operating in malarial parasites. Halofantrine was selected as the candidate antimalarial for these studies based on the initial observations of high levels of activity against both chloroquine-resistant and chloroquine-sensitive parasites, its structural similarity to quinine and mefloquine and its imminent introduction for clinical use.

The use of continuous drug exposure at sub-lethal concentrations to induce parasite resistance is a method which has been used previously (see

Chapter 5.1). We found that this approach resulted in only slight alterations in the IC_{50} values for halofantrine. Small increases in drug concentrations were found completely to eliminate parasites and it was anticipated that in order to achieve a measurable degree of halofantrine resistance would require the sequential exposure of parasites to fractionally higher concentrations over a long period of time. This approach is very different to the conditions which operate in the field and as such it was abandoned in favour of an intermittent exposure pattern.

Brockelman *et al* (1981) has used such a method of intermittent exposure to induce mefloquine tolerance in strains of *P. falciparum*. Although the stability of resistance was not assessed in this study, we have used this approach with modifications and achieved stable halofantrine resistance at each of the drug concentrations selected for at least 10 days with the intermediate concentrations and for over 6 months with the highest concentrations in a chloroquine-sensitive and a chloroquine-resistant strain of *P. falciparum*.

Using this intermittent drug exposure we have been able to achieve a 3.3 fold increase in the IC_{50} for halofantrine against the cloned $T_{9.96}$ parasites and a 9 fold increase against the uncloned K_1 parasites within six months. Oduola *et al* (1988b) required 22 months of continuous drug exposure to produce a 4.3 fold increase in the IC_{50} for mefloquine against a cloned W2 strain. In agreement with Oduola *et al* (1988b) we found that resistance developed more rapidly in uncloned parasites presumably reflecting the genetic heterogeneity and range of individual parasite drug sensitivities in these parasites compared to cloned lines. A similar result was previously reported by Peters *et al* (1978), when

they sought to produce chloroquine-resistance in cloned and uncloned NS strains of *P. berghei*.

Lambros and Notsch (1984) obtained significantly different ID_{50} values for mefloquine against two mefloquine-resistant strains (MR-Camp and MR-Smith) depending on whether the microscopic or radiolabelled hypoxanthine incorporation methods were used. We observed a similar but much smaller discrepancy, but only with $T_{9.96}$HF parasites. The reasons for these findings are unclear.

The cross-resistance patterns observed in the present study raise a number of interesting points. The development of halofantrine resistance was associated with a decrease in parasite drug sensitivity to other antimalarials containing the methanolic function, ie. mefloquine and quinine, suggesting that this functional group is essential for activity or is of importance with respect to the resistance mechanism. This has obvious clinical implications for the introduction and widespread use of halofantrine. Halofantrine resistance in parasites which were originally resistant to chloroquine (K_1) showed significantly increased chloroquine sensitivity which was not apparent in $T_{9.96}$ parasites. It is yet to be evaluated if these K_1HF parasites rapidly regain chloroquine resistance on the reintroduction of chloroquine pressure. Finally the development of halofantrine resistance was associated with a slight decrease in parasite sensitivity to qinghaosu and the sensitivity of $T_{9.96}$HF to pyrimethamine. These findings are in disagreement with those of Robinson *et al* (1986). Using halofantrine-resistant *P. berghei* derived from drug-sensitive (N) and chloroquine-resistant (NS) parasites. They detected cross-resistance to chloroquine as well as to primaquine,

quinine and mefloquine, and suggested on the basis of these results, that halofantrine and chloroquine may share same receptor site. A similar idea was previously suggested by Robinson and Peters (1985). However, an increase in sensitivity to chloroquine in our K_1HF strain of *P. falciparum* may indicate a difference in the mode of action between halofantrine and chloroquine. Robinson *et al* (1986) also reported that resistance to halofantrine in N/Hal and NS/Hal, halofantrine-resistant, strains of *P. berghei* was rapidly lost on removal of drug pressure and following storage in liquid nitrogen. Peters and Porter (1976) demonstrated that WR122, 455 phenanthrenemethanol-resistant N/1102 and NS/1102 *P. berghei* parasites retained some of their resistant capability on removal of drug pressure and following preservation in liquid nitrogen. The halofantrine-resistant strains developed in the present study exhibited stable resistance over 42 days in the absence of drug pressure and were unaffected by cryopreservation in liquid nitrogen. Although the discrepancies between halofantrine-resistant parasites of *P. berghei* and *P. falciparum* are still unclear, the methods used to develop resistance (ie. intermittent -vs- continuous) may determine the stability of the resistance factor.

In conclusion we have been able to rapidly induce stable resistance to halofantrine in both a chloroquine-sensitive and a chloroquine-resistant strain of *P. falciparum*. Cross-resistance appears to be associated predominantly with those drugs containing a methanolic functional group and halofantrine resistance appears to result in increased chloroquine sensitivity in previously resistant parasites.

Table 5.1 **Classification of possible modes of drug resistance. (Peters 1984d)**

A. Resistance primarily dependent upon change of genotype

(i) Mutation

- Spontaneous (the expression of the mutant may be delayed by 'phenomic lag', ie. several generations may elapse between the genetic event and its phenotypic expression
- Induced (usually by non-specific mutagenic agents rather than the drug itself but the latter is possible if subinhibitory drug concentrations are used)

(ii) Genetic exhange (chromosomal or extrachromosomal)

- By gametes (sexual recombination)
- By 'unpackaged' DNA
  - Transduction
  - Transformation

B. Resistance dependent upon non-genetic change of phenotype (ie. inducible organisms)

(i) Induction of a new physiological function

- Production of inducer-inactivating enzymes
  - Single enzyme
  - Chain of enzymes

(ii) Elimination of a cytoplasmic particle

(iii) Accumulation of a drug-inactivating factor

(iv) Selection of an alternative physiological function, eg. selective change in the relative emphasis upon two or more pre-existent enzymatic pathways, leading to the formation of an essential metabolite

(v) 'Reorganisation of the cytoplasm' (a general term quoted from Beale to cover a variety of phenotypic adaptations leading to resistance, eg. altered membrane structure and permeability)

C. Resistance involving no adaptive change

D. Borderline cases

E. Resistance dependent upon composite changes (the most likely process in most cases of drug resistance)

Table 5.3.1 **The ratios of IC_{50} (K_1HF)/IC_{50} (K_1) and IC_{50} ($T_{9.96}HF$)/IC_{50} ($T_{9.96}$) for antimalarial drugs in 48 hour assays**

Strain / Drug	K_1HF	K_1	IC_{50} (K_1HF) / IC_{50} (K_1)	$T_{9.96}HF$	$T_{9.96}$	IC_{50} ($T_{9.96}HF$) / IC_{50} ($T_{9.96}$)
	Mean IC_{50} (nmol/l)	Mean IC_{50} (nmol/l)		Mean IC_{50} (nmol/l)	Mean IC_{50} (nmol/l)	
Halofantrine +	20	2.2	9.09	22	6.6	3.33
Halofantrine *	18	1.9	9.47	26	5.2	5
Mefloquine +	75	29	2.58	166.6	58.3	2.85
Chloroquine +	80	366.6	0.21	29.3	31	0.94
quinine +	386.6	183.3	2.10	112	76.6	1.46
Amodiaquine +	23.2	28.5	0.81	16.5	24	0.68
Qinghaosu +	16.3	9.3	1.75	22	15	1.46
Pyrimethamine +	>1000	>1000	-	54	36.5	1.47

\+ Radiolabelled hypoxanthine incorporation method
* Microscopic examination method

Table 5.3.2 **A comparison of halofantrine and mefloquine IC_{50} values for K_1, K_1HF, $T_{9.96}$ and $T_{9.96}$HF strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Drug	Halofantrine		Mefloquine	
	IC_{50} (nmol/l)		IC_{50} (nmol/l)	
Strain	Individual	Mean±SD	Individual	Mean±SD
K_1	1.7		25	
	2	2.2 ± 0.46	30	29 ± 3.6
	2.3		32	
	2.8			
K_1HF	18		70	
	20	20 ± 1.7	73	75 ± 6.2
	21		82	
	22			
$T_{9.96}$	5.6		53	
	6.2	6.6 ± 1.2	60	58.3 ± 4.7
	8		62	
$T_{9.96}$HF	20		110	
	21	22 ± 2	190	166.6 ± 49.3
	24		200	

Table 5.3.3 **A comparison of chloroquine and quinine IC_{50} values for K_1, K_1HF, $T_{9.96}$ and $T_{9.96}$HF strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Drug	Chloroquine		Quinine	
	IC_{50} (nmol/l)		IC_{50} (nmol/l)	
Strain	Individual	Mean±SD	Individual	Mean±SD
K_1	220		120	
	390	366.6 ± 136.5	140	183.3 ± 92.9
	490		290	
K_1HF	54		370	
	66	80 ± 35.1	390	386.6 ± 15.2
	120		400	
$T_{9.96}$	28		72	
	29	31 ± 4.3	78	76.6 ± 4.1
	36		80	
$T_{9.96}$HF	27		71	
	29	29.3 ± 2.5	85	112 ± 59.3
	32		180	

Table 5.3.4 **A comparison of amodiaquine and qinghaosu IC_{50} values for K_1, K_1HF, $T_{9.96}$ and $T_{9.96}$HF strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Drug	Amodiaquine		Qinghaosu	
	IC_{50} (nmol/l)		IC_{50} (nmol/l)	
Strain	Individual	Mean±SD	Individual	Mean±SD
K_1	18		7.4	
	21	28.5 ± 10.4	7.6	9.3 ± 3.1
	37		13	
	38			
K_1HF	21		13	
	22	23.2 ± 2.2	17	16.3 ± 3
	24		19	
	26			
$T_{9.96}$	18		8	
	19	24 ± 9	17	15 ± 6.2
	34		20	
$T_{9.96}$HF	9.7		13	
	17	16.5 ± 6.6	23	22 ± 8.5
	23		30	

Table 5.3.5 **A comparison of pyrimethamine IC_{50} values for K_1, K_1HF, $T_{9.96}$ and $T_{9.96}HF$ strains of *P. falciparum* in the 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments)**

Drug	Pyrimethamine	
	IC_{50} (nmol/l)	
Strain	Individual	Mean ± SD
K_1	>1000	-
K_1HF	>1000	-
$T_{9.96}$	35	-
	38	
$T_{9.96}HF$	52	-
	56	

Fig.5.3.1 Time course of resistance to 1.7 and 2.5 nmol/l halofantrine in K1 and K1HF1 strains.

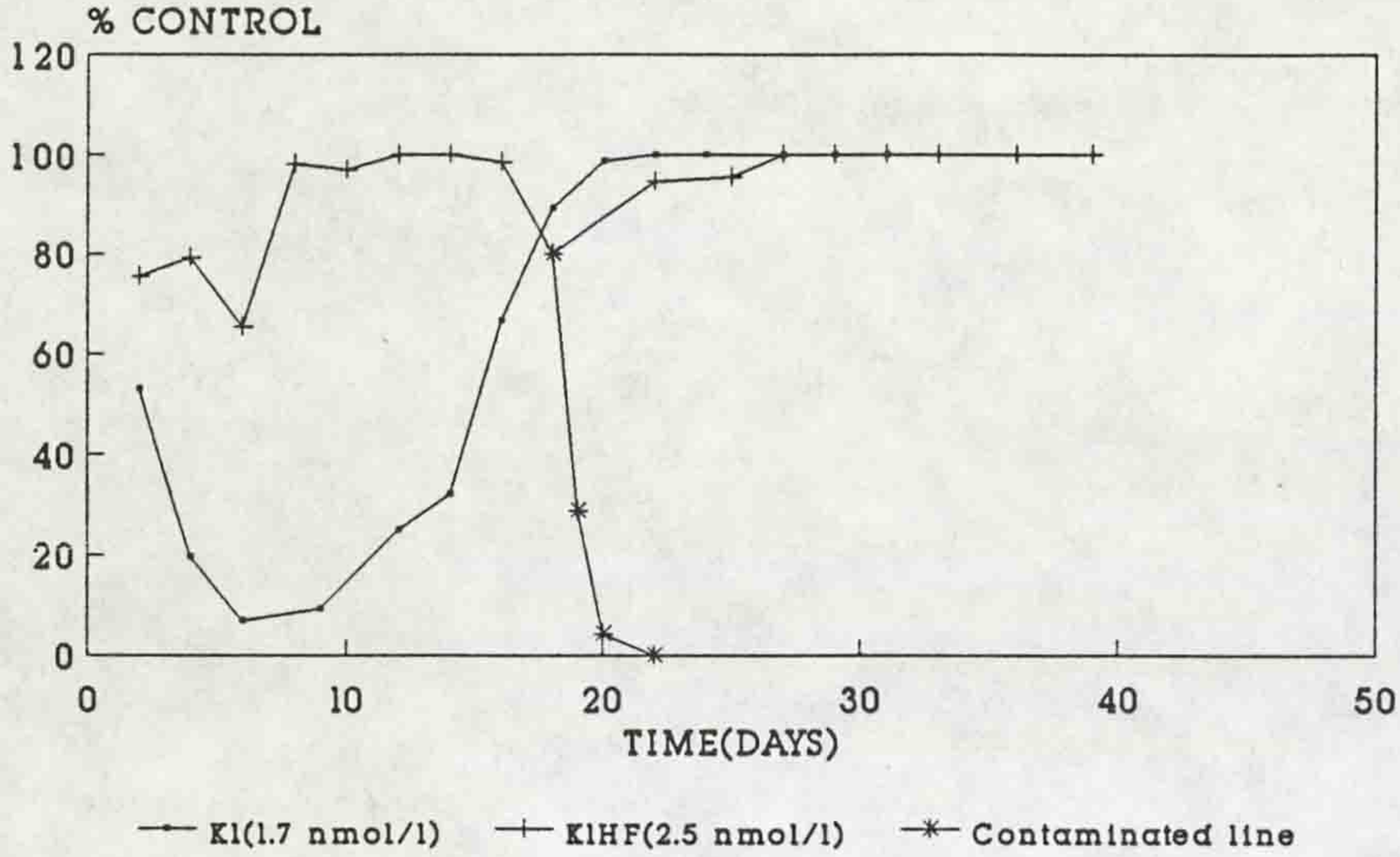


Fig.5.3.2 Time course of resistance to 2.5 and 3.2 nmol/l halofantrine in T9.96 and T9.96HF1 strains.

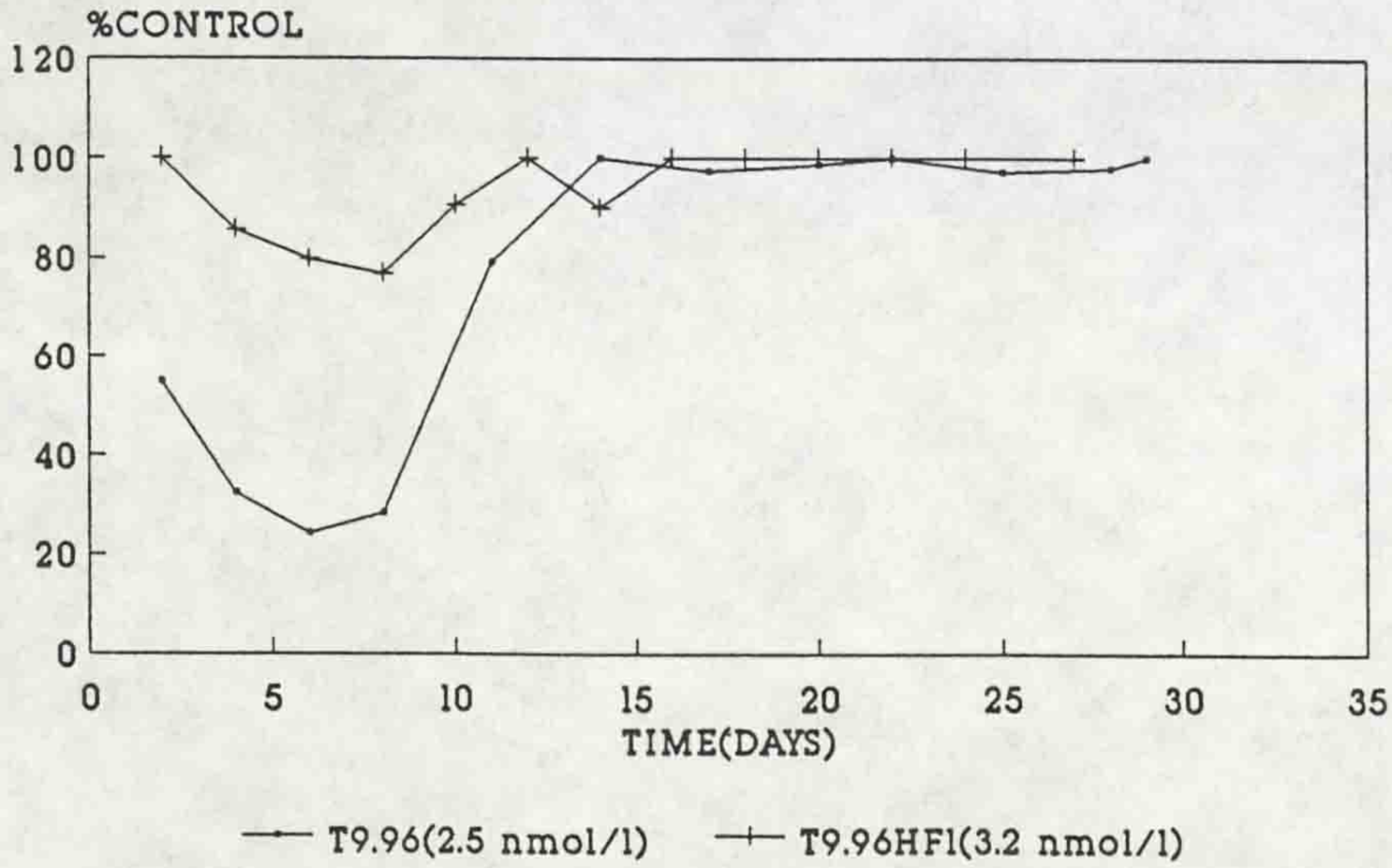


Fig.5.3.3 Time course of response of K1HF2 to 3.5 nmol/l halofantrine.

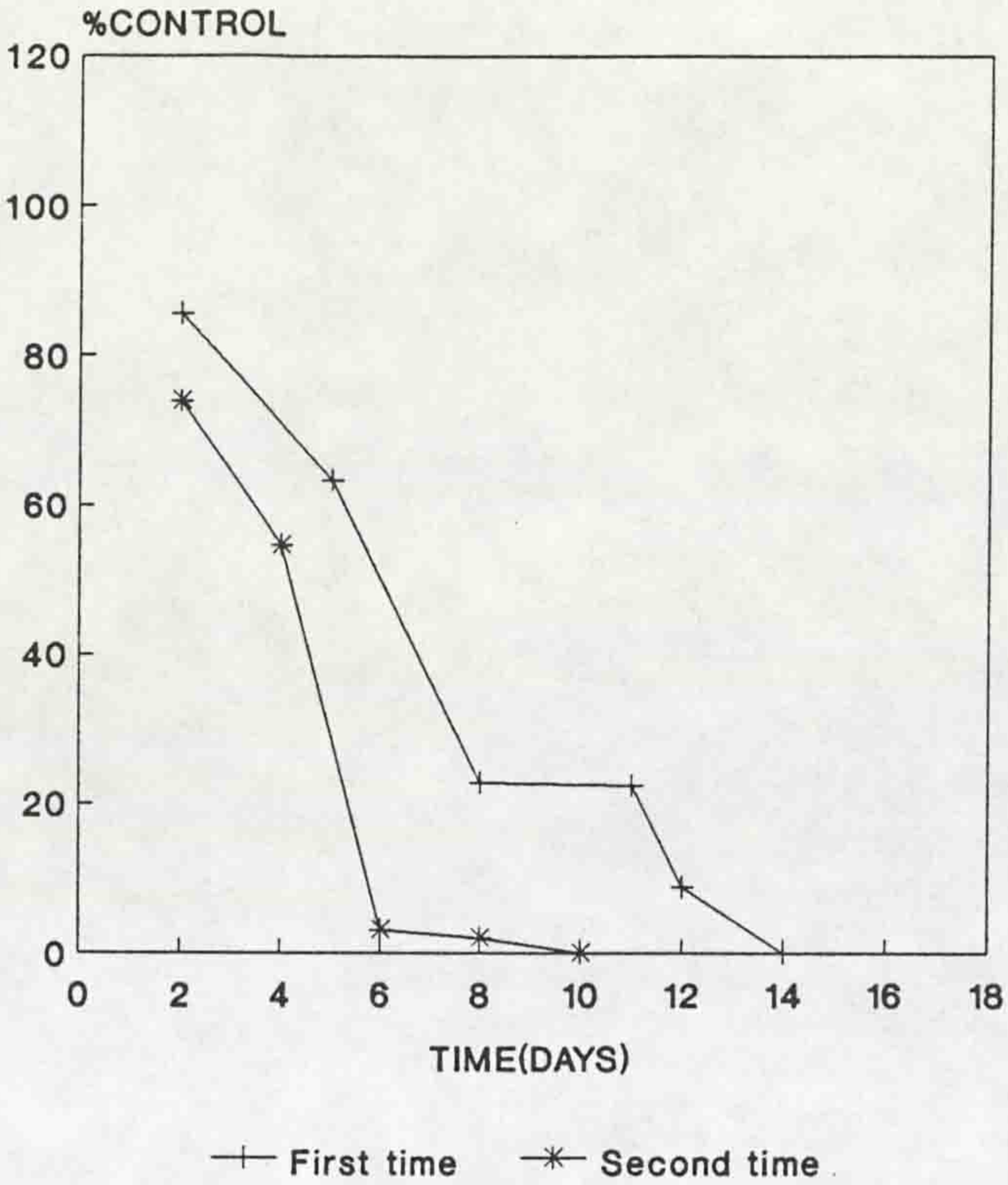


Fig.5.3.4 Time course of response of T9.96HF2 to 4.5nmol/l halofantrine.

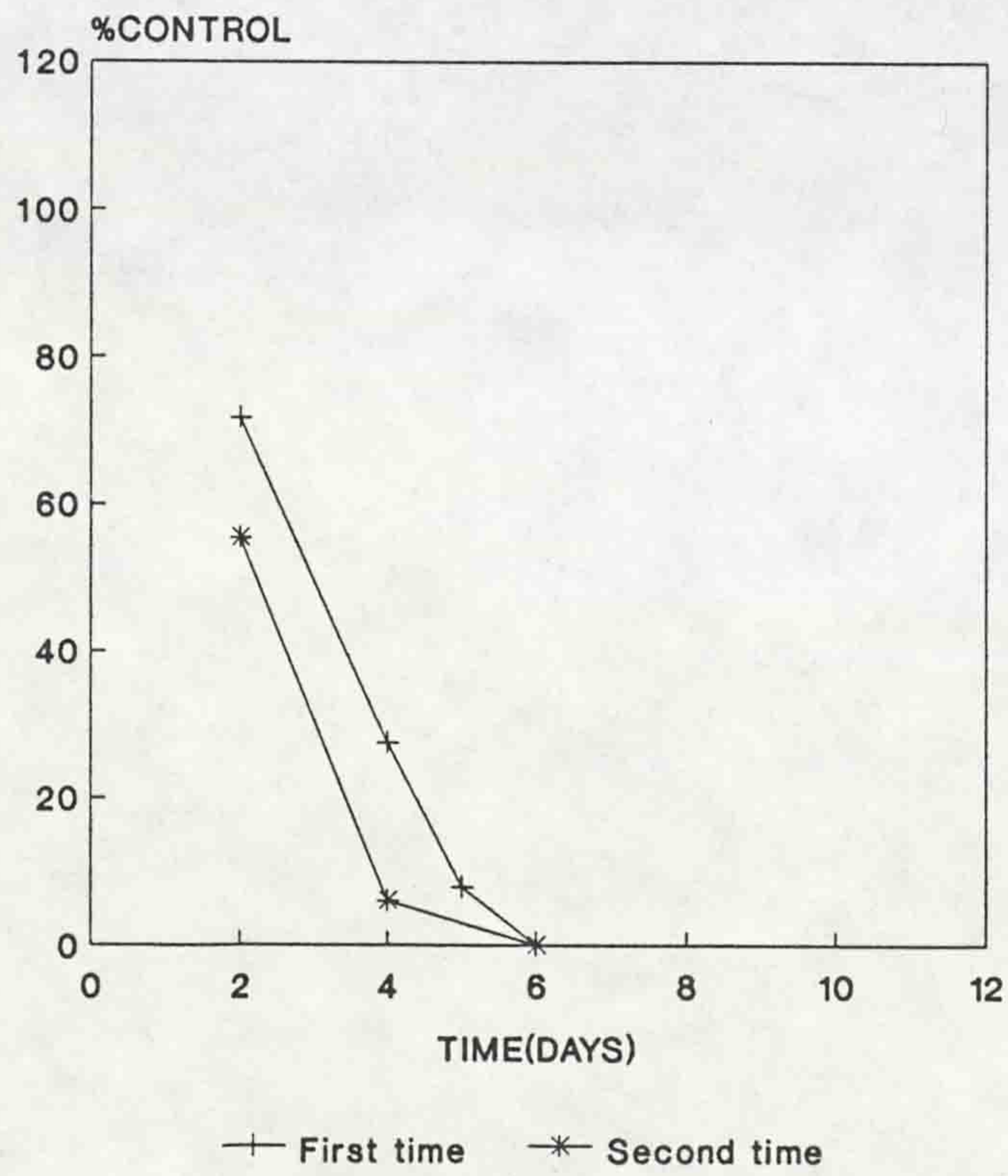


Fig.5.3.5 Time course of resistance to 3.2 nmol/l halofantrine in KlHF2 strain.

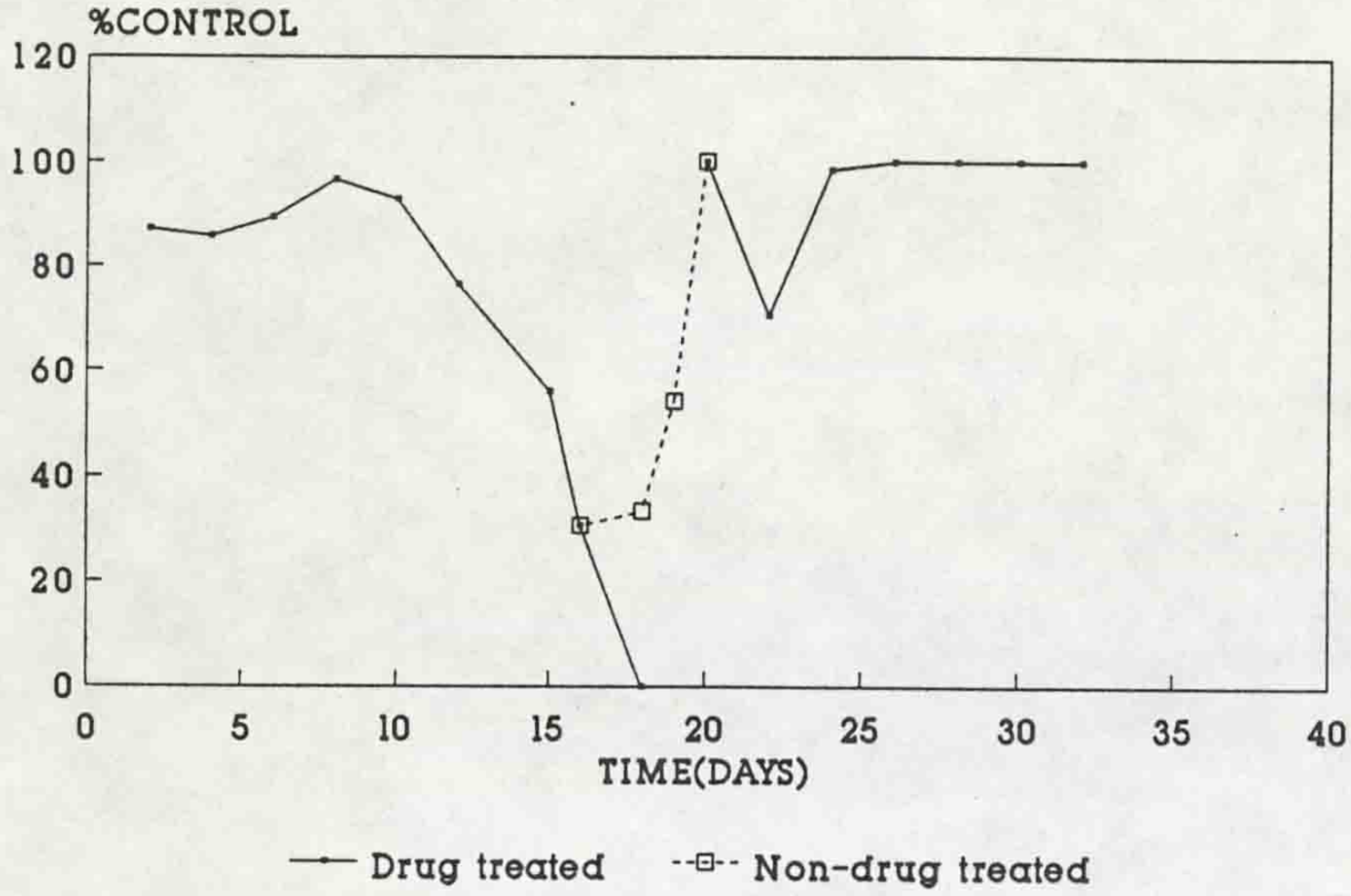


Fig.5.3.6 Time course of resistance to 4.5 nmol/l halofantrine in KlHF3 strain.

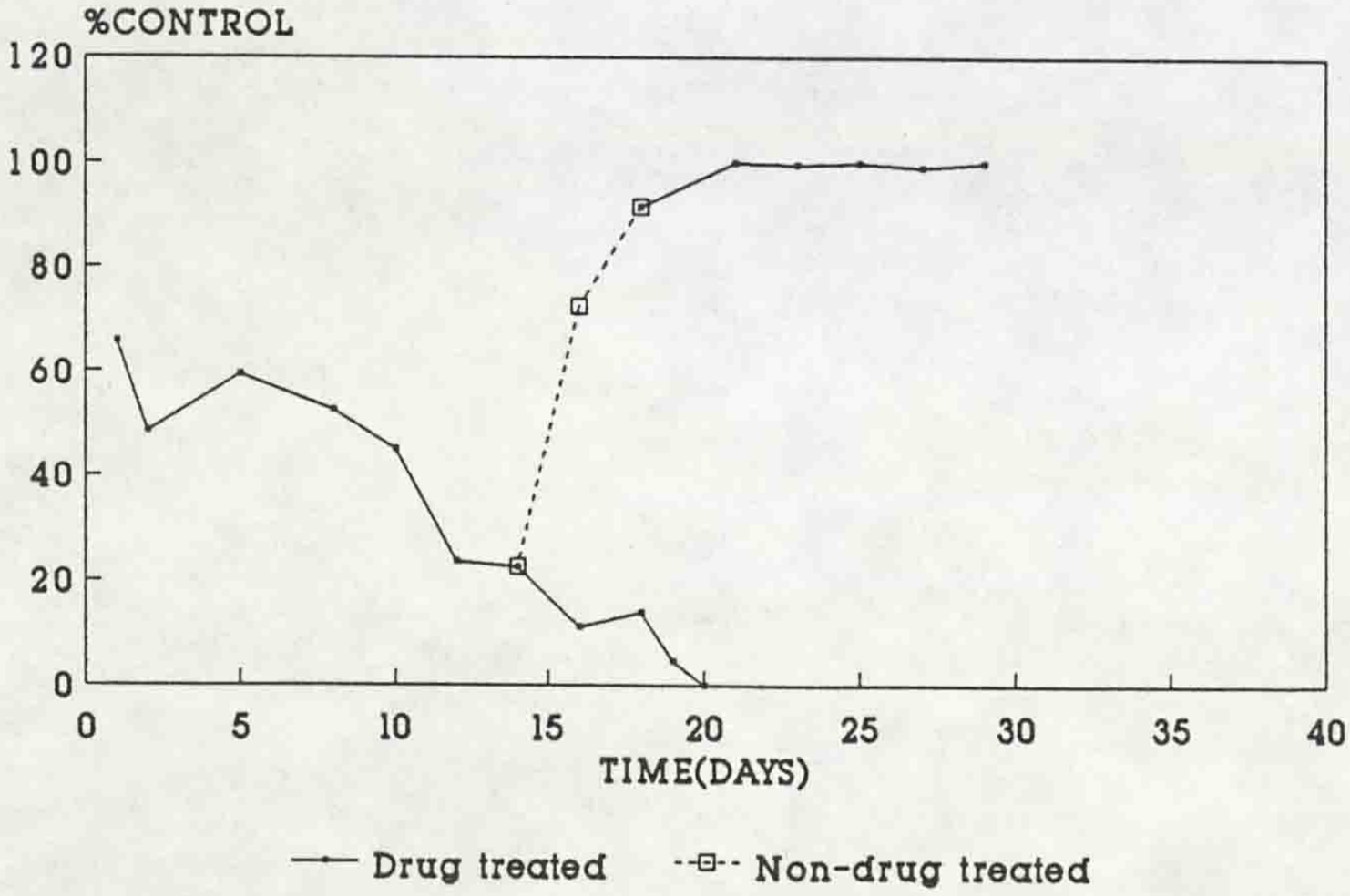


Fig.5.3.7 Time course of resistance to 8 nmol/l halofantrine in KlHF4 strain.

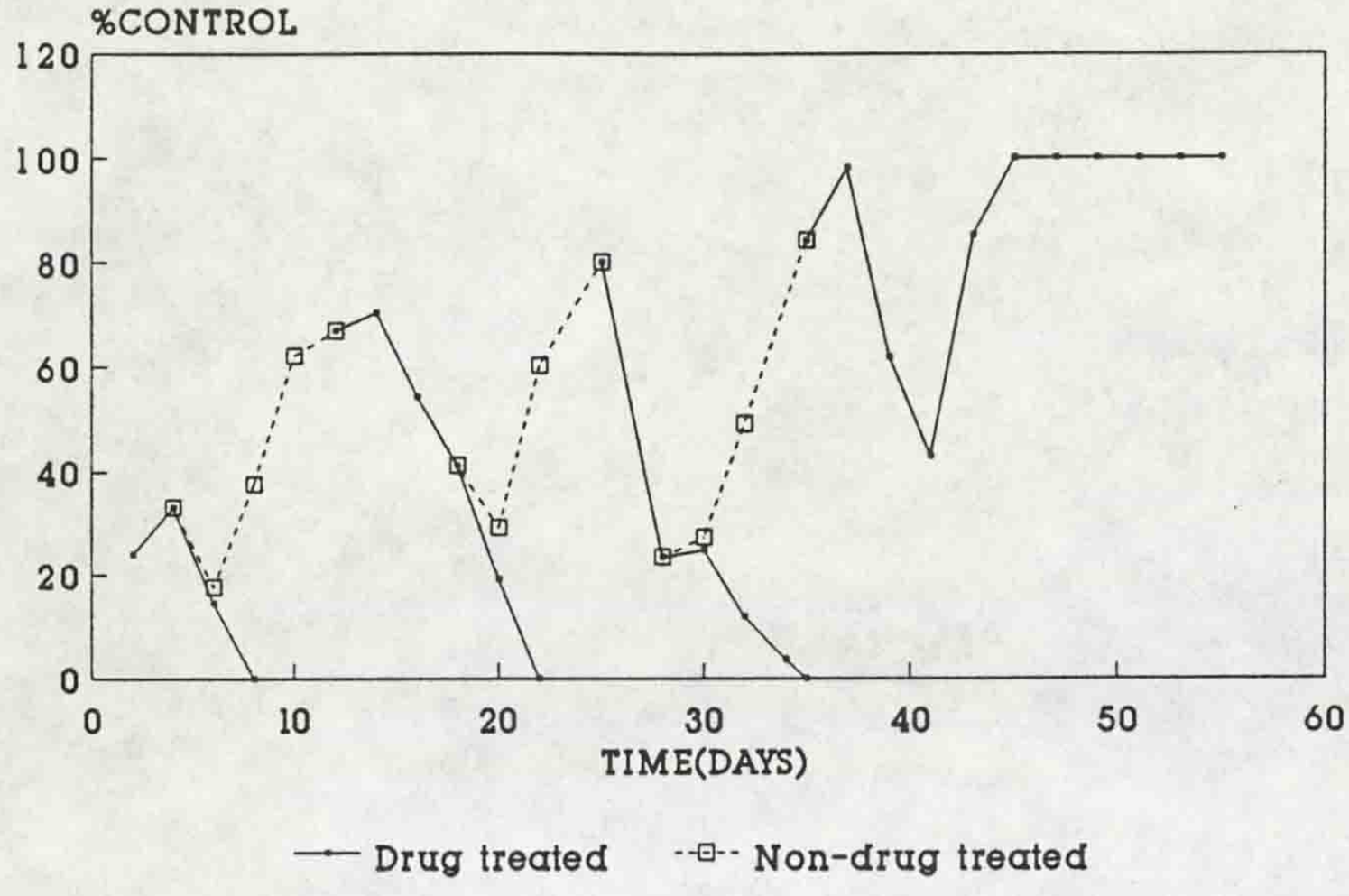


Fig.5.3.8 Time course of resistance to 4.5 nmol/l halofantrine in T9.96HF2 strain.

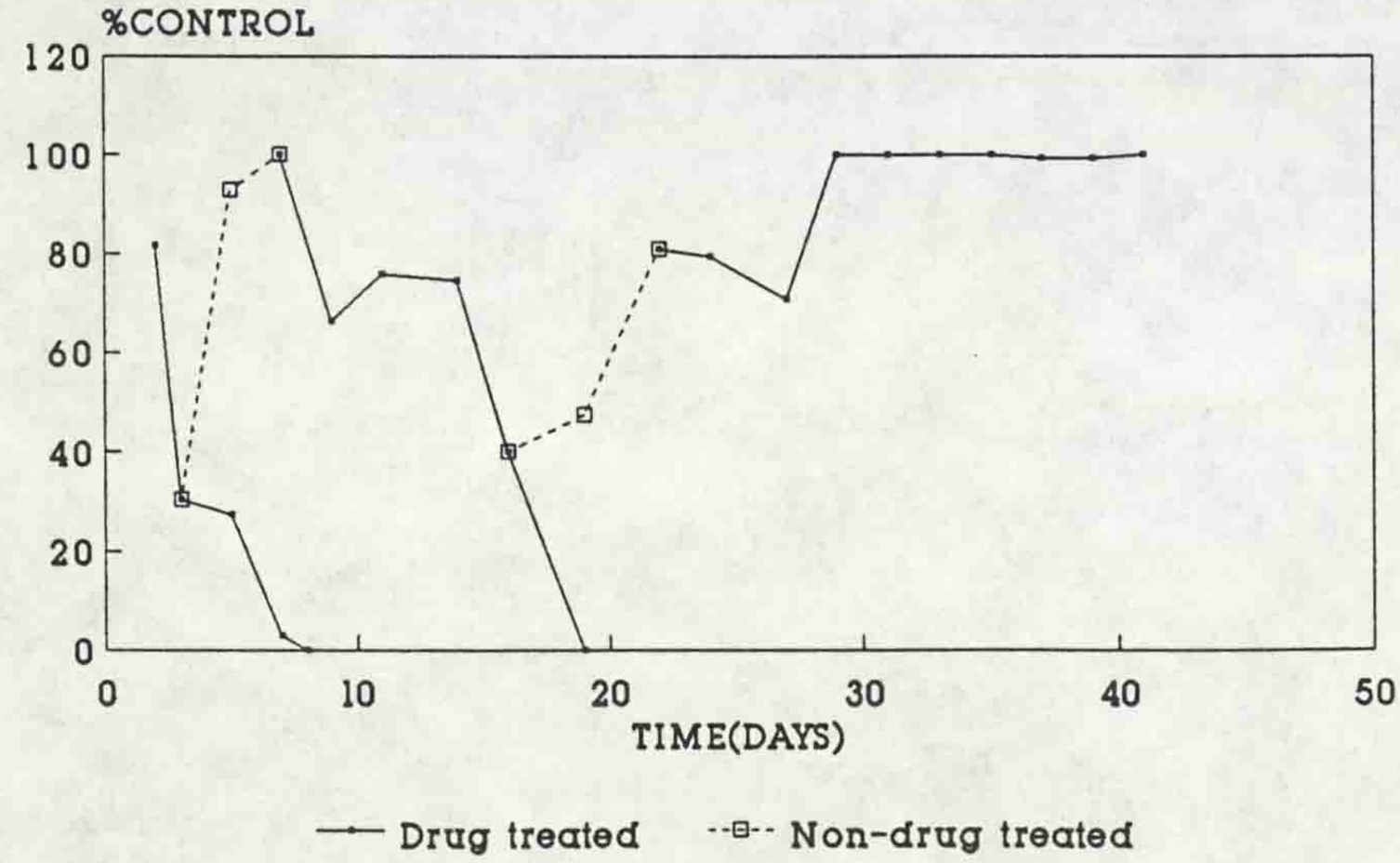


Fig.5.3.9 Time course of resistance to 8 nmol/l halofantrine in T9.96HF3 strain

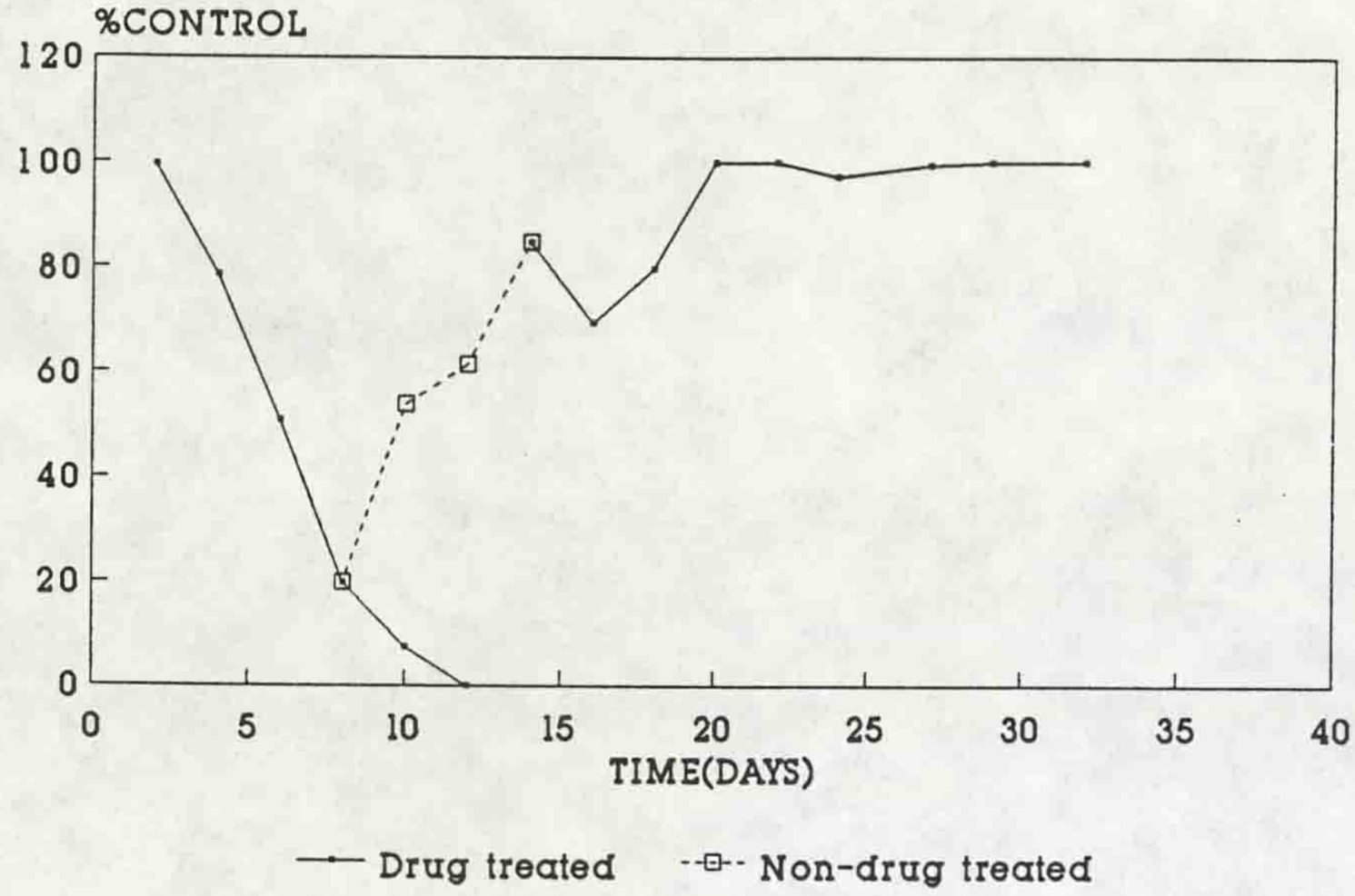
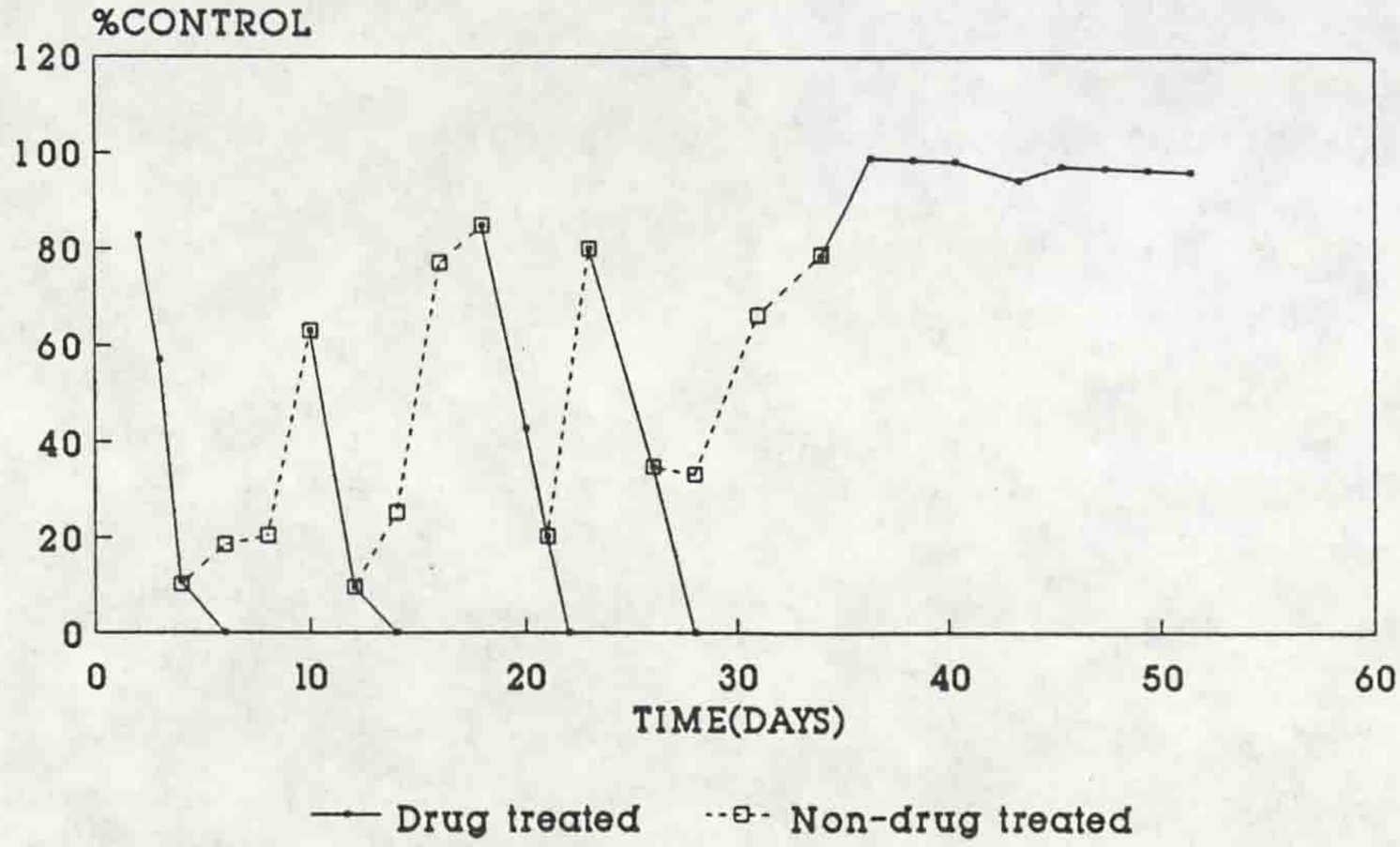


Fig.5.3.10 Time course of resistance to halofantrine in T9.96HF4 strain.



From day 0 -22 parasites intermittently were growing in presence of 20 nmol/l and from 22-51 were growing in 10 nmol/l

Fig.5.3.11 Comparative dose response curves for halofantrine against K1HF and K1 strains(microscopic examination).

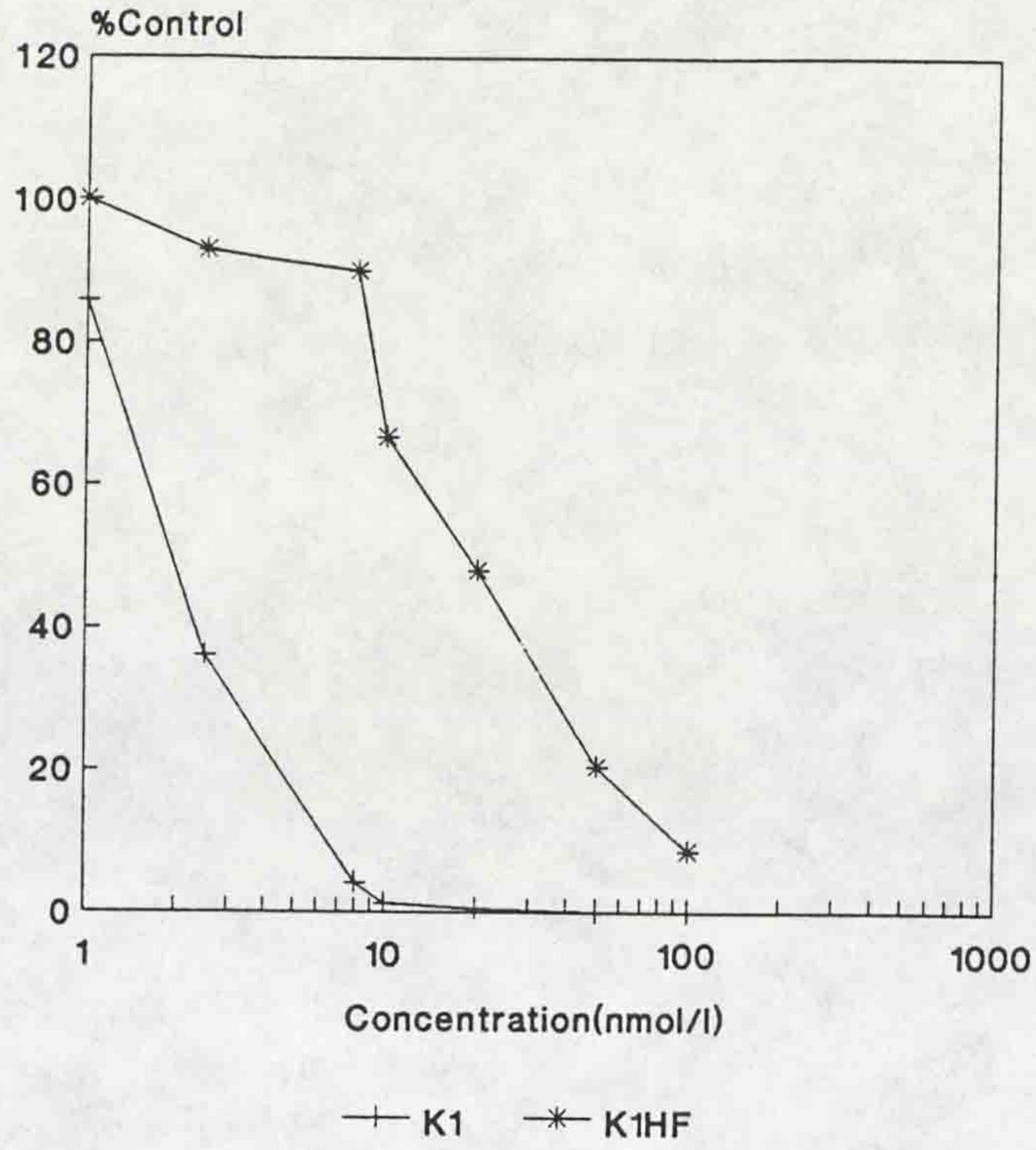


Fig.5.3.12 Comparative dose response curves for halofantrine against T9.96HF and T9.96 strains(microscopic method).

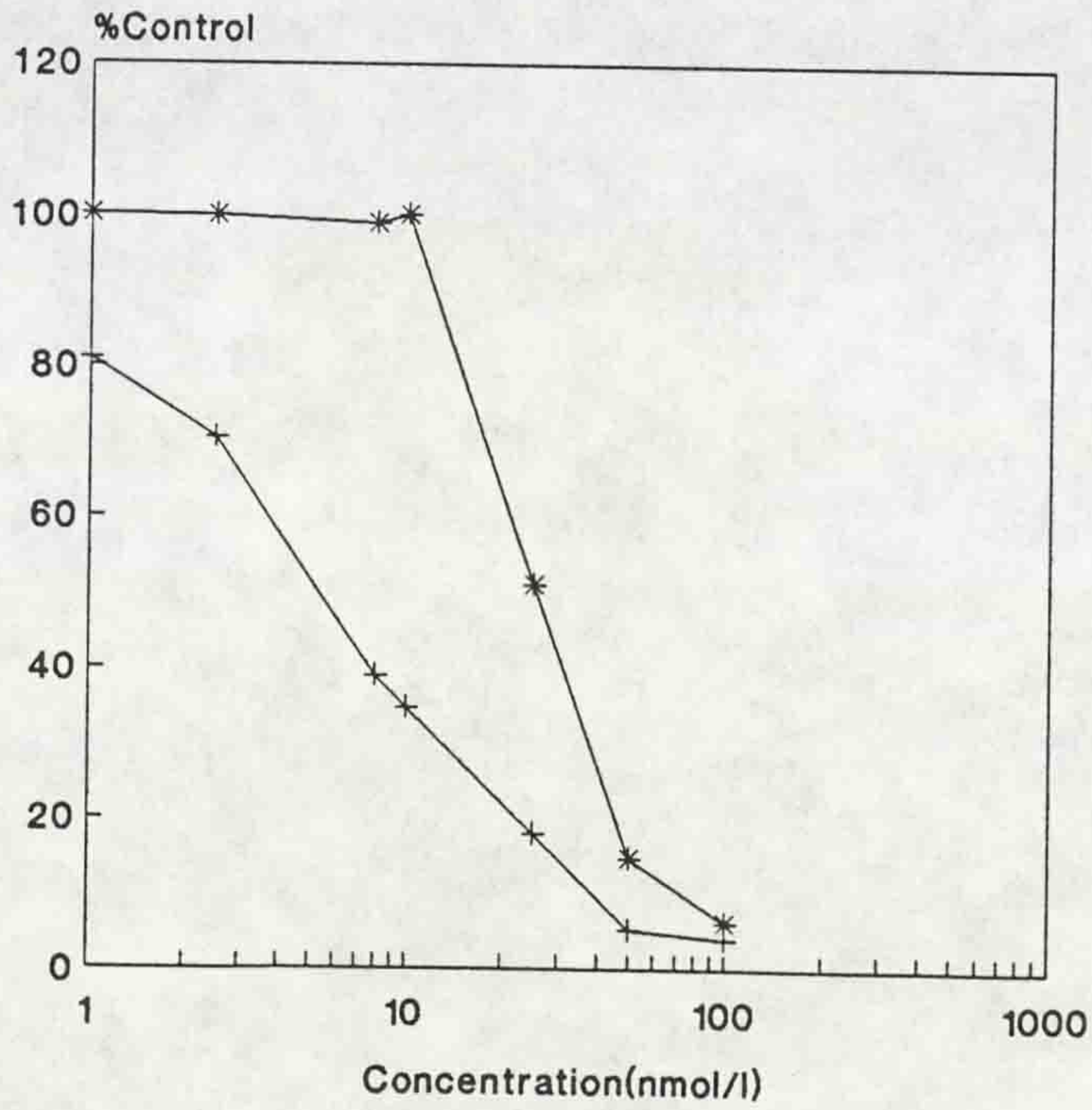
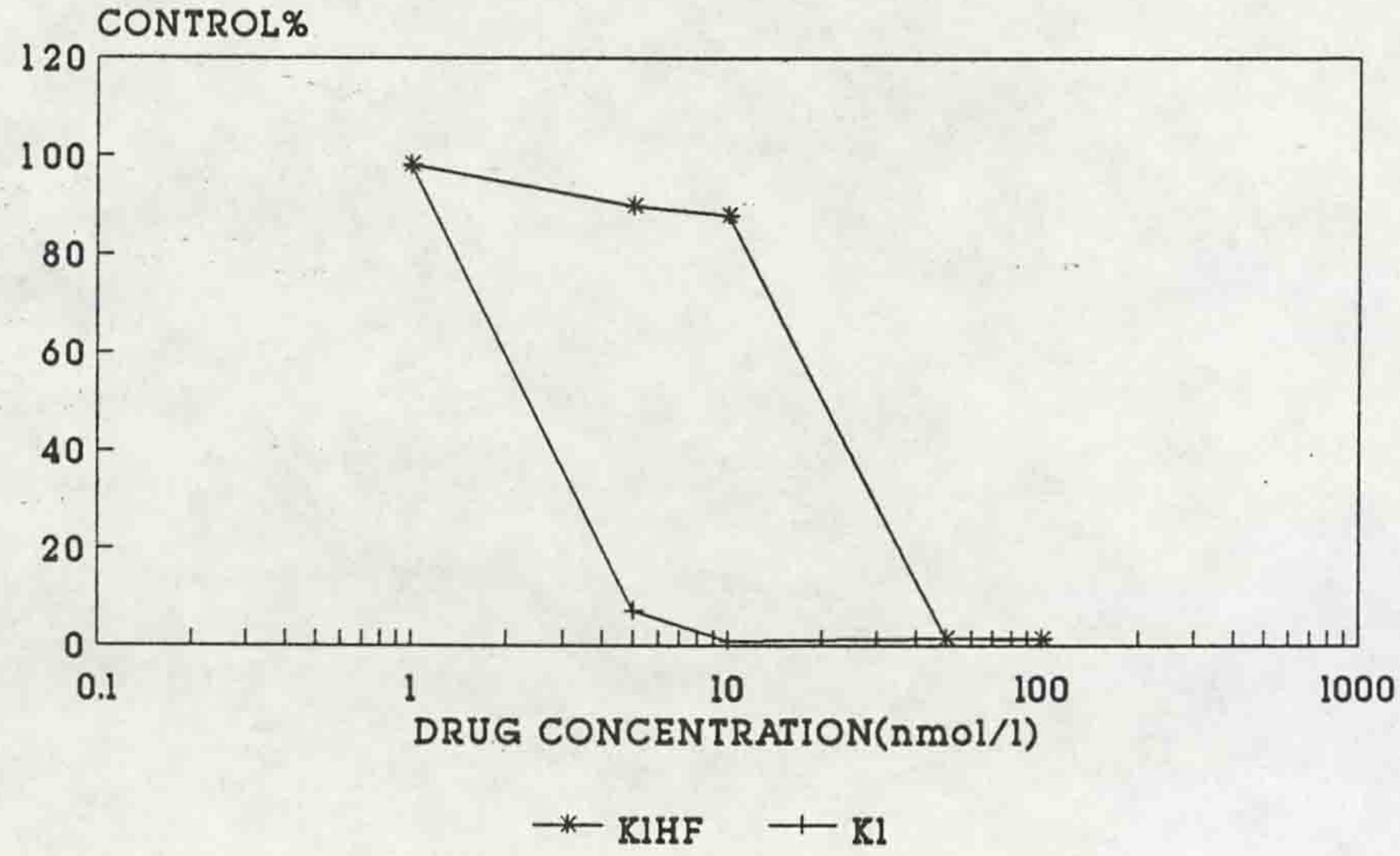
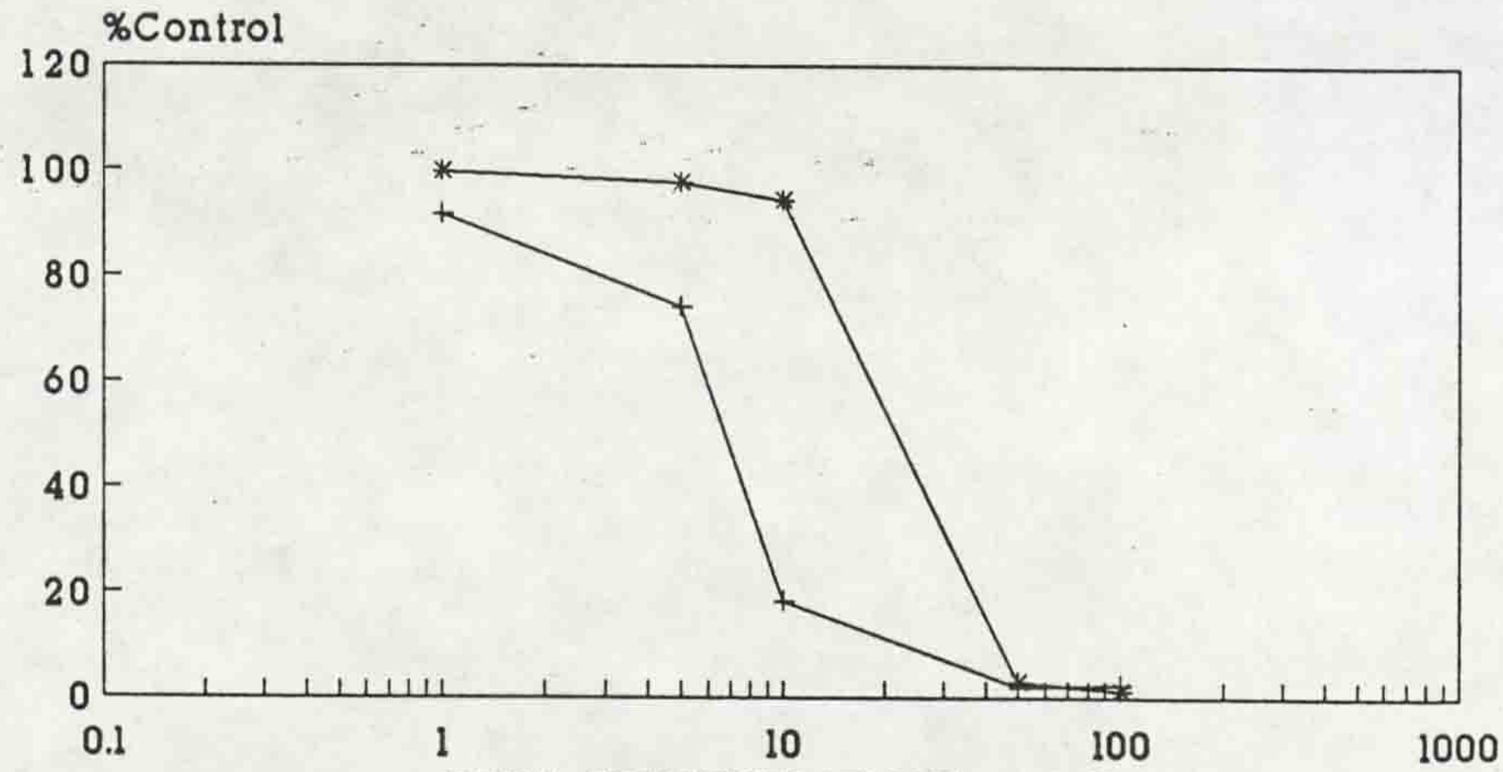


Fig.5.3.13 Comparative dose response curves for HF against KlHF and Kl strains([3H]hypoxanthine incorporation).



HF: halofantrine

Fig.5.3.14 Comparative dose response curves for HF against T9.96HF and T9.96 strains([3H]hypoxanthine incorporation).



HF: halofantrine

Fig.5.3.15 Comparative dose response curves for mefloquine against K1 and K1HF strains.

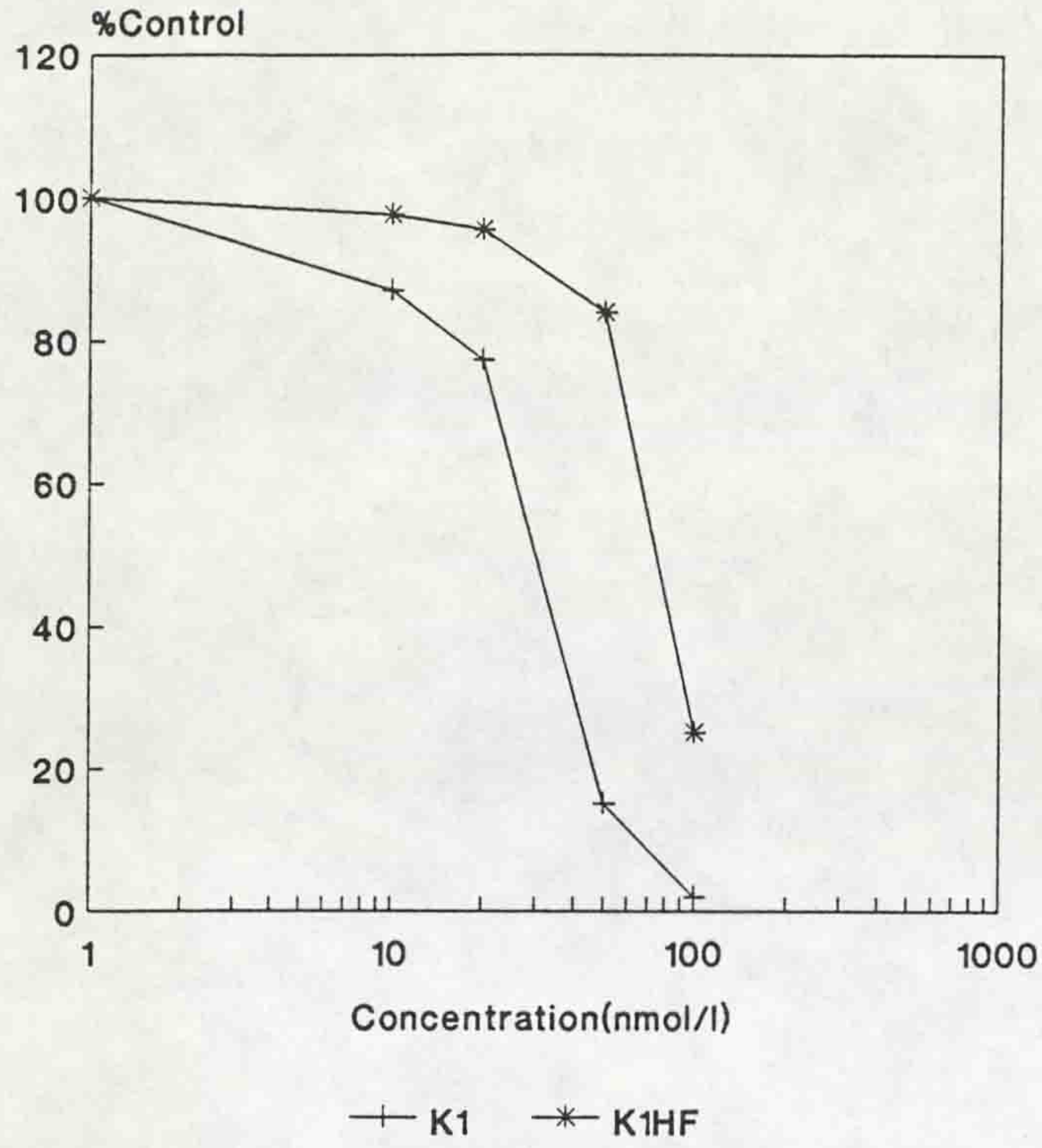


Fig.5.3.16 Comparative dose response curves for chloroquine against K1 and K1HF strains.

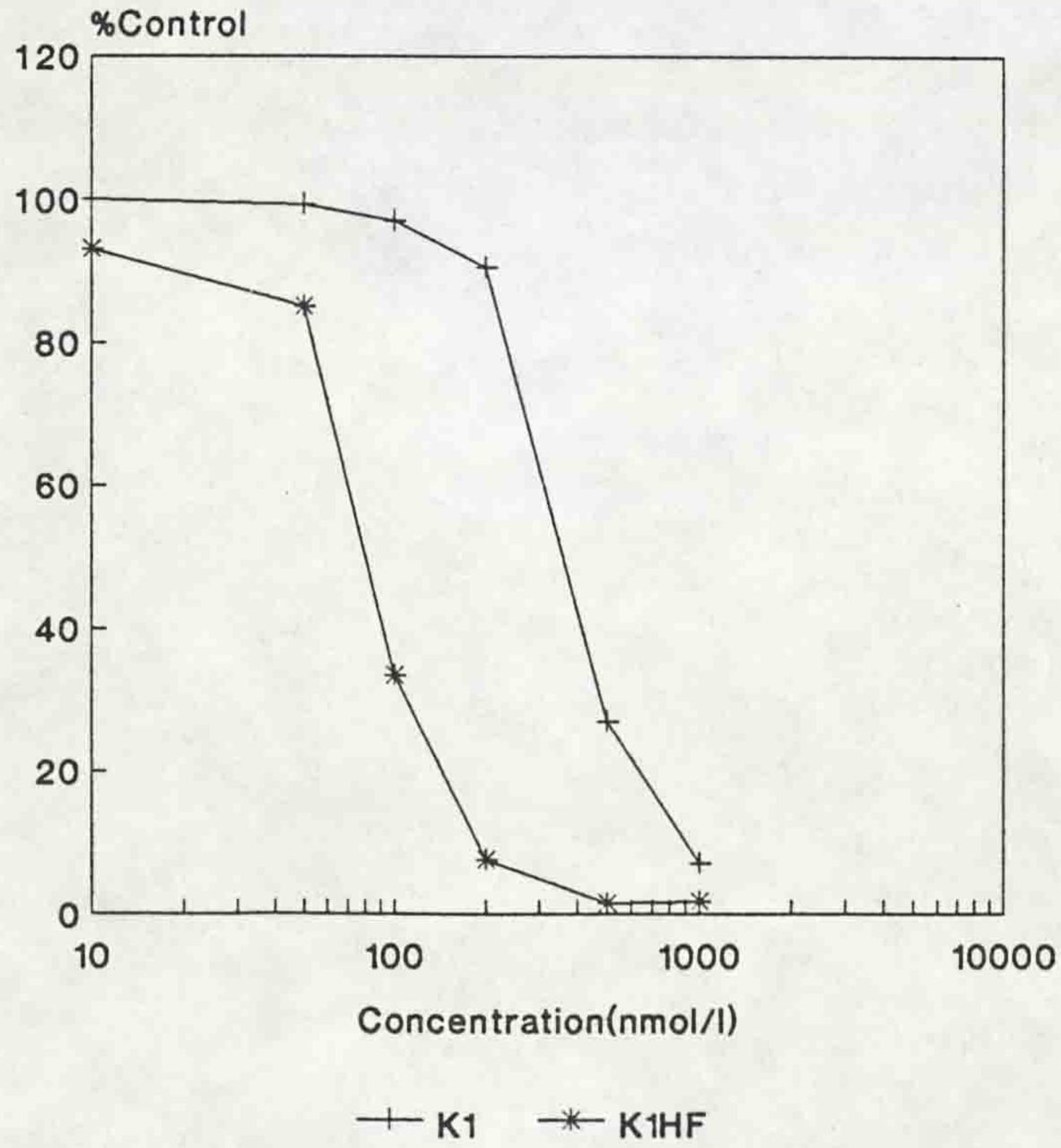


Fig.5.3.17 Comparative dose response curves for quinine against K1 and K1HF strains.

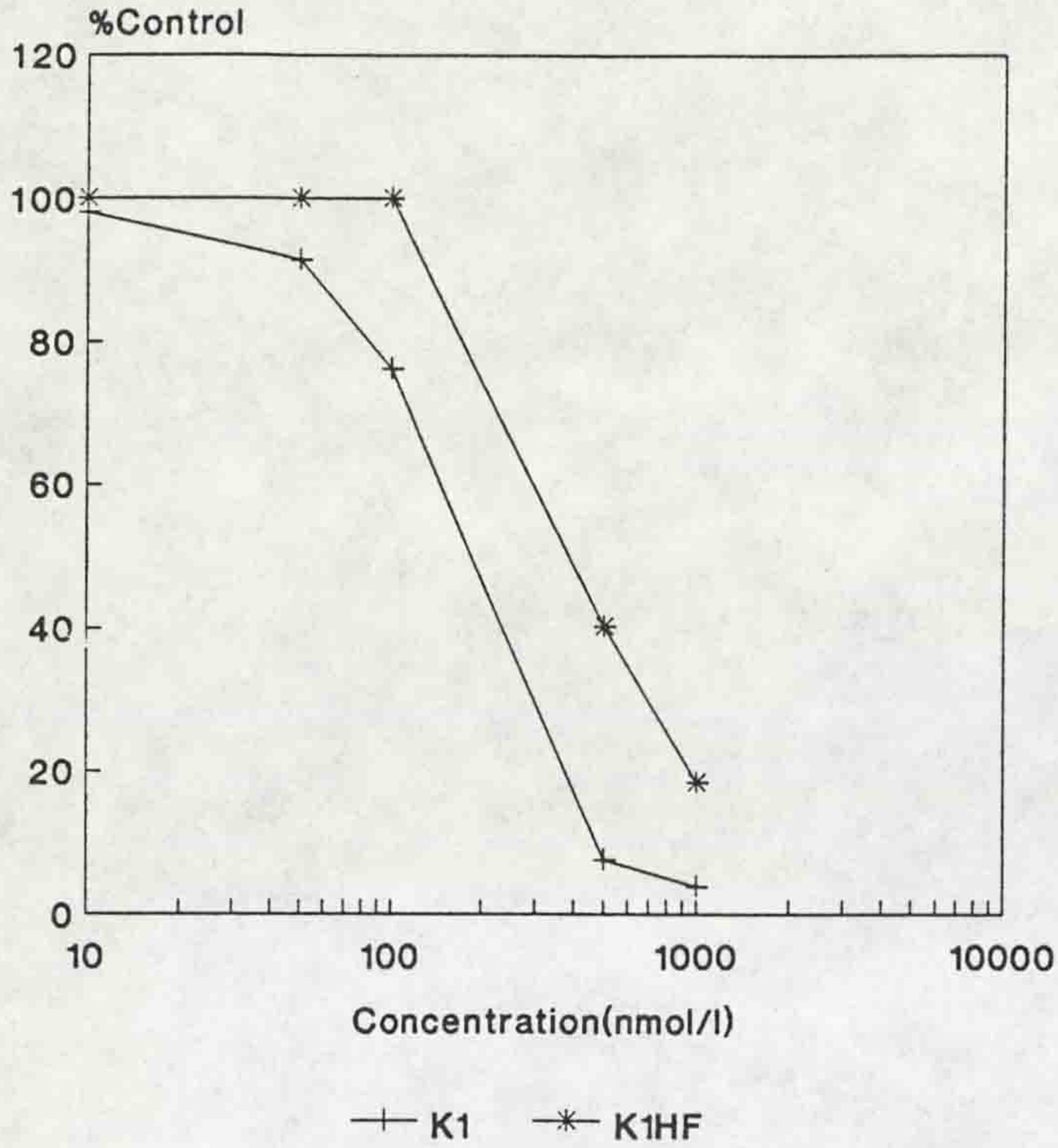


Fig.5.3.18 Comparative dose response curves for amodiaquine against K1 and K1HF strains.

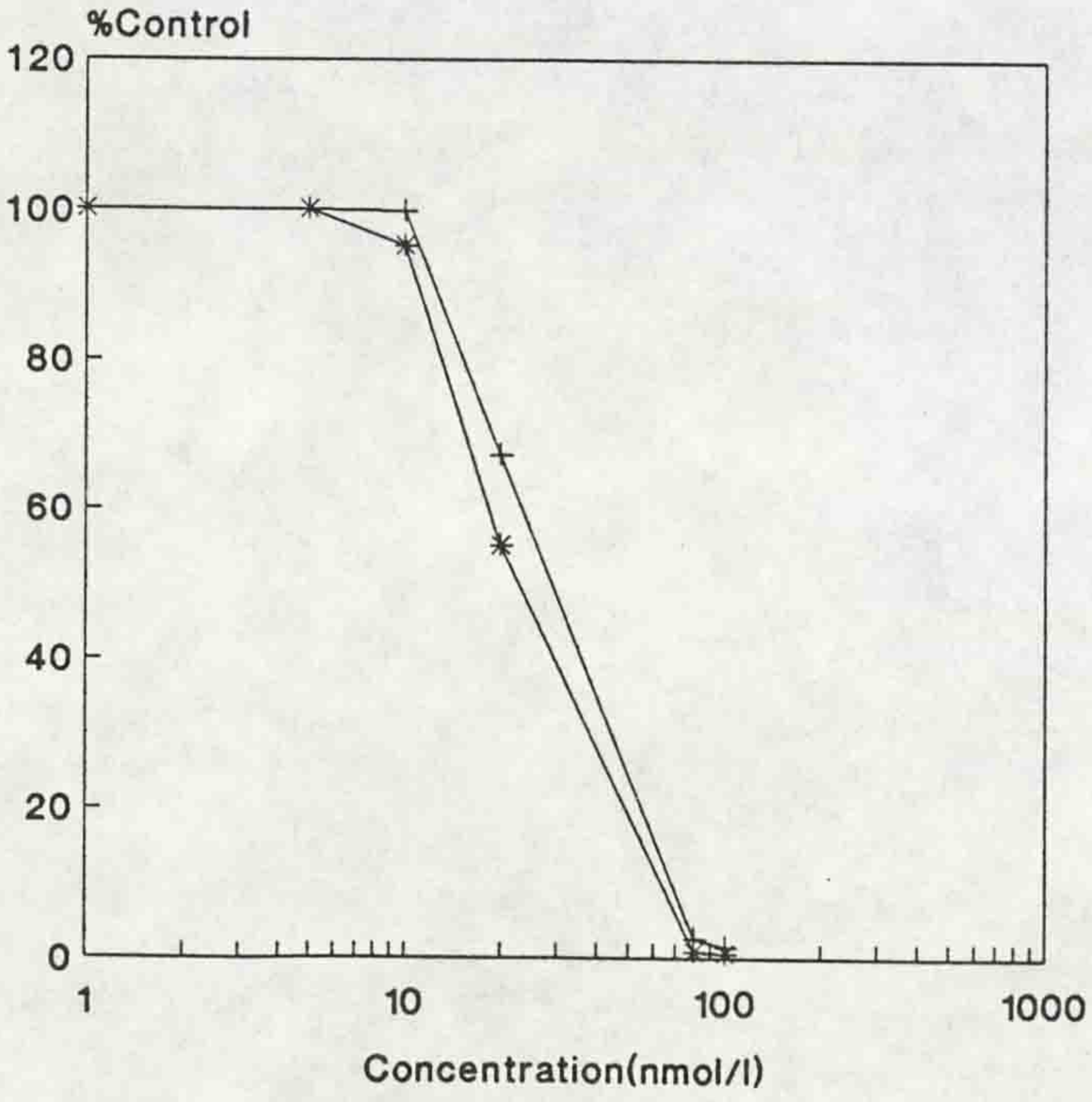


Fig.5.3.19 Comparative dose response curves for qinghaosu against K1 and K1HF strains.

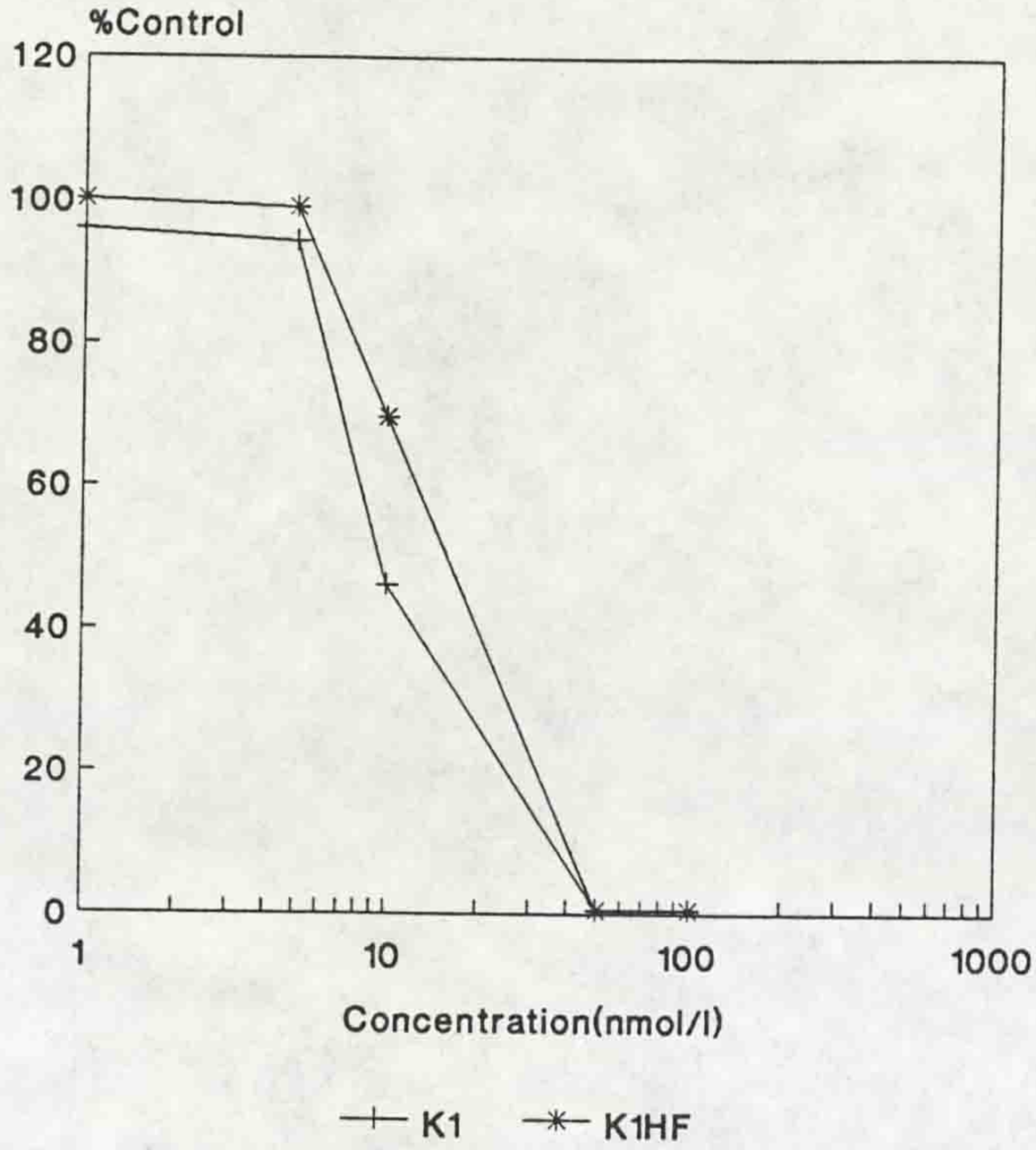


Fig.5.3.20 Comparative dose response curves for mefloquine against T9.96 and T9.96HF strains.

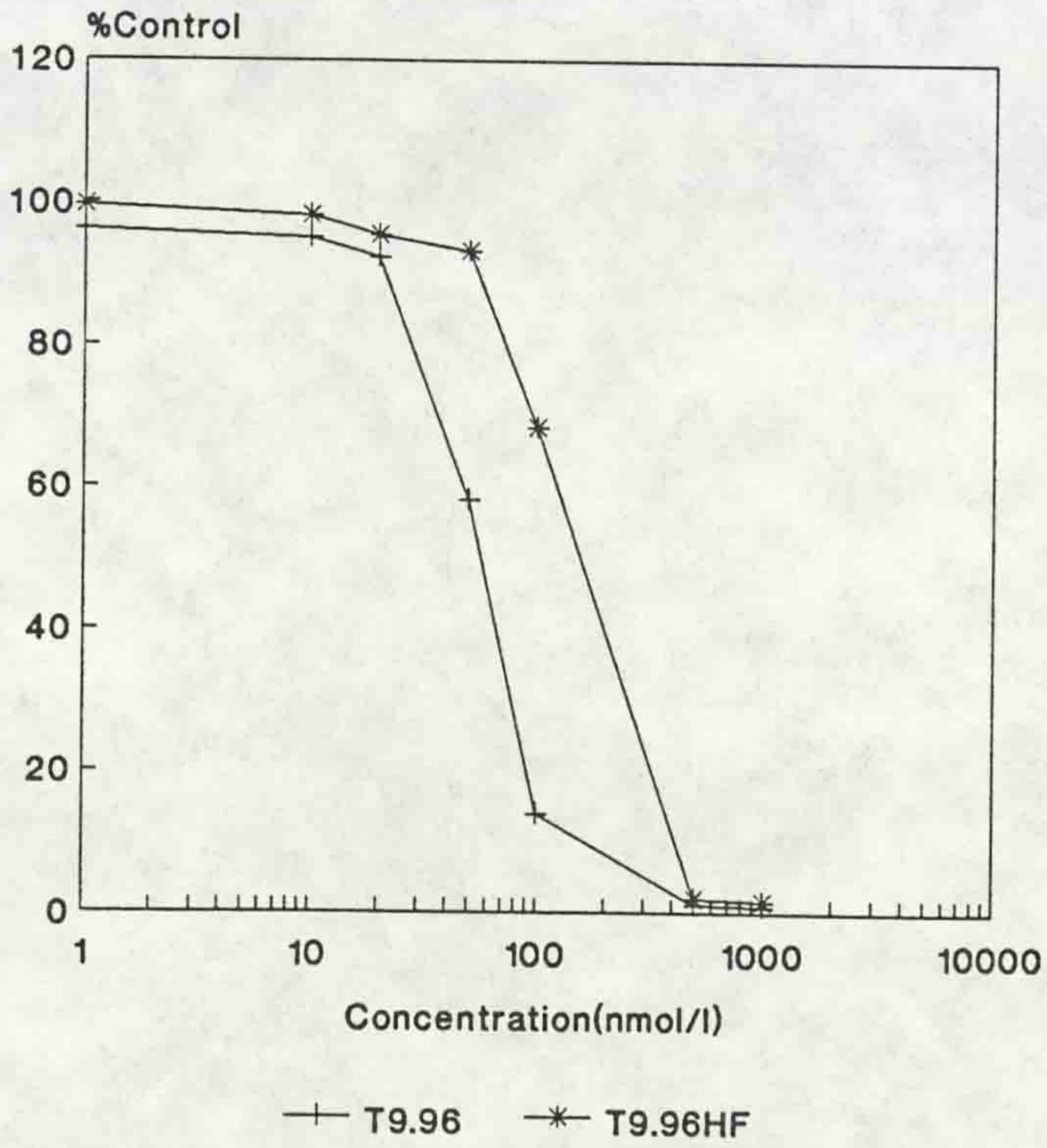


Fig.5.3.21 comparative dose response curves for chloroquine against T9.96 and T9.96HF strains.

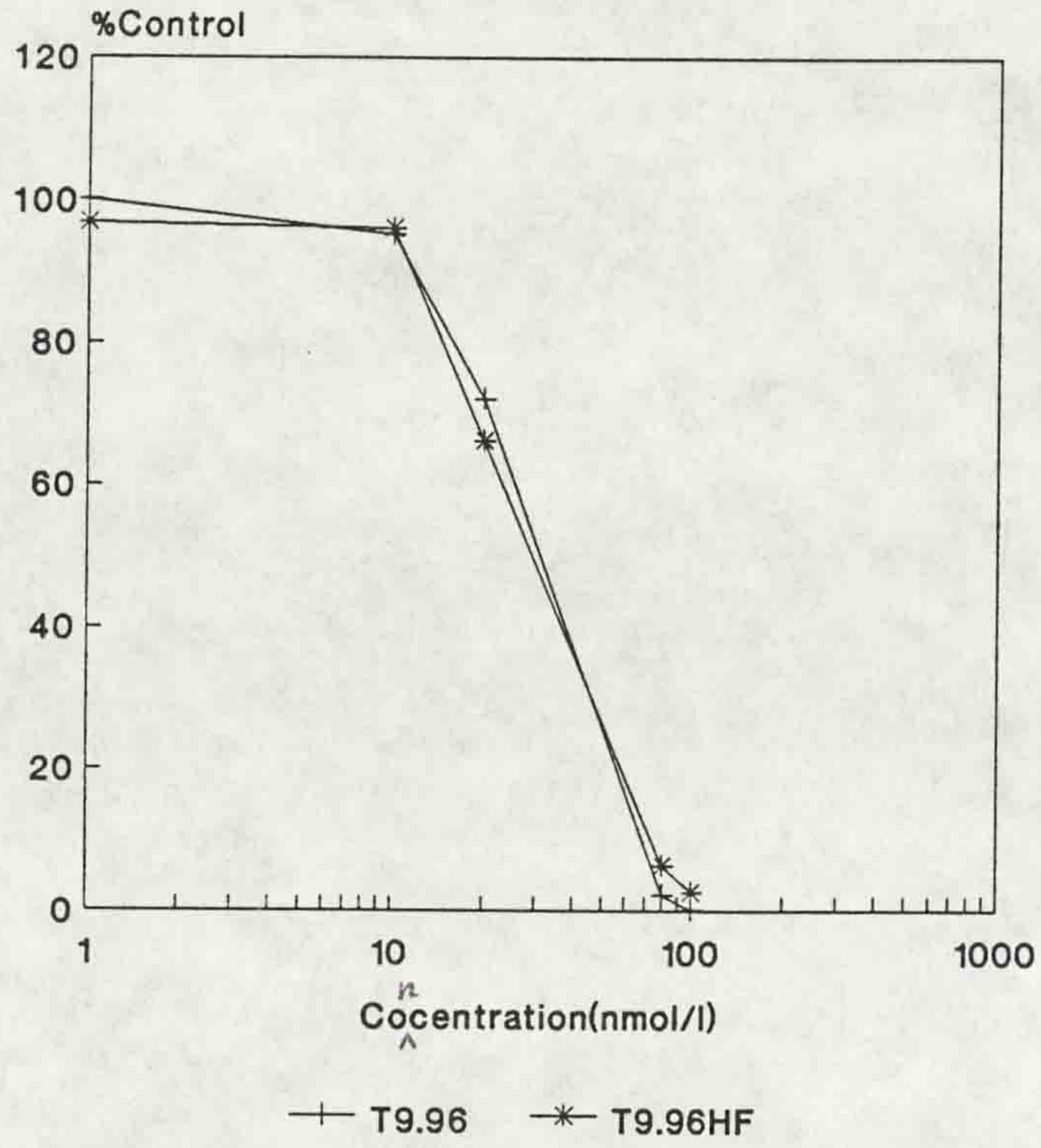


Fig.5.3.22 Comparative dose response curves for quinine against T9.96 and T9.96HF strains.

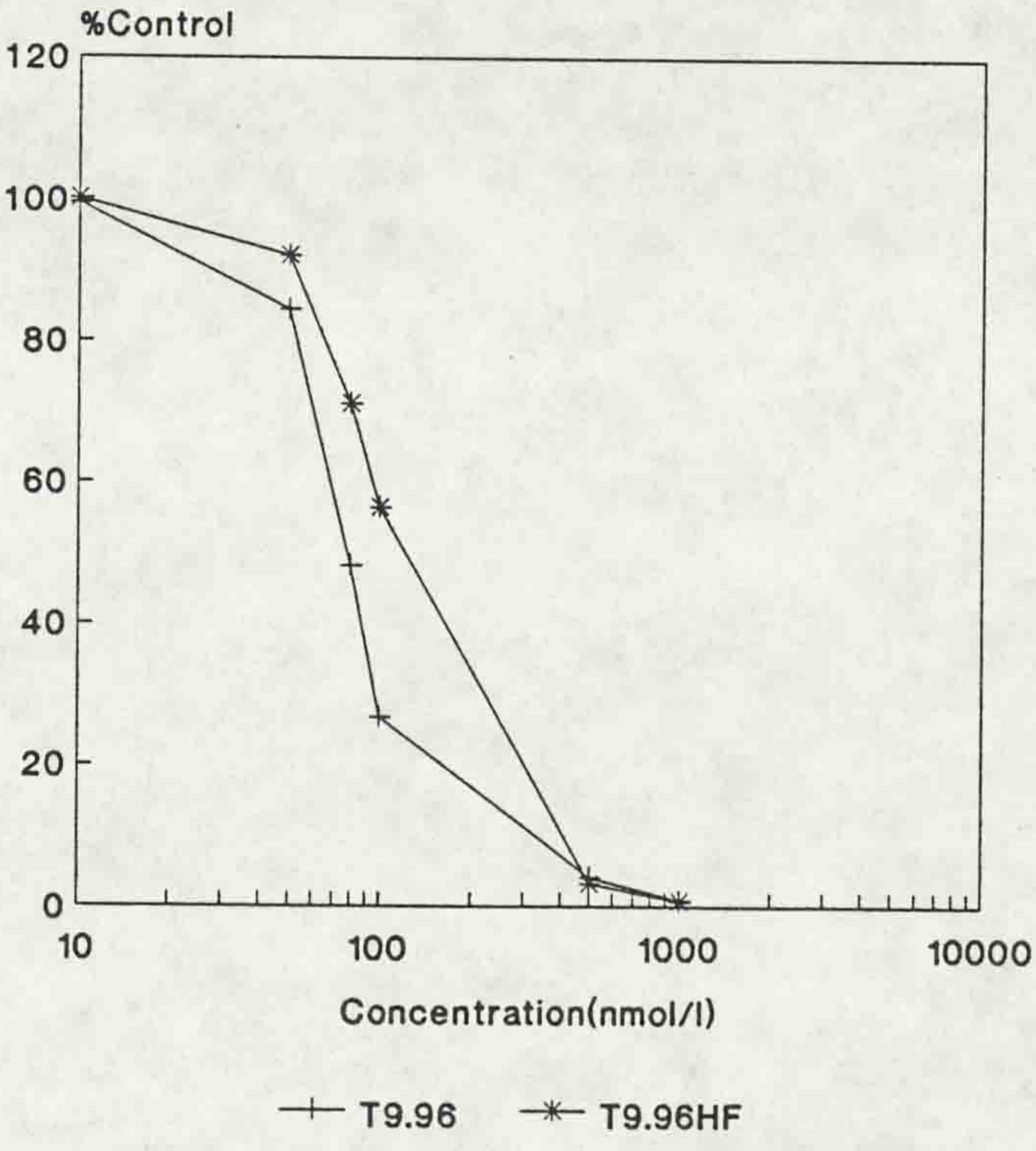


Fig.5.3.23 Comparative dose response curves for amodiaquine against T9.96 and T9.96HF strains.

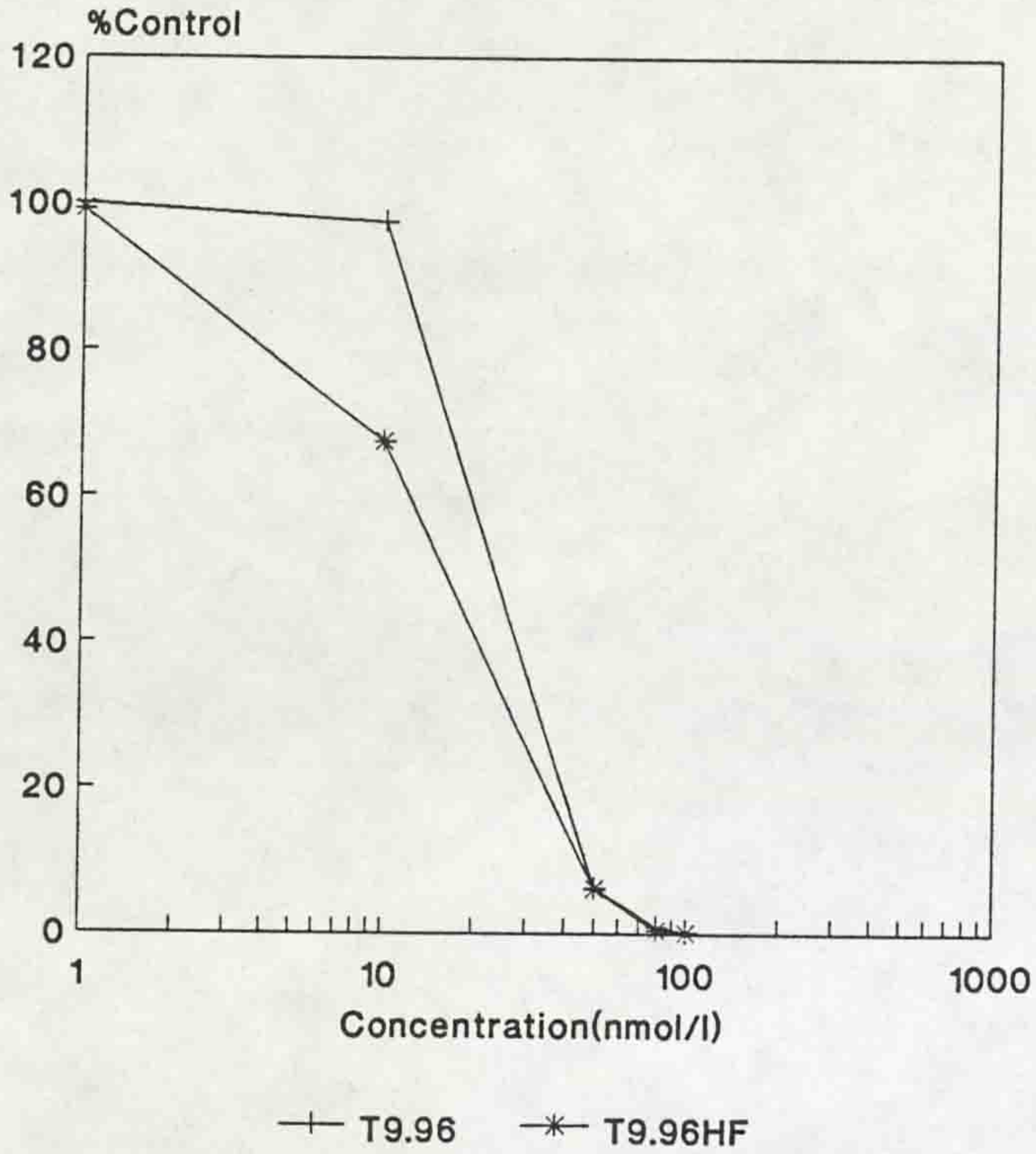


Fig.5.3.24 Comparative dose response curves for qinghaosu against T9.96 and T9.96HF strains.

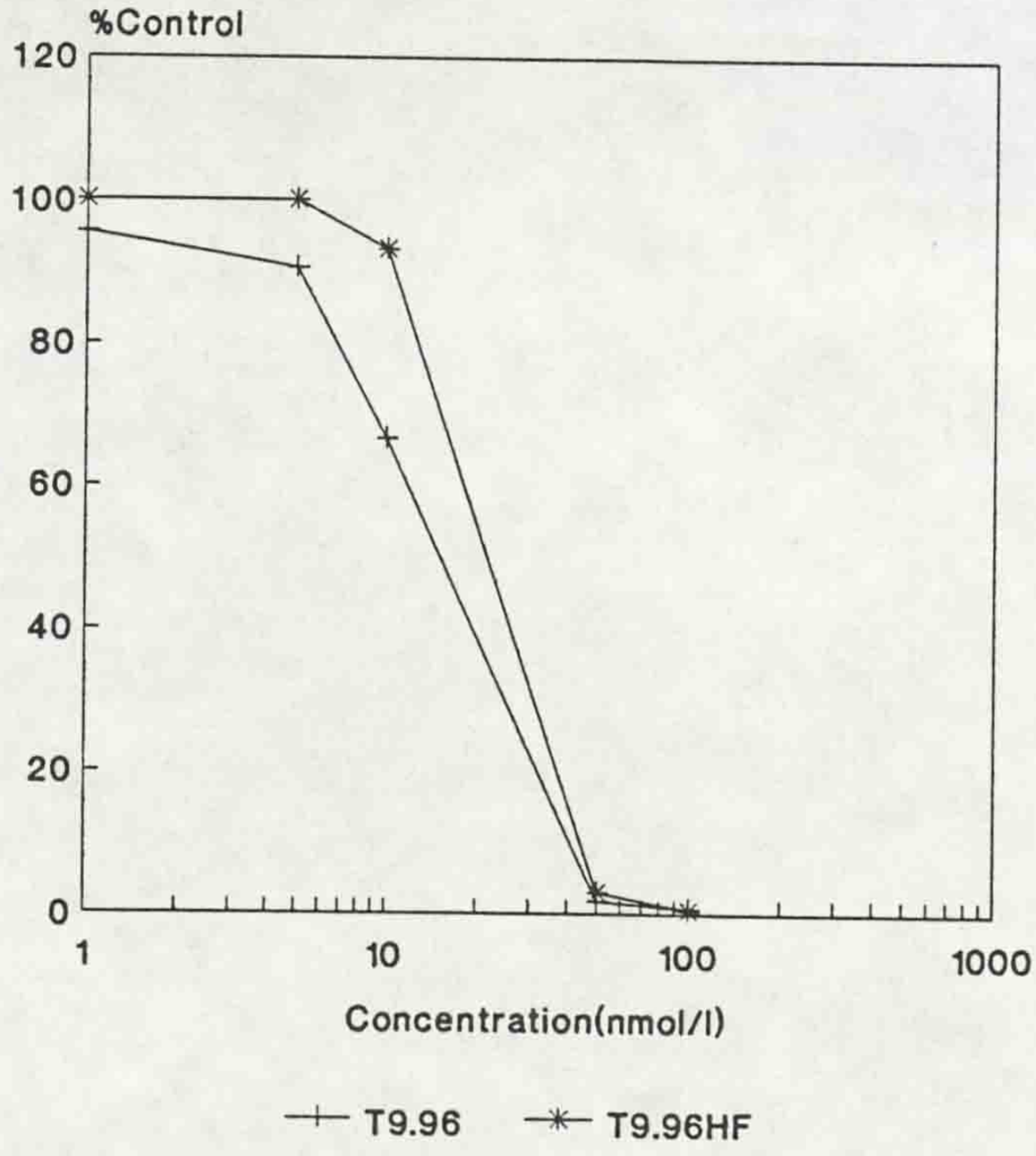


Fig.5.3.25 Comparative dose response curves for pyrimethamine against T9.96 and T9.96HF strains.

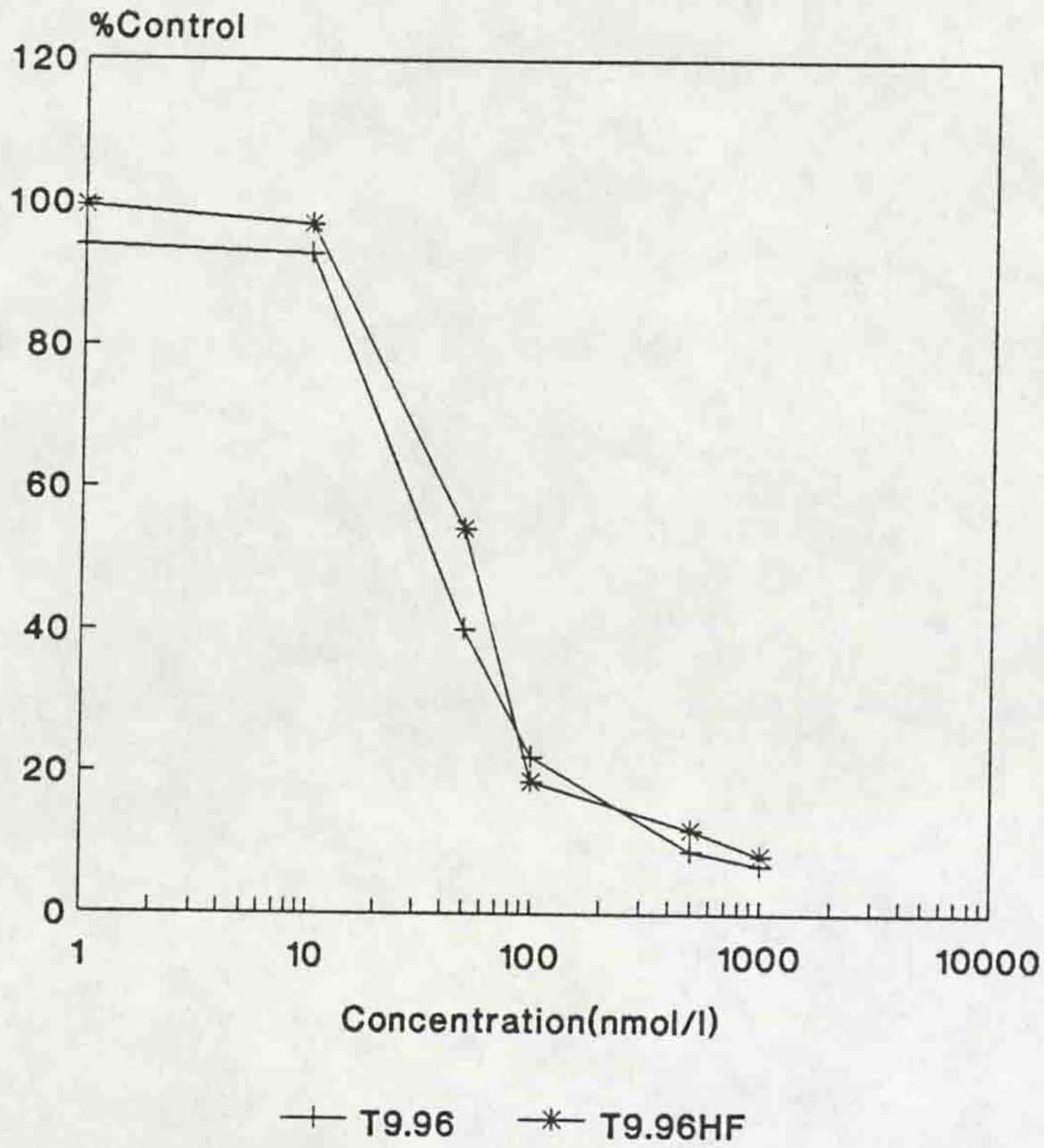


Fig.5.3.26 Comparative dose response curves for halofantrine against K1 and K1HF strains(resistance stability test).

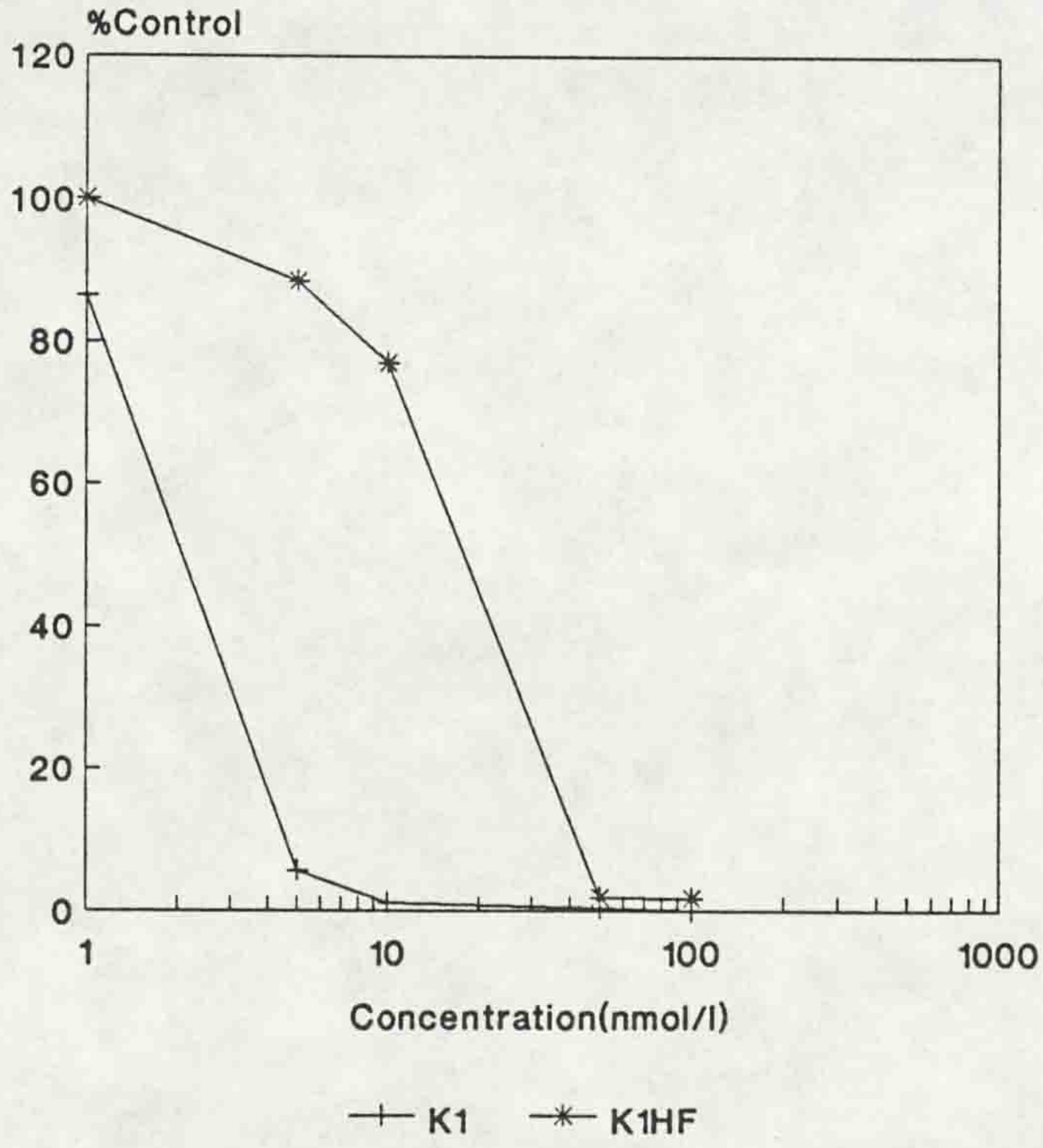


Fig.5.3.27 Comparative dose response curves for halofantrine against T9.96 and T9.96HF(resistance stability test).

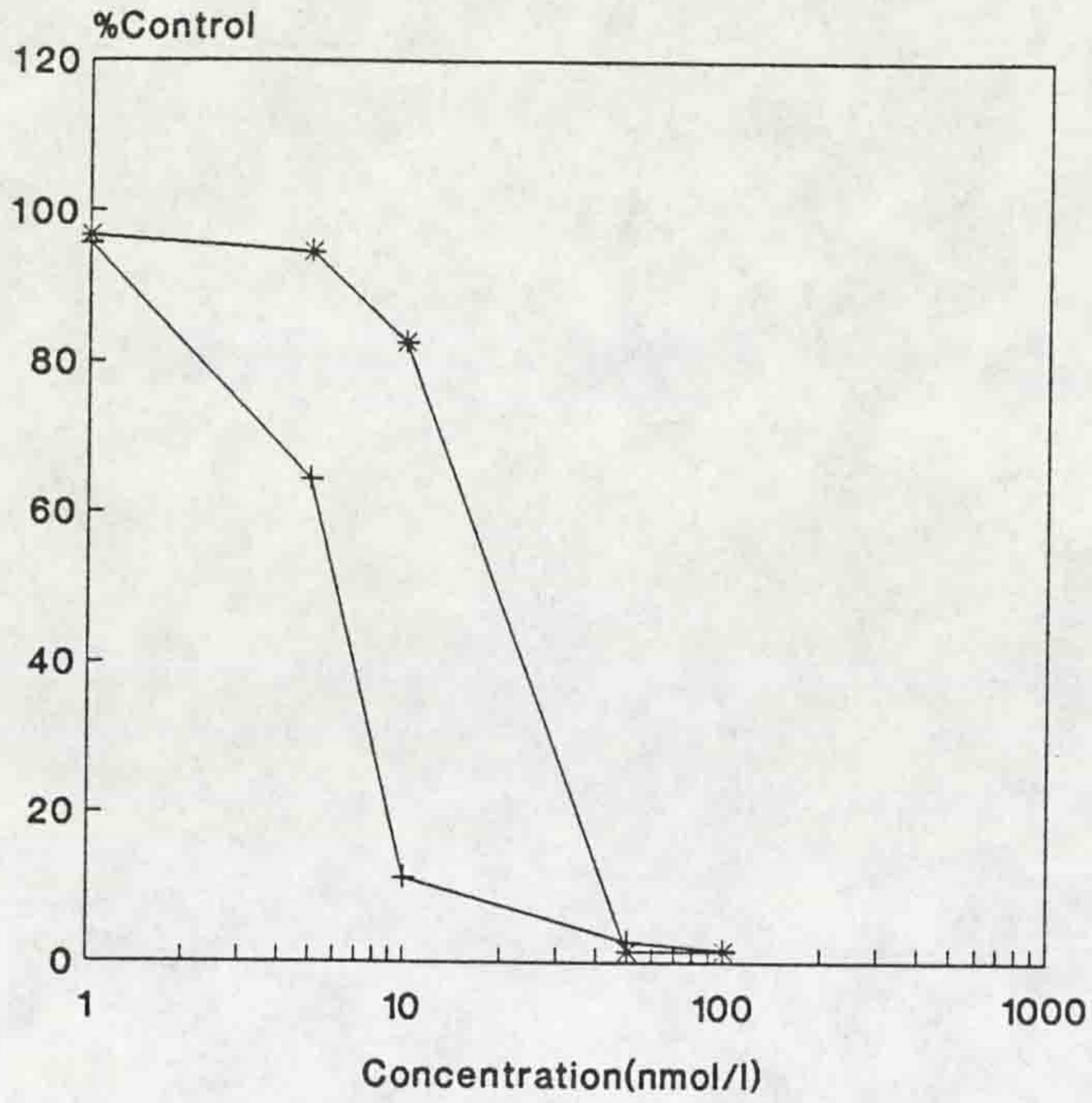
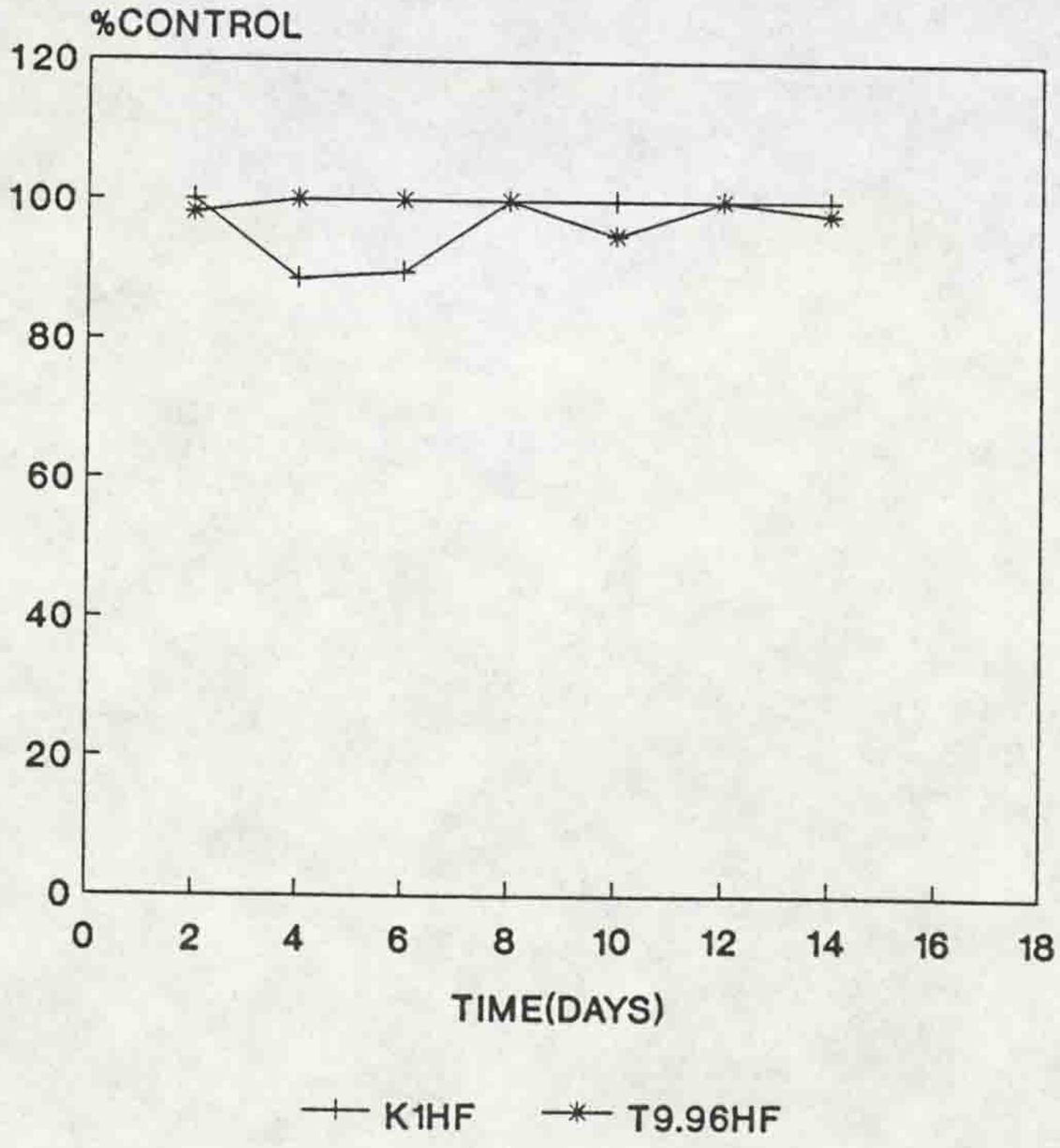


Fig.5.3.28 Time course of resistance stability of K1HF and T9.96HF strains after 6 weeks growing in drug-free medium.



K1HF and T9.96HF were cultured in 8 and 10 nmol/l halofantrine respectively.

CHAPTER 6 : THE EFFECT OF NOVEL DRUG COMBINATIONS AGAINST HALOFANTRINE-RESISTANT STRAINS OF *P. FALCIPARUM*

CHAPTER 6 Page

The effect of novel drug combinations against halofantrine-resistant strains of *P. falciparum*

6.1 Introduction

The development of halofantrine-resistant strains of *P. falciparum* (see Chapter 5) provided the opportunity to study these resistance mechanisms developed in the laboratory in greater detail. In particular we were interested to see if the resistance mechanisms shared any similarities with chloroquine resistance, which can be reversed by a number of drugs with no inherent antimalarial activity (see previous chapters). Although we originally set out to look at this problem from a phenomenological angle the recent observations of treatment failures with halofantrine (Gay *et al*, 1990) make these investigations of much greater practical importance as it is now almost inevitable that halofantrine resistance will become a major problem in the field.

In the last chapter we demonstrated that the development of halofantrine-resistance in chloroquine-resistant K_1 and chloroquine-sensitive $T_{9.96}$ strains of *P. falciparum* was associated with a significant decrease in parasite sensitivity to other antimalarials containing the methanolic function, ie. mefloquine and quinine, and a significant increase in parasite sensitivity to chloroquine. So, in addition to investigating the ability of the non-antimalarial drugs penfluridol, verapamil and fluoxetine to reverse halofantrine resistance in our resistant lines, we also examined their effects on the sensitivity of the parasites to mefloquine, quinine and chloroquine in combination forms.

6.2 Materials and methods

Six strains, K_1HF, $T_{9.96}$HF, $T_{9.96}$HF4, W2-mef, K_1 and $T_{9.96}$, were used during this study. Cultivation of parasites and measurement of drug

susceptibility were performed as in Chapter 2.1.2 and 2.2.3.1, respectively. The interaction between antimalarial drugs and non-antimalarial compounds was assessed on the basis of the fixed ratios method as described in Chapter 4.2. Additionally, the graphical analysis was conducted as in Chapter 4.2.

6.3 Results

6.3.1 Comparative dose response of K_1HF, $T_{9.96}$HF, $T_{9.96}$HF4, K_1 and $T_{9.96}$ parasites to penfluridol, verapamil, fluoxetine and mefloquine

The IC_{50} and IC_{90} values for penfluridol, verapamil, fluoxetine and mefloquine against K_1HF, $T_{9.96}$HF, $T_{9.96}$HF4, K_1 and $T_{9.96}$ strains are tabulated in Tables 6.3.1 to 6.3.4. The K_1HF and $T_{9.96}$HF parasites showed a significant decrease in sensitivity to penfluridol compared to the original strains K_1 and $T_{9.96}$. The results showed 4.68 and 3.51-fold increases in the IC_{50} values and 2.13 and 2.47-fold increases in the IC_{90} values for penfluridol for K_1HF and $T_{9.96}$HF strains respectively, compared to the parent strains (Tables 6.3.1 and 6.3.2). A significant decrease in sensitivity to verapamil exhibited by the K_1HF and $T_{9.96}$HF with 1.98 and 6.16 fold increases in the IC_{50} values compared to the K_1 and $T_{9.96}$ parasites (Tables 6.3.1 and 6.3.2). The K_1HF strain showed a slight increase in parasite sensitivity to fluoxetine, but there was no significant difference between the IC_{50} values for $T_{9.96}$HF and $T_{9.96}$ strains to the drug.

Penfluridol exhibited an IC_{50} of 1800nmol/l against W2-mef strain which is comparable to that obtained for the $T_{9.96}$HF strain (Table 6.3.3). The results are also graphically illustrated in Figs. 6.3.1 to 6.3.5.

6.3.2 Combinations of halofantrine, quinine or chloroquine with penfluridol, verapamil or fluoxetine against K_1 and K_1HF strains

The results are graphically presented in Figs. 6.3.6 to 6.3.14. A combination of penfluridol with halofantrine or chloroquine exhibited an antagonistic effect against the K_1 strain. There was a loss of synergy between chloroquine and verapamil against the K_1HF parasites, particularly at the ratios of 50:50, 30:70 and 10:90 (with 44.26, 40.34 and 36.03 percent inhibition respectively) compared to the parent strain K_1 (Fig. 6.3.12).

Fluoxetine antagonised the activity of chloroquine markedly, at the ratios of 30:70 and 10:90 (chloroquine:fluoxetine) (with 39.63 and 31.26 percent inhibition respectively) against the K_1HF parasites compared to the K_1 parasites (Fig. 6.3.13).

The combination of penfluridol with quinine was associated with antagonism against K_1HF parasites (Fig. 6.3.14) similar to the findings for penfluridol plus halofantrine (Fig. 6.3.8). The greatest antagonism occurred with a combination of penfluridol and chloroquine, particularly in the ratios of 70:30, 50:50 and 30:70 (penfluridol:chloroquine; with 12.07, 9.91 and 12.39 percent inhibition respectively) against the K_1HF strain (Fig. 6.3.9). The interaction between halofantrine and verapamil against K_1HF showed potentiation at the ratios of 90:10, 70:30 and 50:50 (with 83.85, 71.08 and 53.95 percent inhibition respectively) (Fig. 6.3.10). There was no significant discrepancy between the response of the K_1HF and K_1 parasites to the combination of halofantrine with fluoxetine (Fig. 6.3.11).

6.3.3 Combinations of halofantrine, mefloquine or quinine with penfluridol, verapamil or fluoxetine against $T_{9.96}$, $T_{9.96}$HF and $T_{9.96}$HF4 strains

The results of penfluridol + halofantrine and penfluridol + mefloquine combinations against $T_{9.96}$ parasites are graphically presented in Figs. 6.3.15 and 6.3.16. An additive effect was observed for the combination of penfluridol with halofantrine and an antagonism for the combination of penfluridol with mefloquine especially notable at the ratios 30:70 and 10:90 with 28.55 and 27.78 percent inhibition, respectively. Penfluridol also produced an additive effect on the $T_{9.96}$HF4 strain in combination with mefloquine (Fig. 6.3.17).

Figs. 6.3.18 to 6.3.20 illustrate the results of combinations of penfluridol with halofantrine, mefloquine and quinine against $T_{9.96}$HF parasites. Potentiation was obtained for the combination of penfluridol with halofantrine especially at the ratios of 50:50 and 30:70 with 80.64 and 80.69 percent inhibition, respectively. As such penfluridol increased the activity of mefloquine against the $T_{9.96}$HF parasites. In contrast, the interaction between penfluridol and quinine was one of antagonism particularly at the ratios of 70:30 and 50:50 with 10.35 and 9.79 percent inhibition, respectively.

Using a combination of halofantrine with verapamil resulted in an antagonistic effect against $T_{9.96}$HF as did the fluoxetine + halofantrine combination especially at the ratio of 50:50 with 38.83 percent inhibition. The results are graphically presented in Figs. 6.3.21 and 6.3.22.

6.3.4 The combination of penfluridol with mefloquine against the W2-mef strain

The interaction between penfluridol and mefloquine produced an additive effect on mefloquine-resistant W2-mef parasites in all ratios except the ratio of 10:90 which showed slight antagonism. The results are graphically shown in Fig. 6.3.23.

A summary of the results of drug combination experiments against K_1HF, $T_{9.96}$HF, $T_{9.96}$HF4, W2-mef, K_1 and $T_{9.96}$ strains of *P. falciparum* are tabulated in Tables 6.3.5 to 6.3.7.

6.4 Discussion

The results obtained from sensitivity tests in this study indicated that halofantrine resistance was associated with a decreased susceptibility to penfluridol and verapamil, but not to fluoxetine.

The reduced sensitivity to penfluridol and verapamil in the K_1HF and $T_{9.96}$HF parasites correlated with the degree of resistance developed to halofantrine and mefloquine. It is most interesting that combinations of penfluridol with halofantrine and mefloquine exhibited potentiation against the $T_{9.96}$HF parasites, as will be discussed later on.

Penfluridol [R16341; 4-(4-chloro-α, α, α-trifluoro-m-tolyl)-1-[4, 4-bis (p-fluorophenyl) butyl]-4-piperidinol] is a potent and long-acting neuroleptic drug. This drug is a white microcrystalline tertiary amine. The pharmacology, pharmacokinetics and toxicity of penfluridol has been extensively studied by Janssen *et al* (1970). They demonstrated that penfluridol is relatively non-toxic

in normal dosages. The occasional side-effects are restricted to common neurological symptoms (Janssen *et al*, 1970).

The actions of penfluridol on parasites is unknown. Gietzen *et al* (1980) reported that penfluridol like chlorpromazine can inhibit the activation of erythrocyte Ca^{2+}-transporting ATPase via calmodulin. In another study, Lavoie (1987) examined the effect of penfluridol on fast axonal transport in the nerve of the bullfrog *in vitro*. The author suggested that inhibition of axonal transport might be connected with inhibition of the action of calmodulin by penfluridol. However, it is, more or less, clear that penfluridol can antagonise the action of calmodulin, but the actual mechanism of this inhibition remains unsolved.

Results obtained for the combination of halofantrine with penfluridol produced a slight antagonism against the K_1HF strain, but a considerable potentiation against the $T_{9.96}$HF parasites. Although the reason for this discrepancy is not clear, it may be speculated that the $T_{9.96}$HF and K_1HF strains have inherently different responses to the combination of halofantrine with penfluridol or to either drug alone. In agreement with this Ringwald and co-workers (1990) isolated 178 strains of *P. falciparum* with a wide range of sensitivity to halofantrine from thirteen African countries. Additionally, the variable response of strains of *P. falciparum* to the similar drug combinations has been reported by a number of investigators. For instance, using combinations of chloroquine with desipramine or cyproheptadine against chloroquine-resistant FCR_3 parasites by Bitonti and McCann (1989) and against cloned multidrug-resistant FCM29/Cameroon parasites by Basco and Le Bras (1990a) produced significantly different results. Basco and Le Bras (1990a) suggested that the

difference between these results may depend on genetic and biochemical differences, or different sensitivity levels in these strains to the drug combinations.

The results indicated that combination of mefloquine with penfluridol produced potentiation against the $T_{9.96}$HF parasites, but only an additive effect against $T_{9.96}$HF4 (which has a lower level of resistance to mefloquine than $T_{9.96}$HF) and W2-mef (mefloquine-resistant) strains. The difference between $T_{9.96}$HF and $T_{9.96}$HF4 parasites in response to mefloquine + penfluridol combinations presumably depends on the level of resistance to mefloquine. In other words, the combination of mefloquine with penfluridol against the $T_{9.96}$ (mefloquine-sensitive strain), $T_{9.96}$HF4 and $T_{9.96}$HF produced antagonisms, an additive effect and potentiation, respectively. As such the discrepancy between $T_{9.96}$HF and W2-mef parasites in response to the combination may relate to the same situation as stated above or follow a similar pattern to those already described for the combination of halofantrine with penfluridol against the K_1HF and $T_{9.96}$HF parasites.

Although penfluridol potentiated the activity of mefloquine and halofantrine against the $T_{9.96}$HF parasites, in combination with quinine (another member of methanolic functional group) it showed a remarkable antagonism. The reason for this discrepancy is not clear.

The results obtained from the combination halofantrine + verapamil indicated that verapamil considerably increased the sensitivity of the K_1HF parasites to halofantrine compared to the parent parasites (K_1). Whereas there was no significant difference between response of the $T_{9.96}$HF and $T_{9.96}$ parasites to the combination of halofantrine with verapamil. Such differences

between K_1HF and $T_{9.96}$HF parasites in response to combination halofantrine + verapamil may follow a similar pattern to those described above for halofantrine + penfluridol combination.

Our data would suggest that different resistance mechanisms to halofantrine have been developed in our laboratory strains, despite using the same protocol to induce resistance. Halofantrine resistance in K_1HF parasites may have a common basis with chloroquine resistance as both appear to be reversed with verapamil. However, in our $T_{9.96}$HF parasites sensitivity was unaltered by verapamil but reversed by penfluridol which has no effect on chloroquine sensitivity in resistant parasites.

In conclusion penfluridol appears to potentiate the activity of halofantrine and mefloquine against halofantrine-resistant $T_{9.96}$HF parasites. The activity of halofantrine was increased against K_1HF parasites when employed in combination with verapamil. Additionally, the combination of chloroquine with verapamil or fluoxetine lost considerably its potentiation against K_1HF parasites compared to the parent parasites (K_1).

Table 6.3.1 **The IC_{50} (K_1HF)/IC_{50} (K_1) ratios and IC_{90} (K_1HF)/IC_{90} (K_1) ratios for penfluridol, verapamil and fluoxetine in the 48-hour assay, using [3H] hypoxanthine incorporation**

Strain / Drug	K_1HF Mean IC_{50} (nmol/l)	K_1 Mean IC_{50} (nmol/l)	IC_{50} (K_1HF) / IC_{50} (K_1)	K_1HF Mean IC_{90} (nmol/l)	K_1 Mean IC_{90} (nmol/l)	IC_{90} (K_1HF) / IC_{90} (K_1)
Penfluridol	1850	395	4.68	4800	2250	2.13
Verapamil	13900	7017	1.98	66000	>10000	-
Fluoxetine	15500	21667	0.71	42500	75667	0.56

Table 6.3.2 **The IC_{50} ($T_{9.96}HF$)/IC_{50} ($T_{9.96}$) and IC_{90} ($T_{9.96}HF$)/IC_{90} ($T_{9.96}$) ratios for penfluridol, verapamil and fluoxetine in 48-hour assay, assessed by [3H] hypoxanthine incorporation**

Strain / Drug	$T_{9.96}HF$ Mean IC_{50} (nmol/l)	$T_{9.96}$ Mean IC_{50} (nmol/l)	IC_{50} ($T_{9.96}HF$) / IC_{50} ($T_{9.96}$)	$T_{9.96}HF$ Mean IC_{90} (nmol/l)	$T_{9.96}$ Mean IC_{90} (nmol/l)	IC_{90} ($T_{9.96}HF$) / IC_{90} ($T_{9.96}$)
Penfluridol	1733.3	493.3	3.51	4200	1700	2.47
Verapamil	26500	4300	6.16	78000	56000	1.39
Fluoxetine	17500	18500	0.94	44500	41500	1.07

Table 6.3.3 **Penfluridol IC_{50} and IC_{90} values for K_1, K_1HF, $T_{9.96}$, $T_{9.96}$HF, W2-mef and $T_{9.96}$HF4 strains of *P. falciparum* in the 48-hour hypoxanthine incorporation assay (data from individual experiments and mean ± SD)**

Strain	IC_{50} (nmol/l)		IC_{90} (nmol/l)	
	Individual	Mean ± SD	Individual	Mean ± SD
K_1	330	-	2100	-
	460		2400	
K_1HF	1700	-	4700	-
	2000		4900	
$T_{9.96}$	450		1400	
	500	493.3 ± 40.41	1700	1700 ± 300
	530		2000	
$T_{9.96}$HF	1100		2900	
	1700	1733.3 ± 650.6	4200	4200 ± 3000
	2400		4500	
W2-mef	1600	-	5000	-
	2000		5800	
$T_{9.96}$HF4	1200	-	3700	-
	1800		4500	

Table 6.3.4 **Verapamil, fluoxetine and mefloquine IC_{50} and IC_{90} values for K_1HF, $T_{9.96}HF$, W2-mef and $T_{9.96}HF4$ strains of *P. falciparum* in 48-hour [3H] hypoxanthine incorporation assay (data from individual experiments)**

		IC_{50} (nmol/l)		IC_{90} (nmol/l)	
Verapamil	Strain	Individual	Mean ± SD	Individual	Mean ± SD
	K_1HF	8800	-	56000	-
		19000		76000	
	$T_{9.96}HF$	22000	-	76000	-
		31000		80000	
Fluoxetine	K_1HF	11000	-	39000	-
		20000		46000	
	$T_{9.96}HF$	16000	-	42000	-
		19000		47000	
Mefloquine	W2-mef	70		300	
		85	95 ± 31.2	400	407 ± 110.1
		130		520	
	$T_{9.96}HF4$	70	-	260	-
		110		480	

Table 6.3.5 Summary of results of drug combinations against the K_1HF strain of *P. falciparum*, using [3H] hypoxanthine incorporation in 48-hour assays

An: Antagonism; Po: Potentiation; Ad: Additive effect

Drug	Penfluridol	Verapamil	Fluoxetine
Halofantrine	An.	Po.; Ad.*	An.
Mefloquine	-	-	-
Quinine	An.	-	-
Chloroquine	An.	Po.: Ad.*	Po.; Ad.x

* The interaction between halofantrine, chloroquine and verapamil showed potentiation at the ratios of 90:10 and 70:10, but additive effect in the others.

x The interaction between chloroquine and fluoxetine showed potentiation at the ratios of 90:10 , 70:30 and 50:50 but antagonism in the others.

Table 6.3.6 Summary of results of drug combinations against the $T_{9.96}$HF strain of *P. falciparum* using [3H] hypoxanthine incorporation in 48-hour assays

Drug	Penfluridol	Verapamil	Fluoxetine
Halofantrine	Po.	An.	An.
Meflfoquine	Po.	-	-
Quinine	An.	-	-

Table 6.3.7 **Summary of results of drug combinations against the K_1, $T_{9.96}$, $T_{9.96}$HF4 and W2-mef strains of *P. falciparum*, employing [3H] hypoxanthine incorporation in 48-hour assays**

Strains / Combinations	K_1	$T_{9.96}$	$T_{9.96}$HF4	W2-mef
Penfluridol + Halofantrine	An.	Ad.	-	-
Penfluridol + Mefloquine	-	An.	Ad.	Ad.
Penfluridol + Chloroquine	An.	-	-	-

Fig.6.3.1 Comparative dose response curves for penfluridol against K1 and K1HF strains.

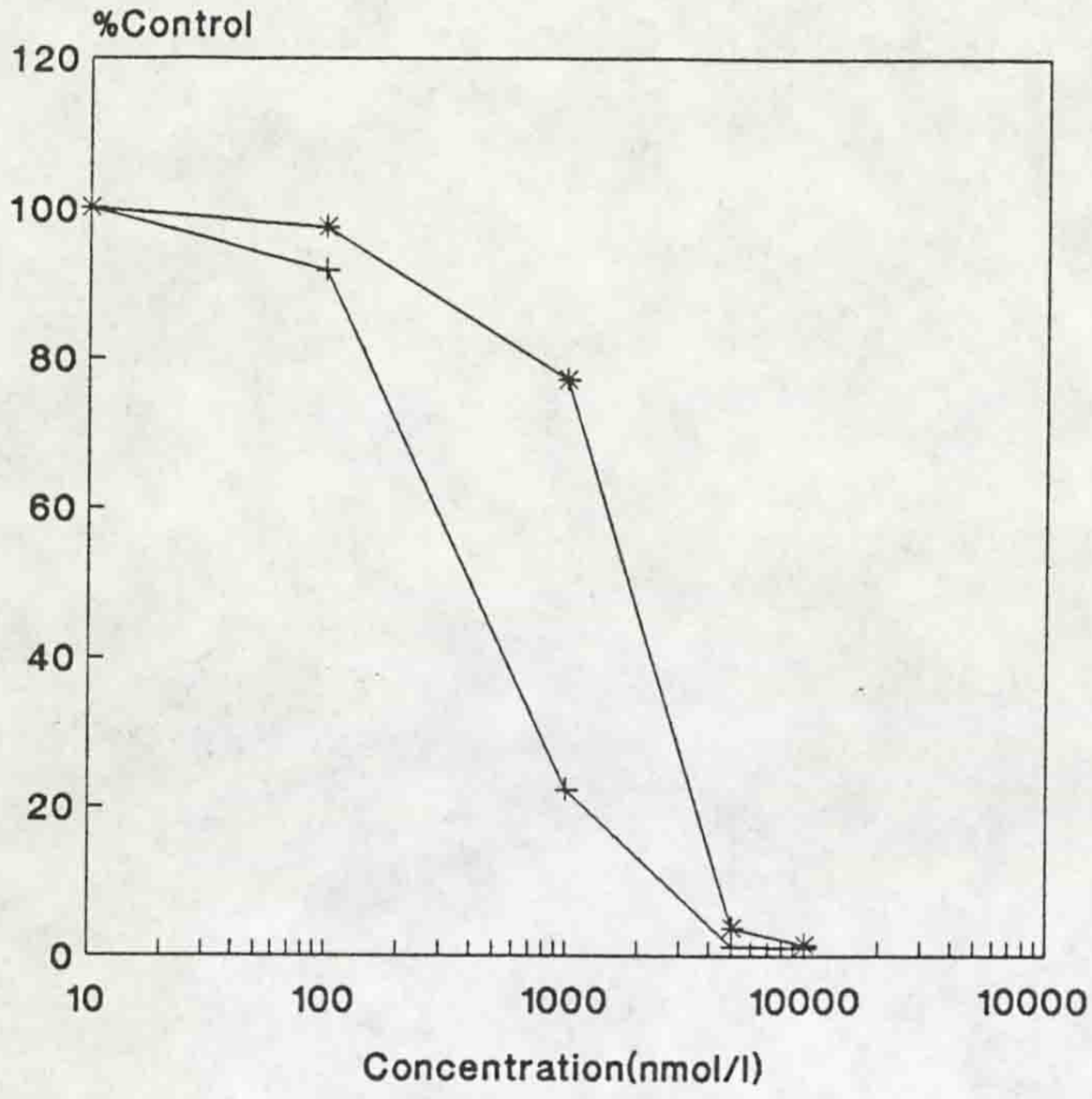


Fig.6.3.2 comparative dose response curves for penfluridol against T9.96, T9.96HF and W2-mef strains.

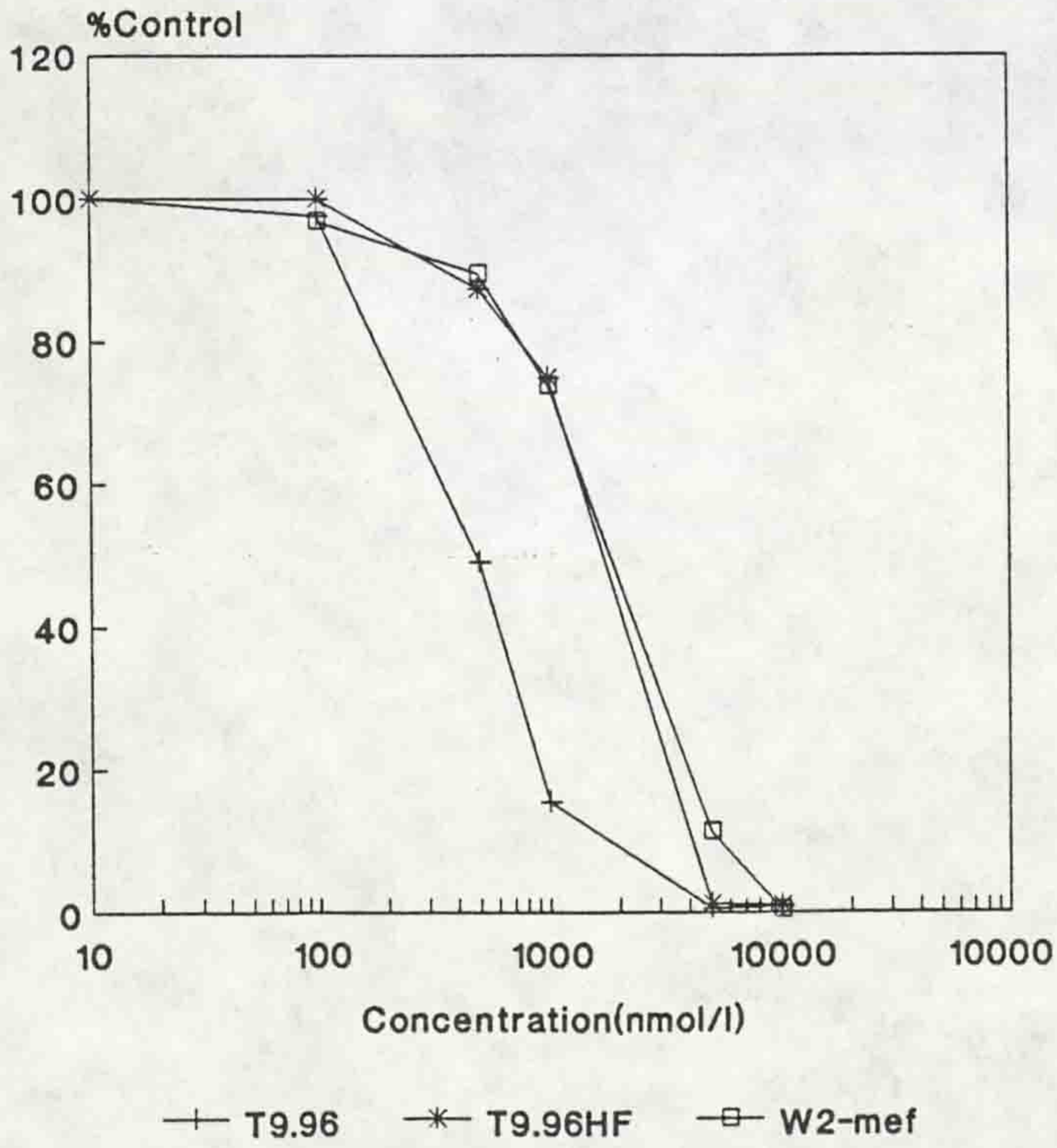


Fig.6.3.3 comparative dose response curves for verapamil and fluoxetine against K1HF strain.

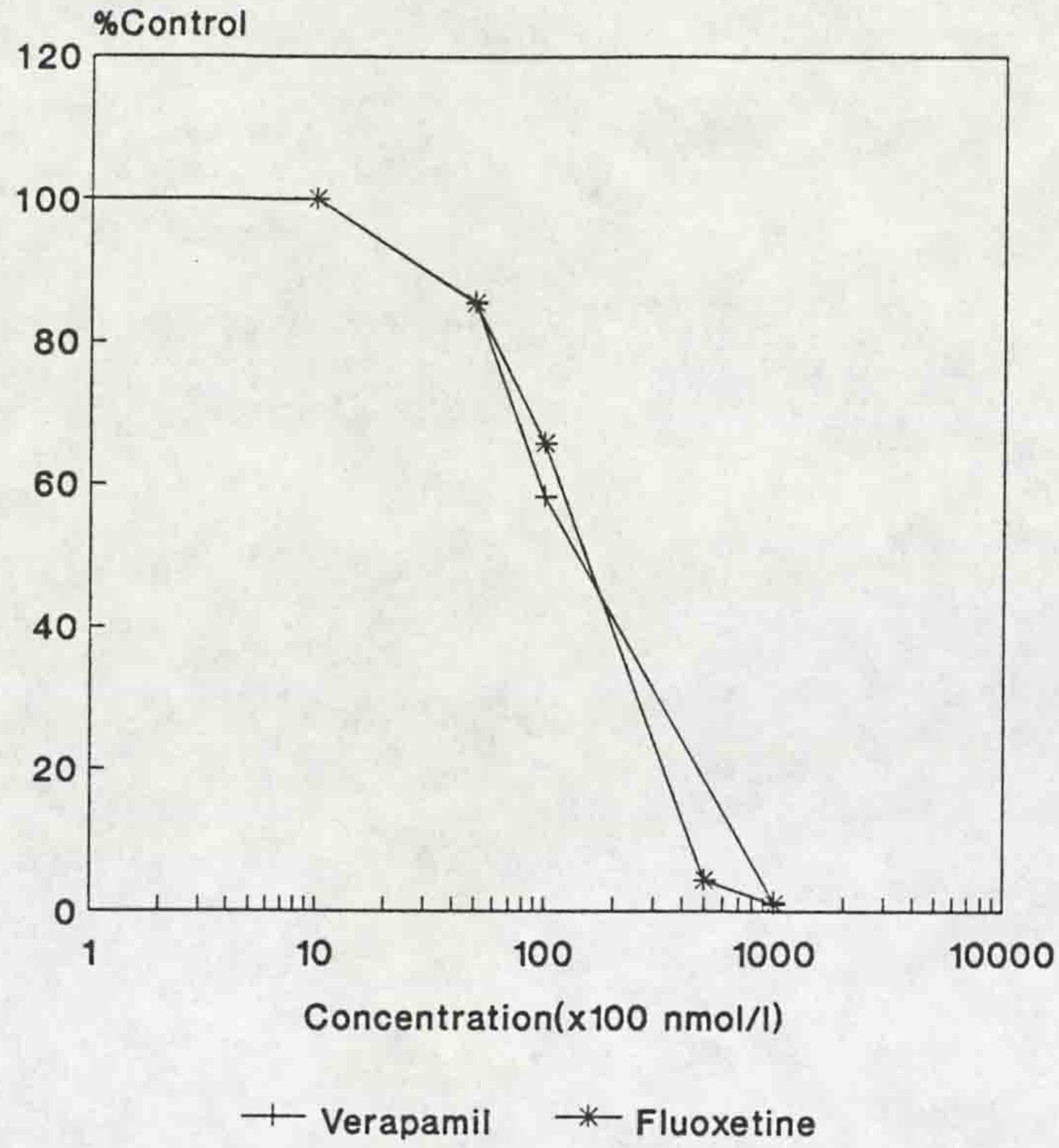


Fig.6.3.4 comparative dose response curves for verapamil and fluoxetine against T9.96HF strain.

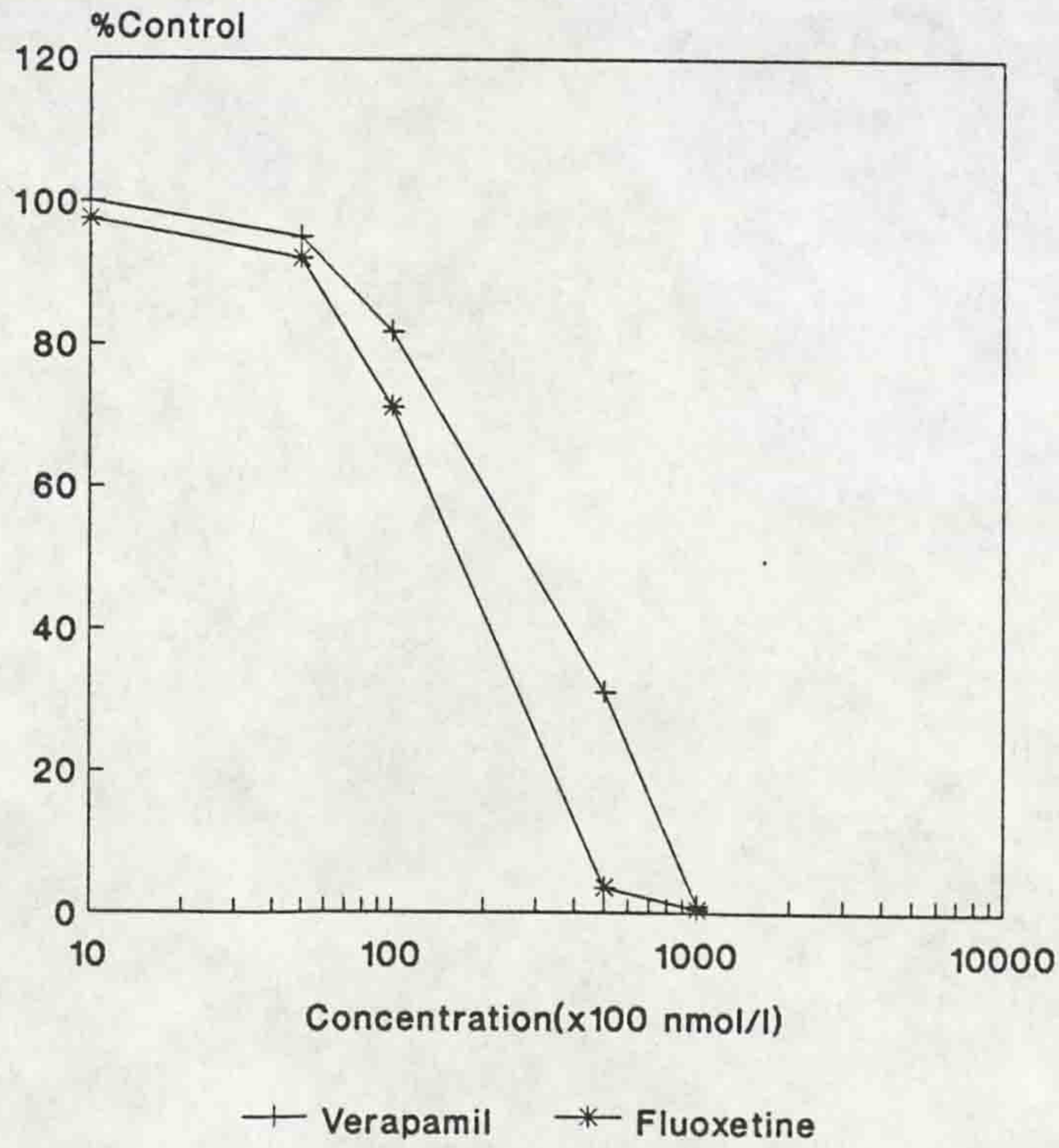


Fig.6.3.5 comparative dose response curves for mefloquine and penfluridol against T9.96HF4 strain.

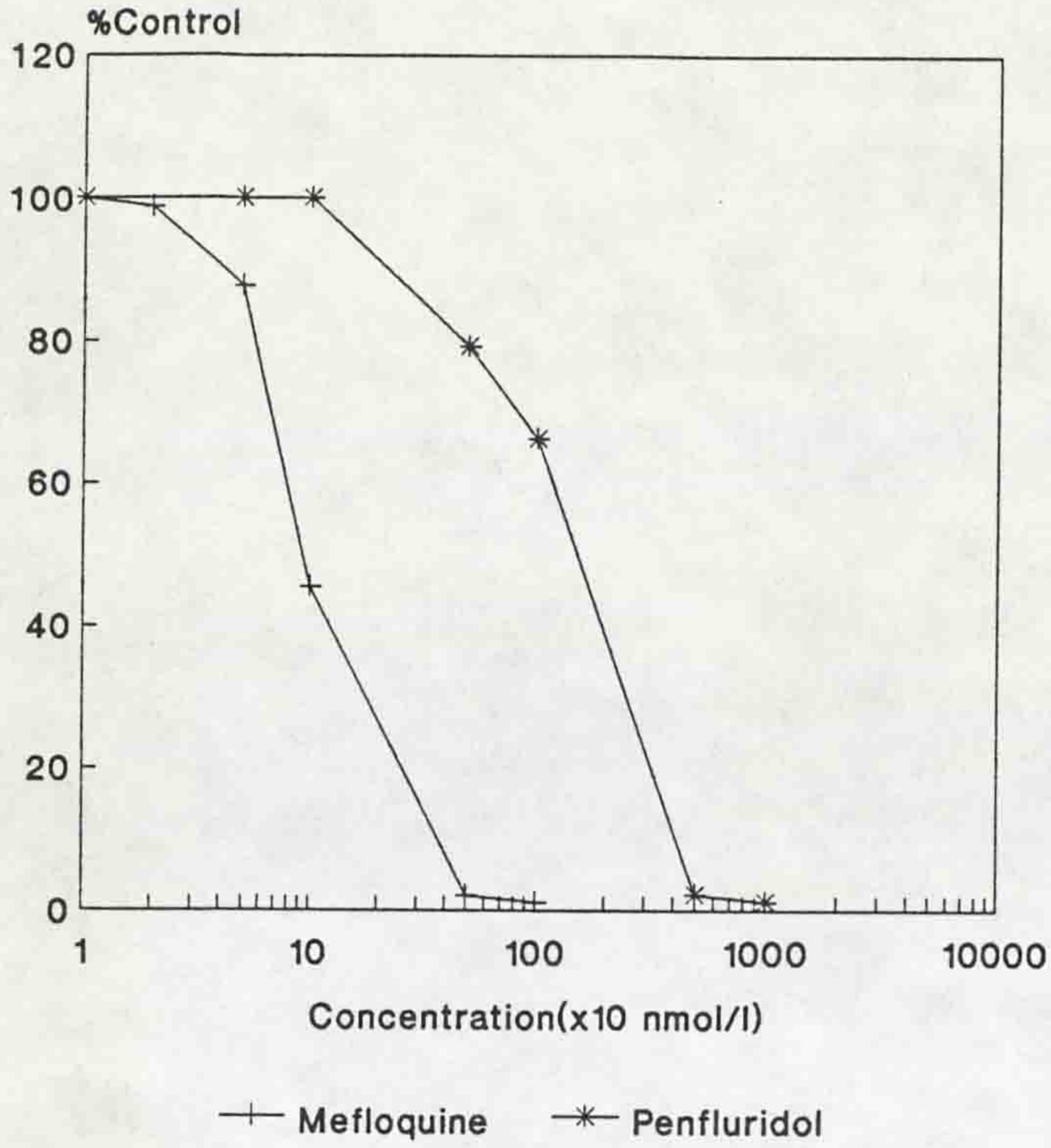


Fig.6.3.6 Interaction between penfluridol(PF) and chloroquine (CQ) on K1 strain.

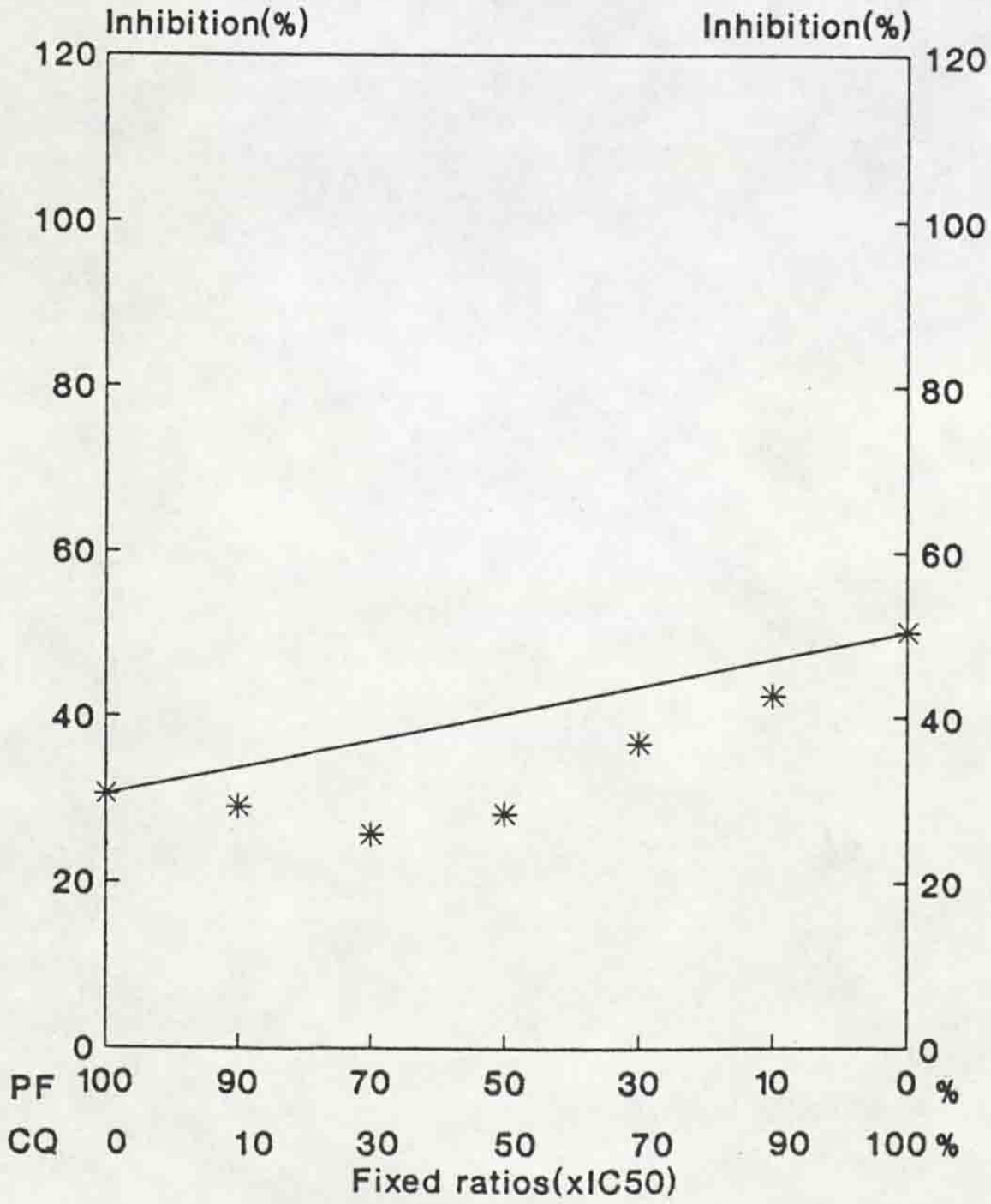


Fig.6.3.7 Interaction between penfluridol(PF) and halofantrine (HF) on K1 strain.

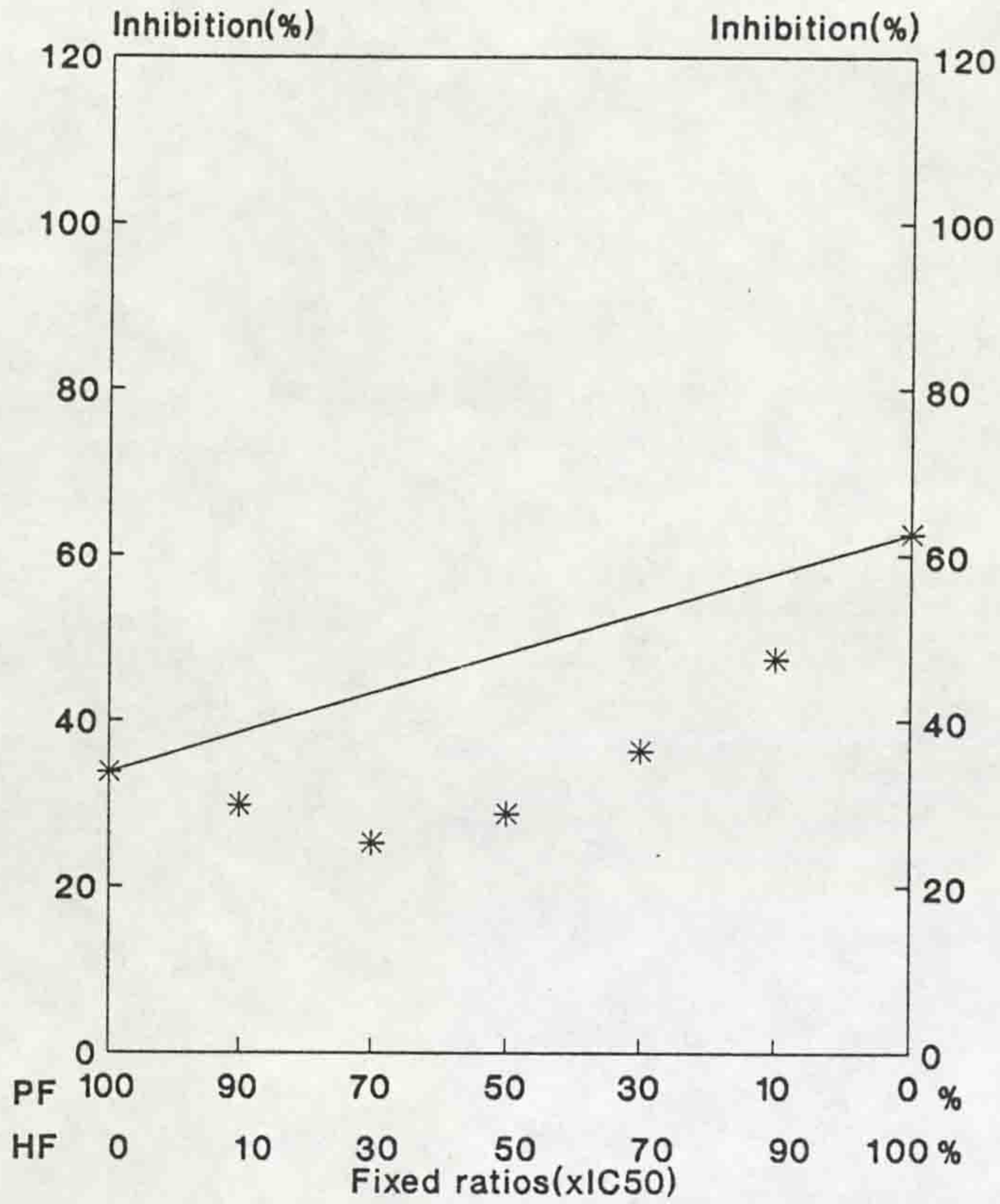


Fig.6.3.8 Interaction between penfluridol(PF) and halofantrine (HF) on K1HF strain.

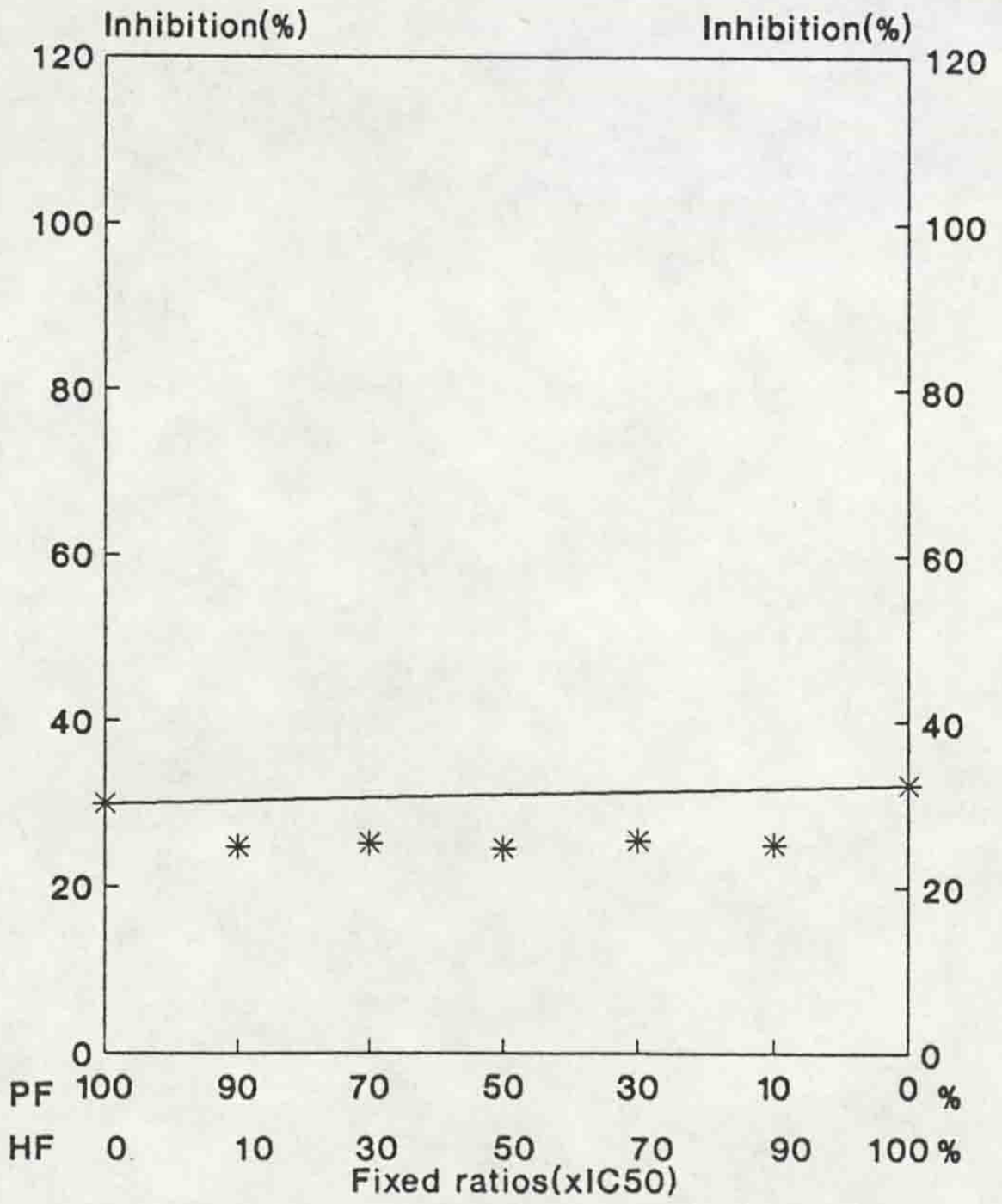


Fig.6.3.9 Interaction between penfluridol(PF) and chloroquine (CQ) on K1HF strain.

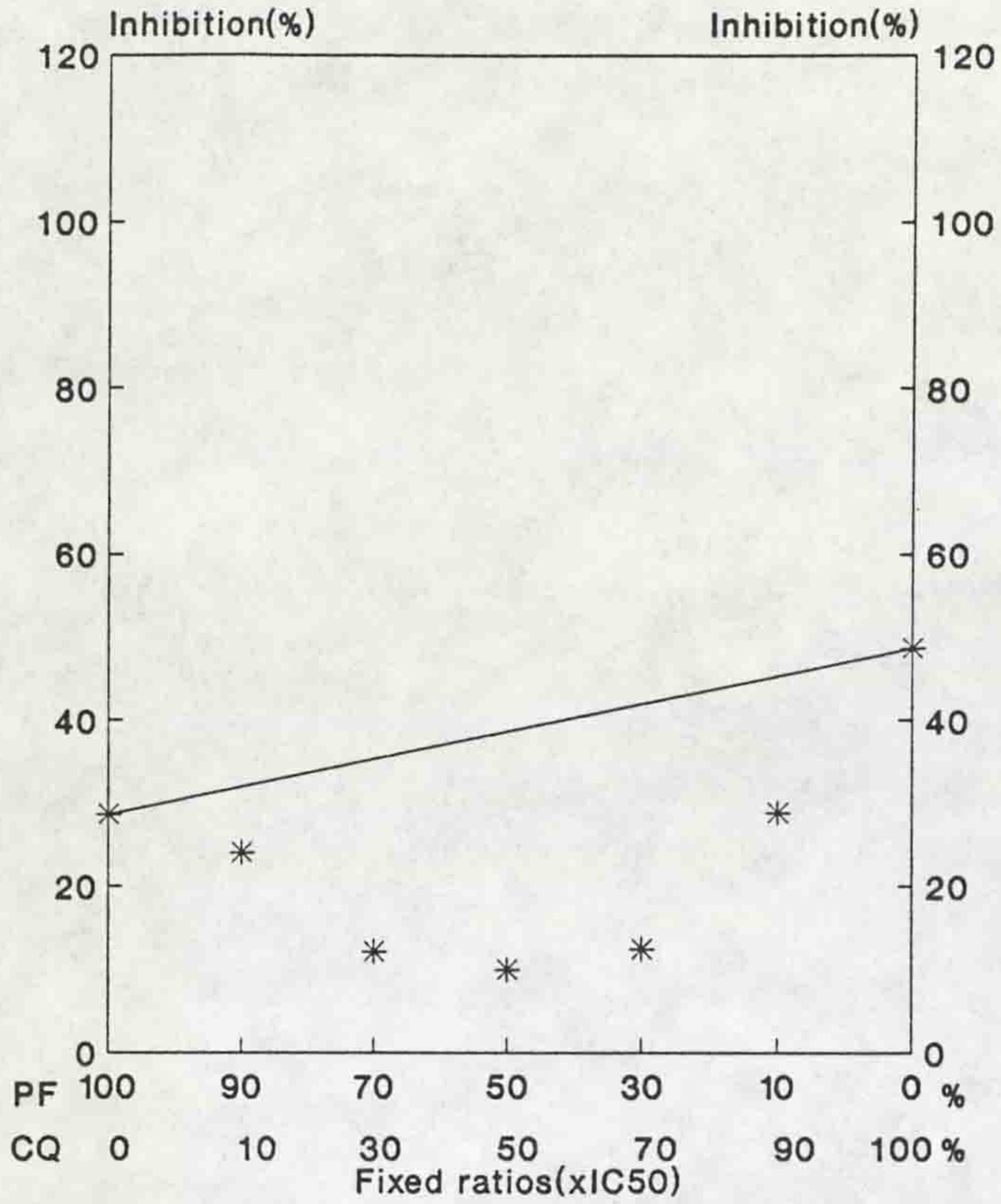


Fig.6.3.10 Interaction between halofantrine(HF) and verapamil(VP) on K1(--+-) and K1HF(—*-) strains.

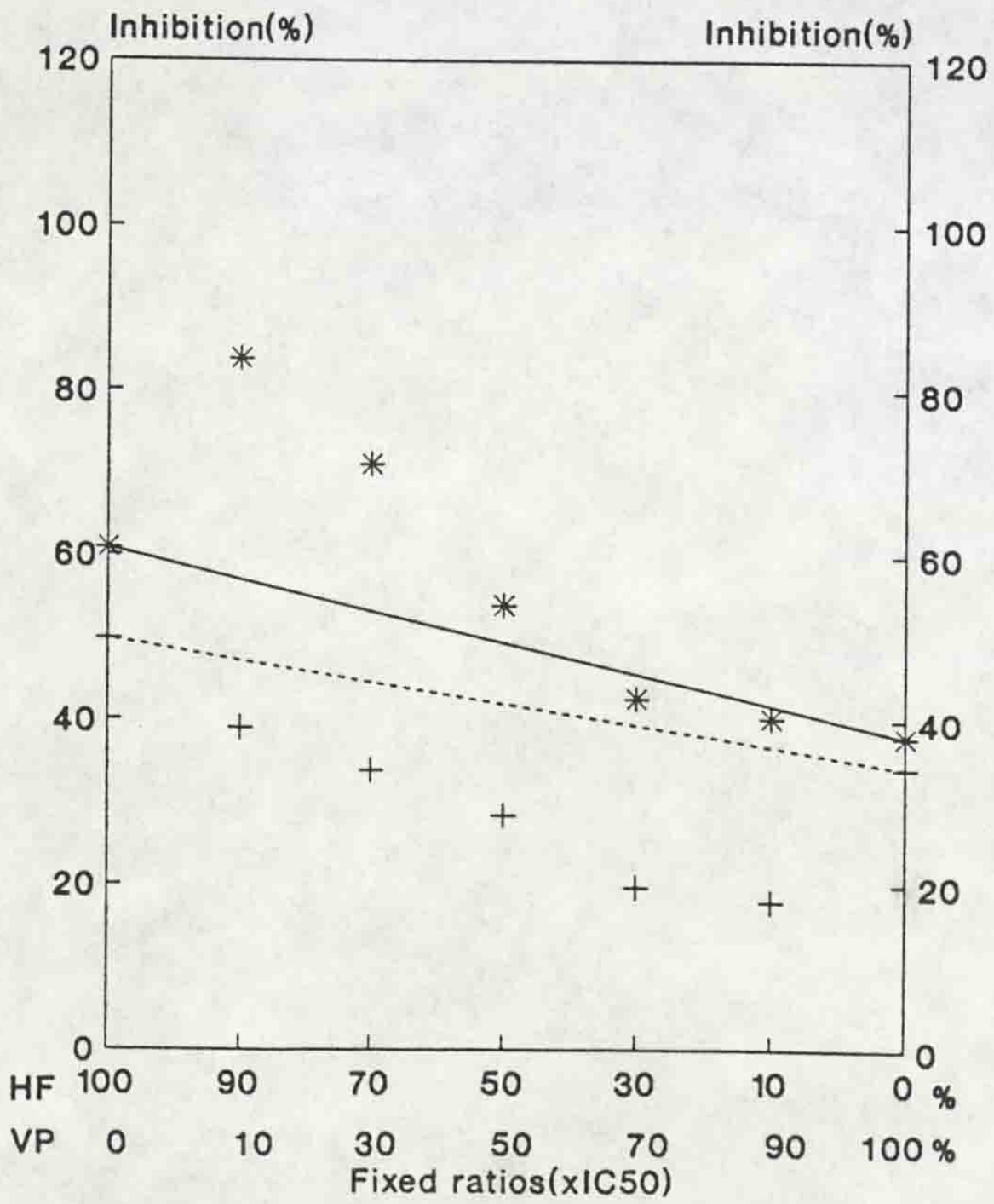


Fig.6.3.11 interaction between halofantrine(HF) and fluoxetine(FX) on K1(--+-) and K1HF(—*-) strains.

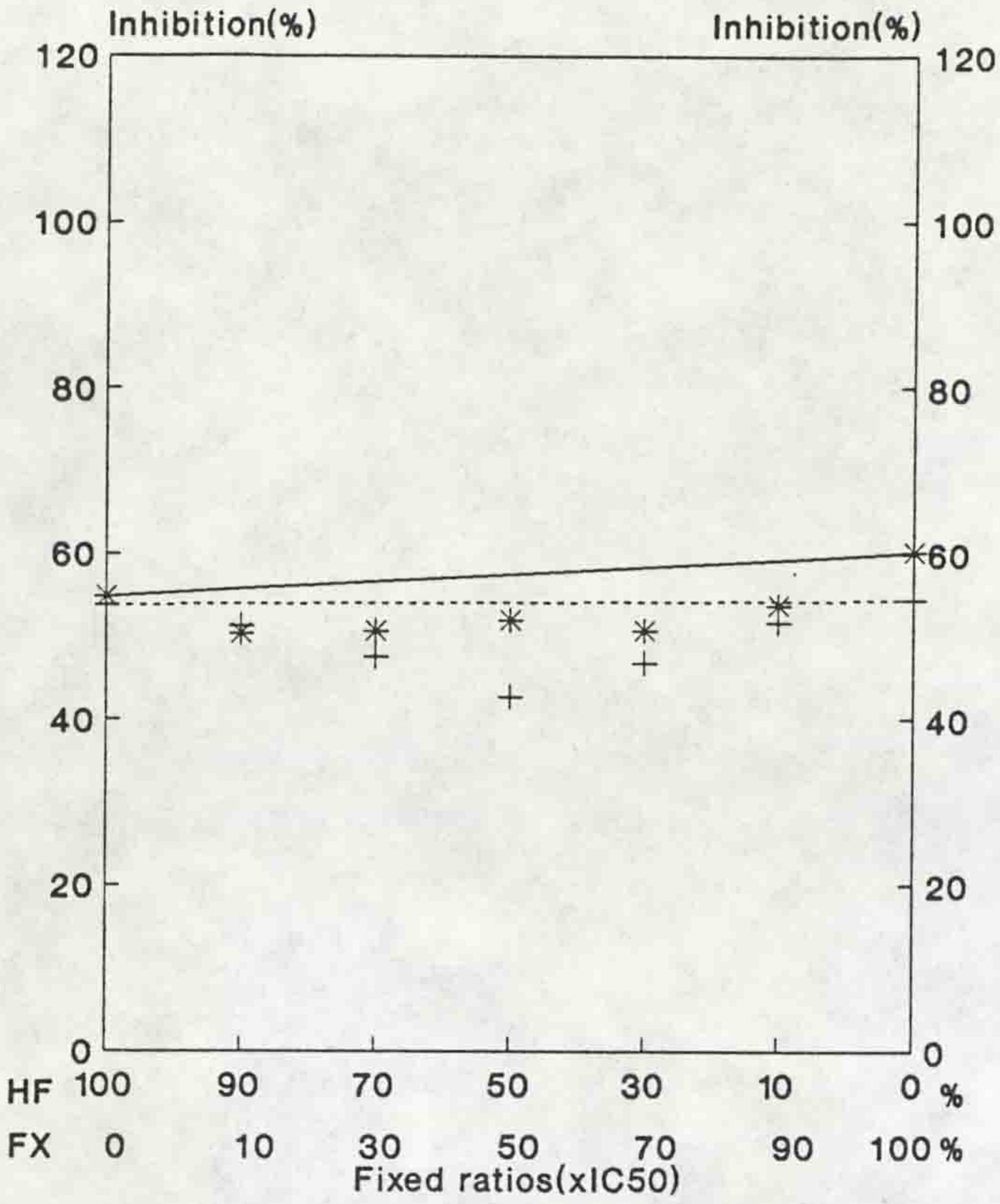


Fig.6.3.12 interaction between chloroquine(CQ) and verapamil(VP) on K1(--+-) and K1HF(—*-) strains.

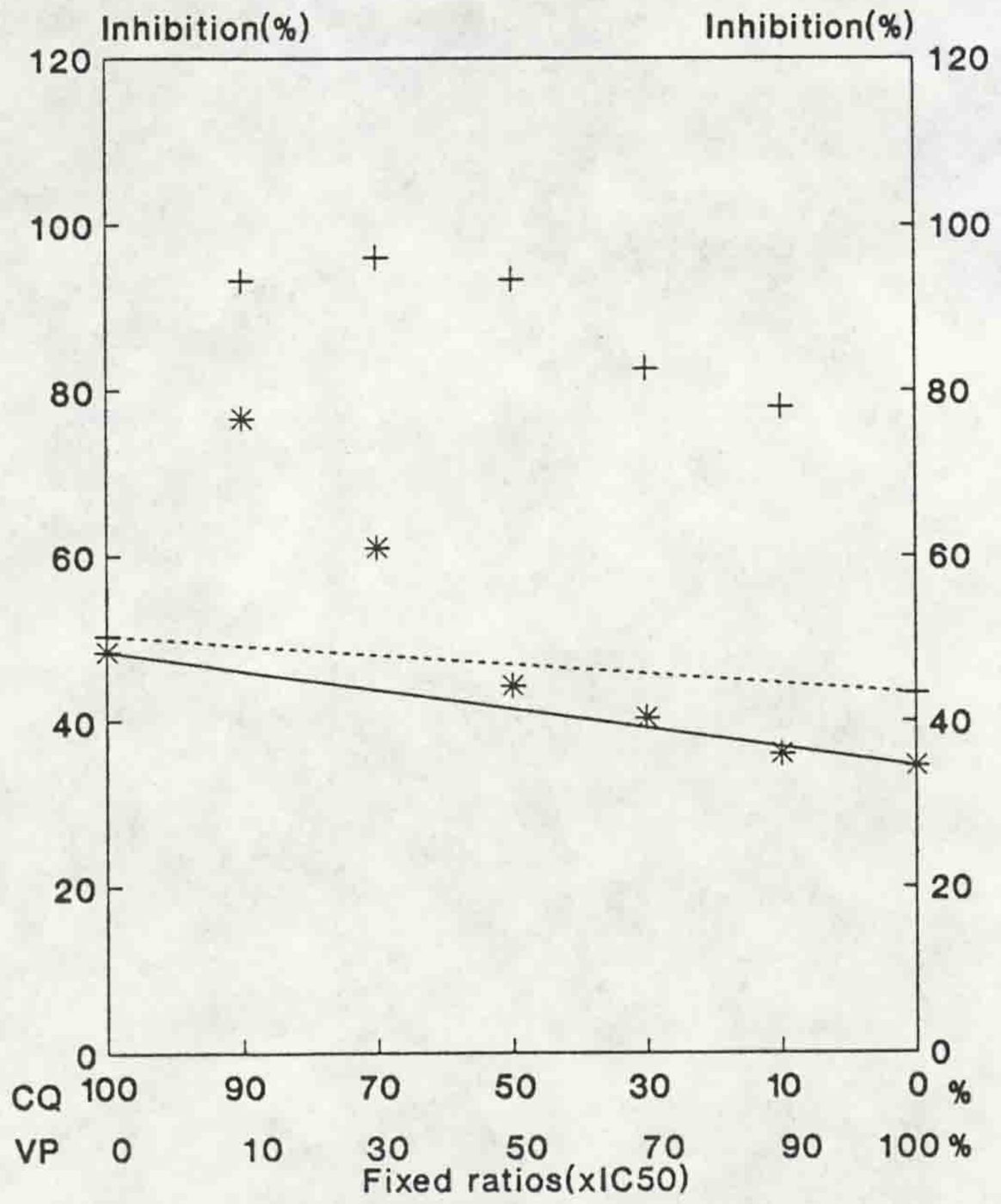


Fig.6.3.13 interaction between chloroquine(CQ) and fluoxetine(FX) on K1(--+-) and K1HF(—*-) strains.

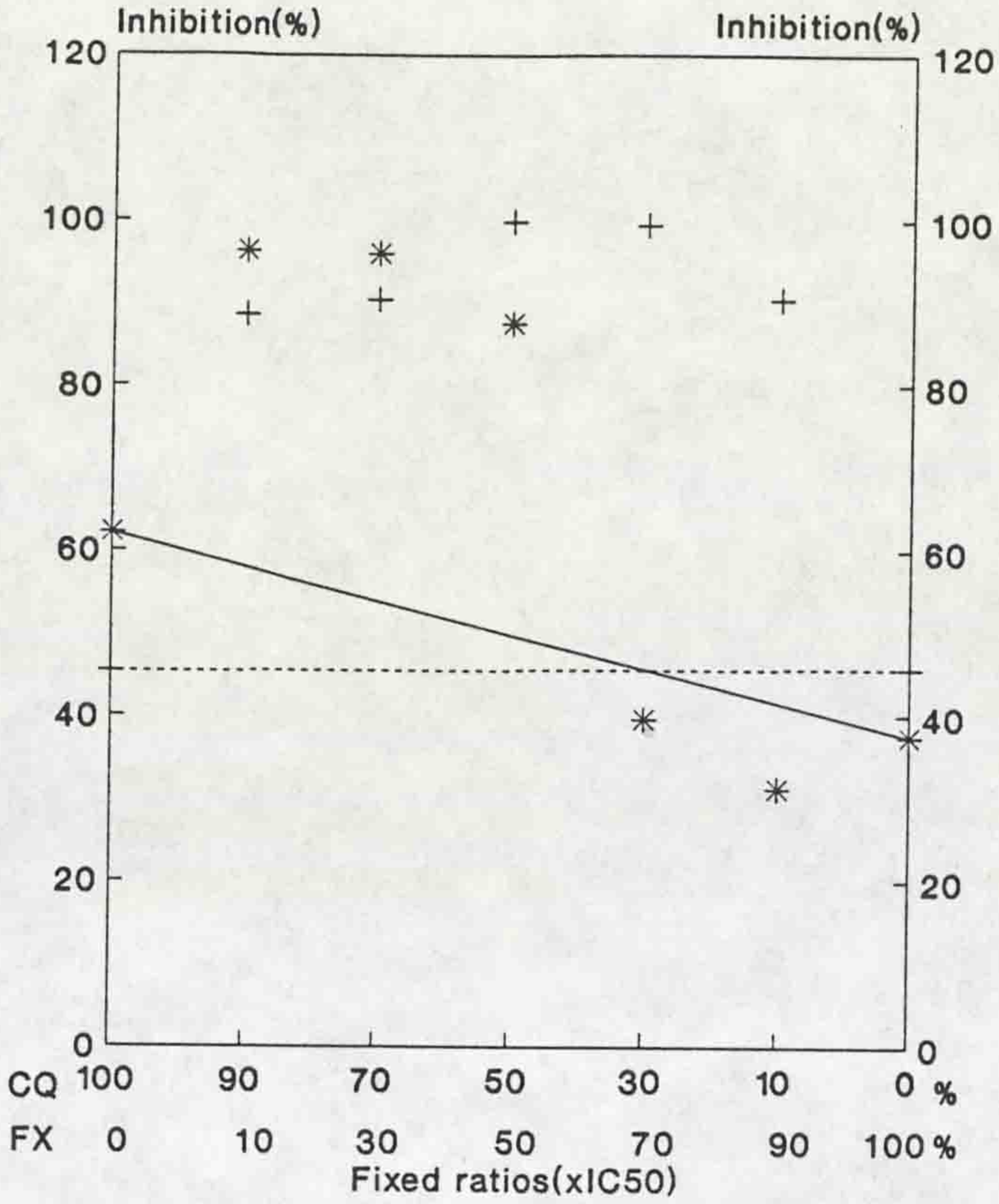


Fig.6.3.14 Interaction between penfluridol(PF) and quinine(QN) on K1HF strain.

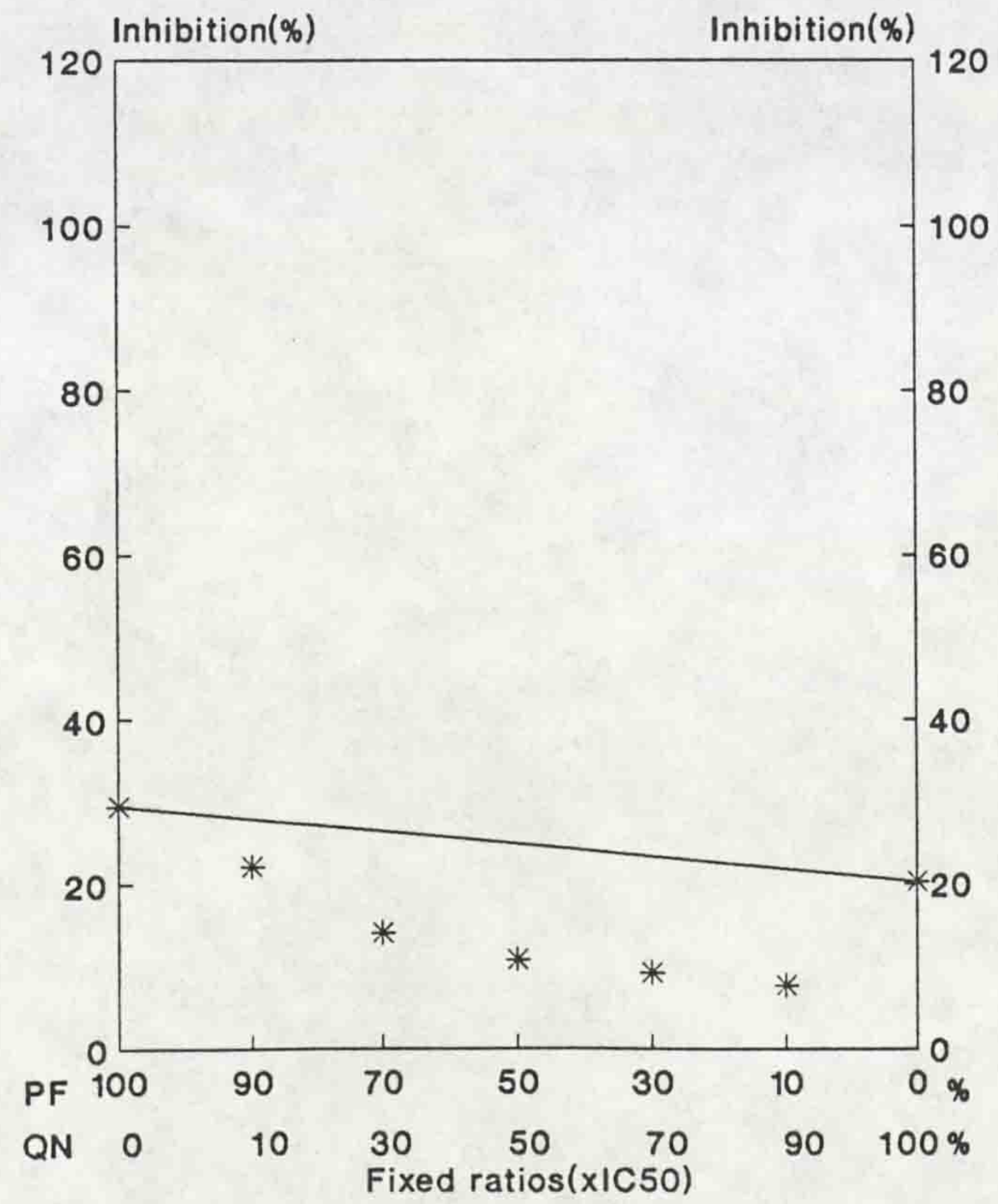


Fig.6.3.15 Interaction between penfluridol(PF) and halofantrine (HF) on T9.96 strain.

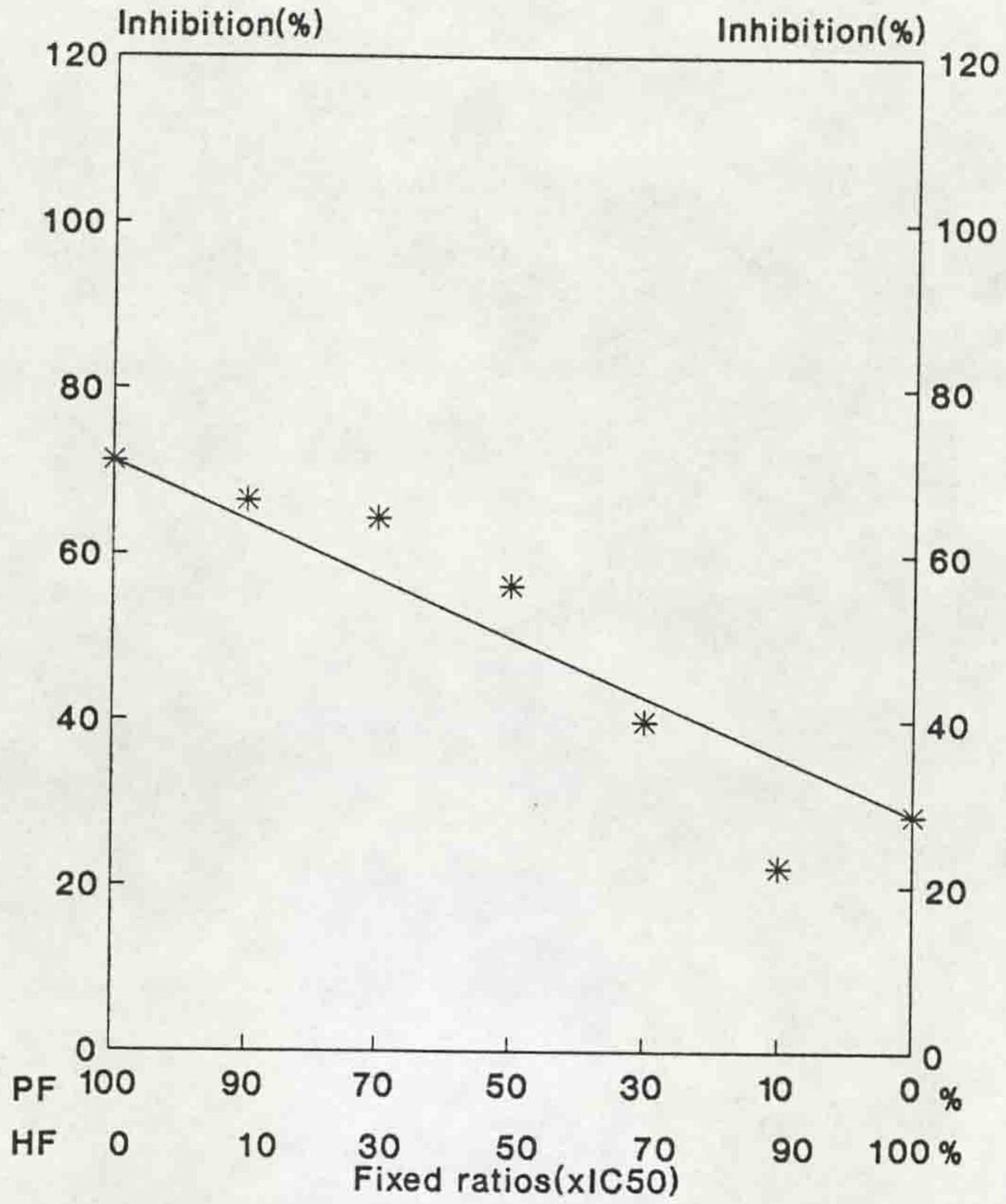


Fig.6.3.16 Interaction between penfluridol(PF) and mefloquine(MF) on T9.96 strain.

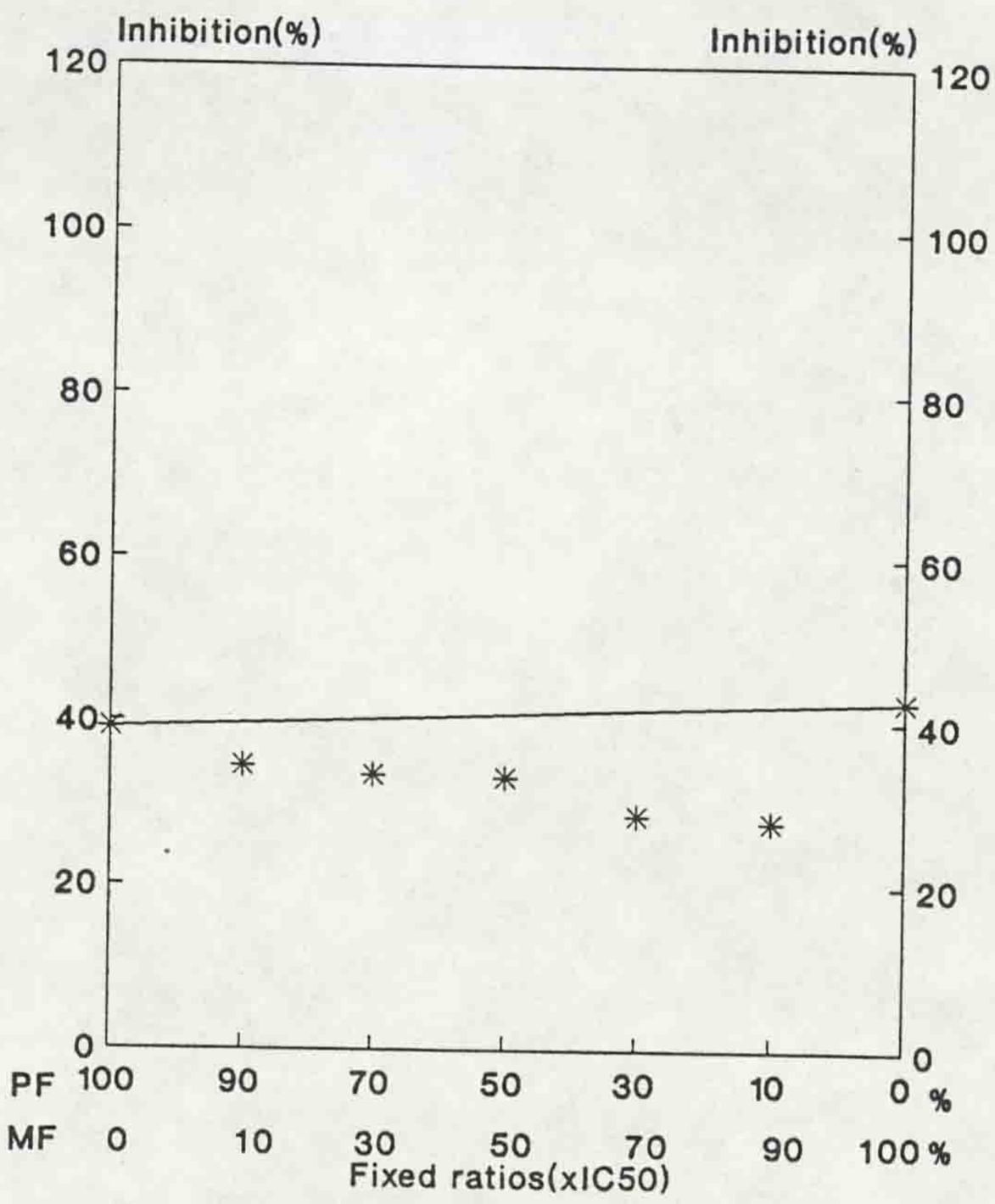


Fig.6.3.17 Interaction between penfluridol(PF) and mefloquine(MF) on T9.96HF4 strain.

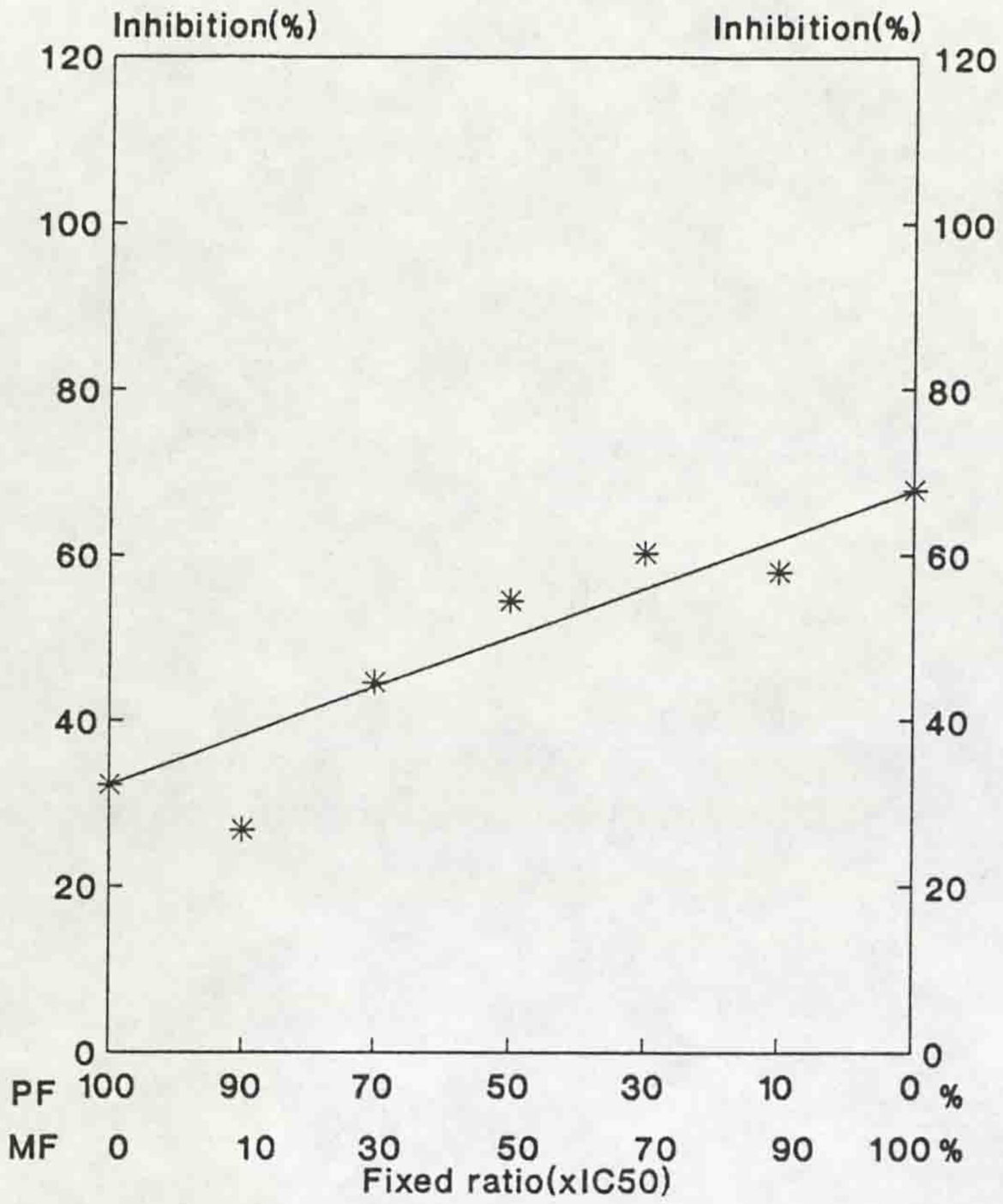


Fig.6.3.18 Interaction between penfluridol(PF) and halofantrine (HF) on T9.96HF strain.

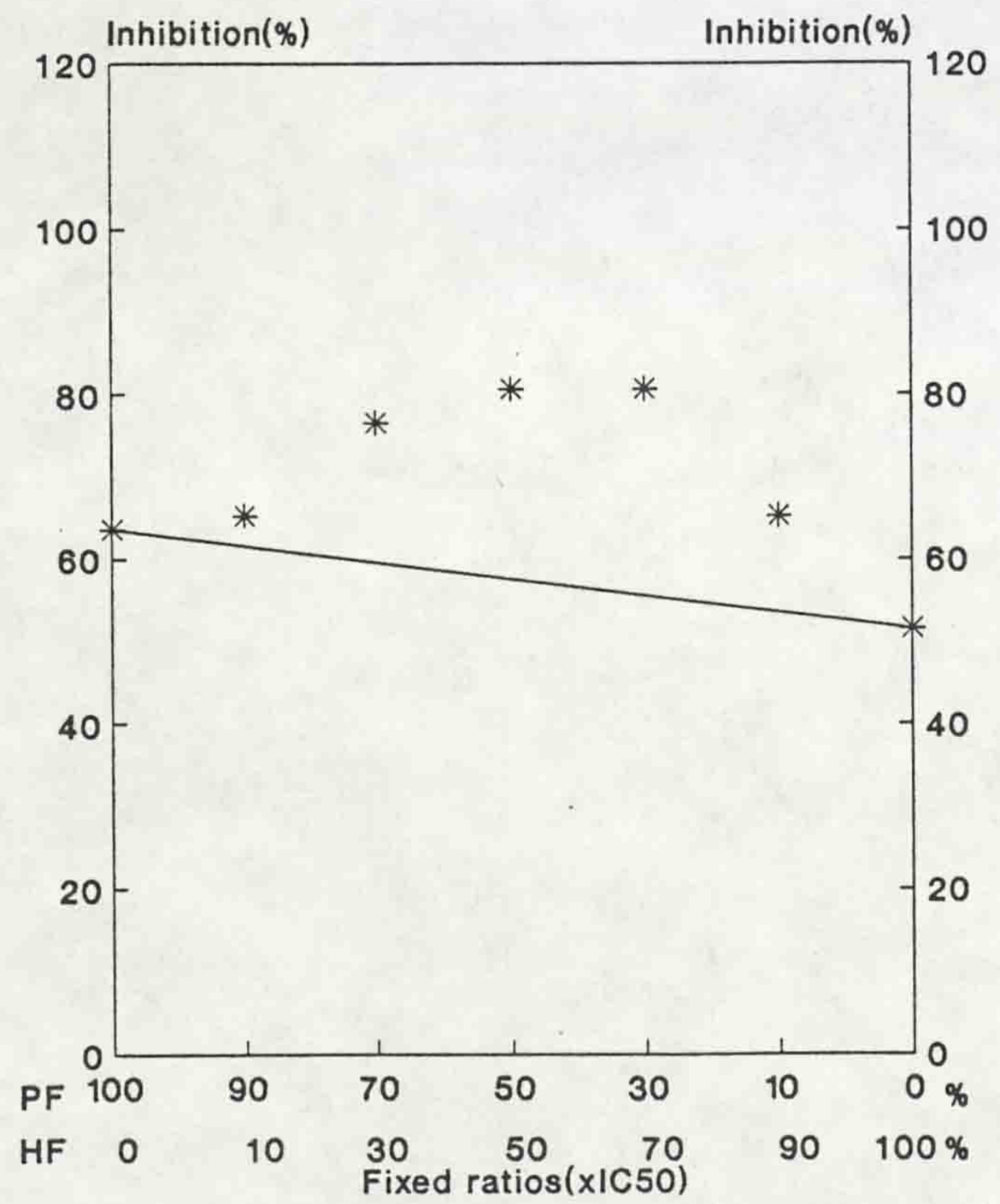


Fig.6.3.19 Interaction between penfluridol(PF) and mefloquine(MF) on T9.96HF strain.

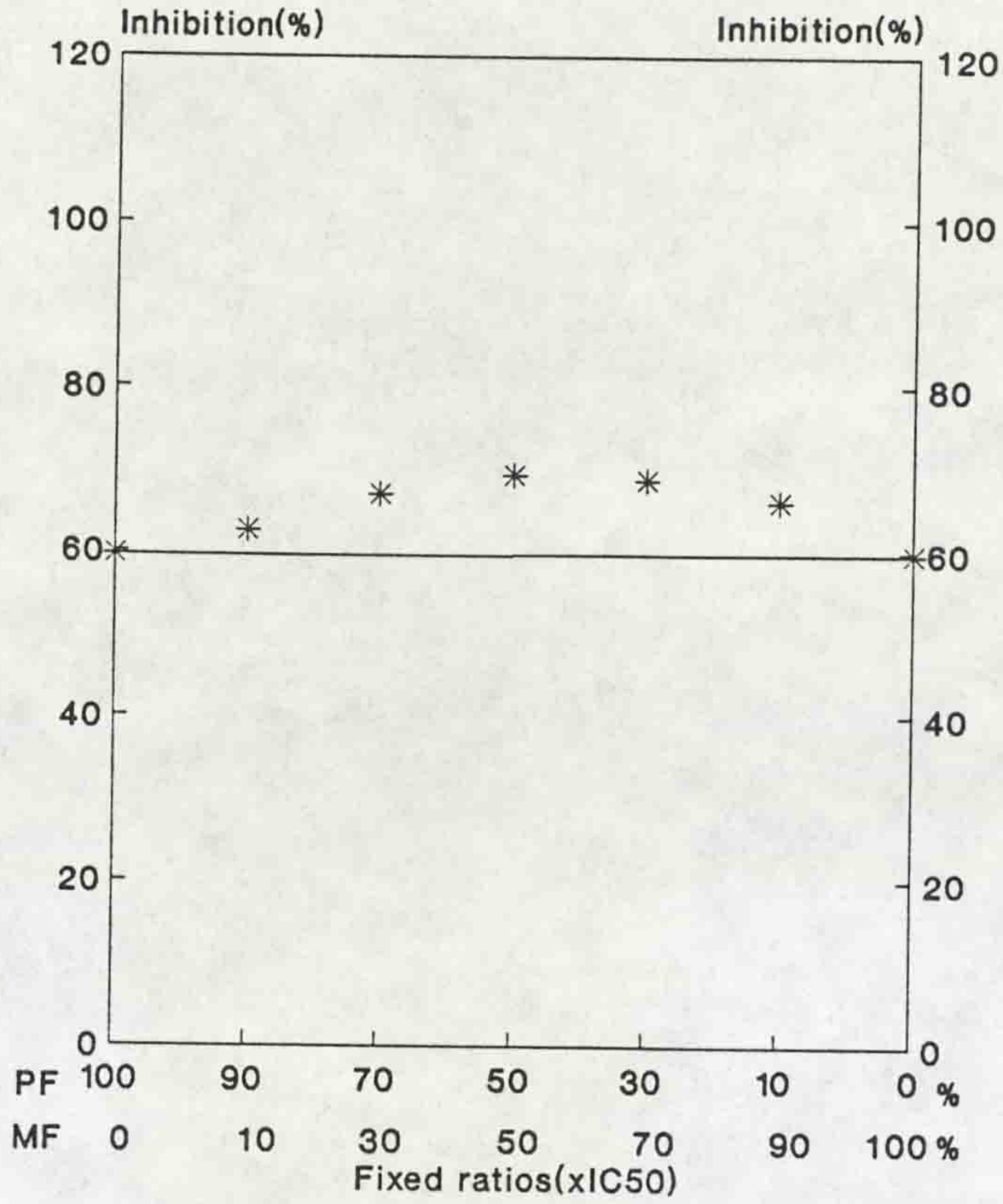


Fig.6.3.20 Interaction between penfluridol(PF) and quinine(QN) on T9.96HF strain.

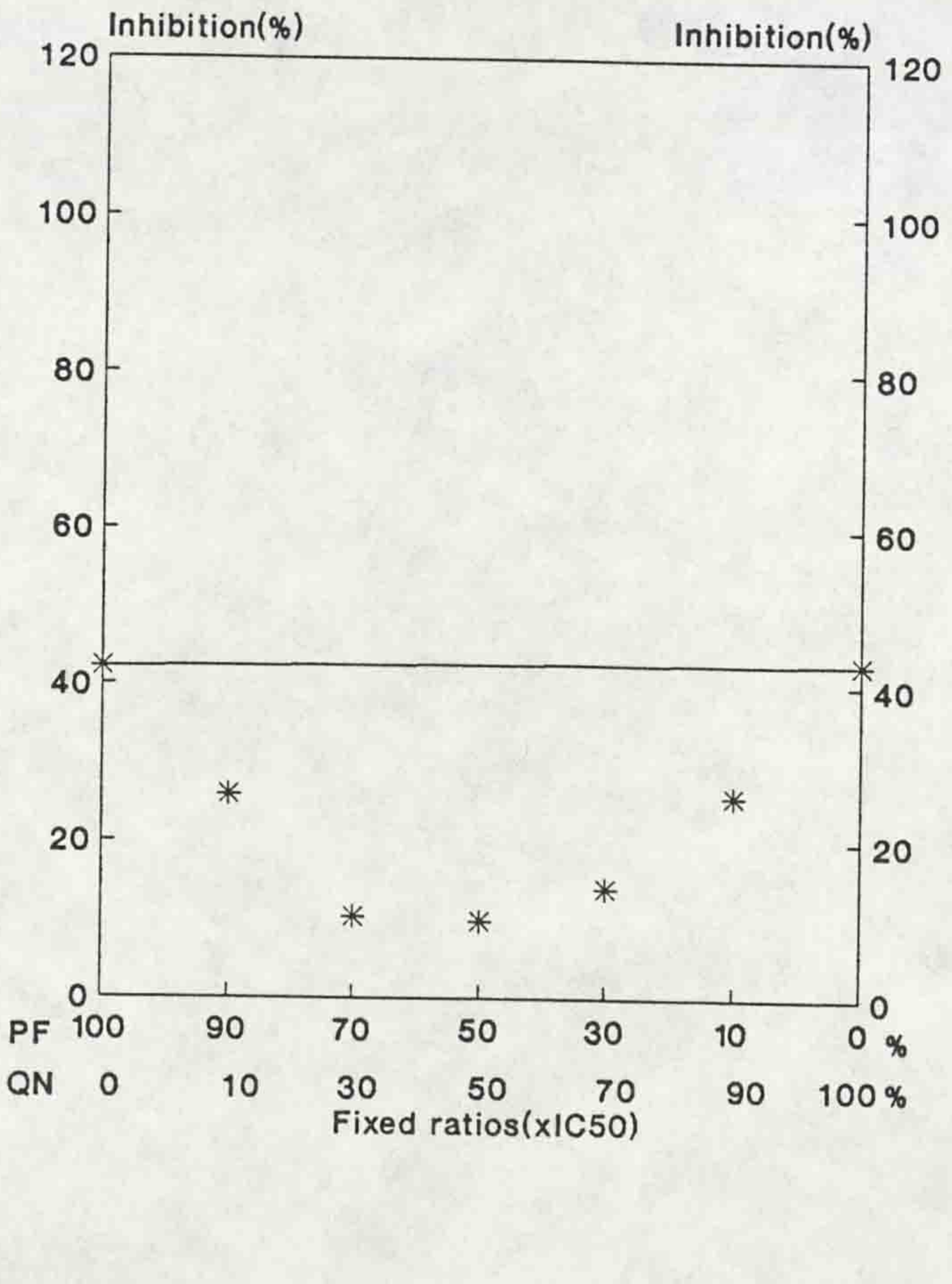


Fig.6.3.21 interaction between halofantrine(HF) and verapamil(VP) on T9.96(--+-) and T9.96HF(—*-).

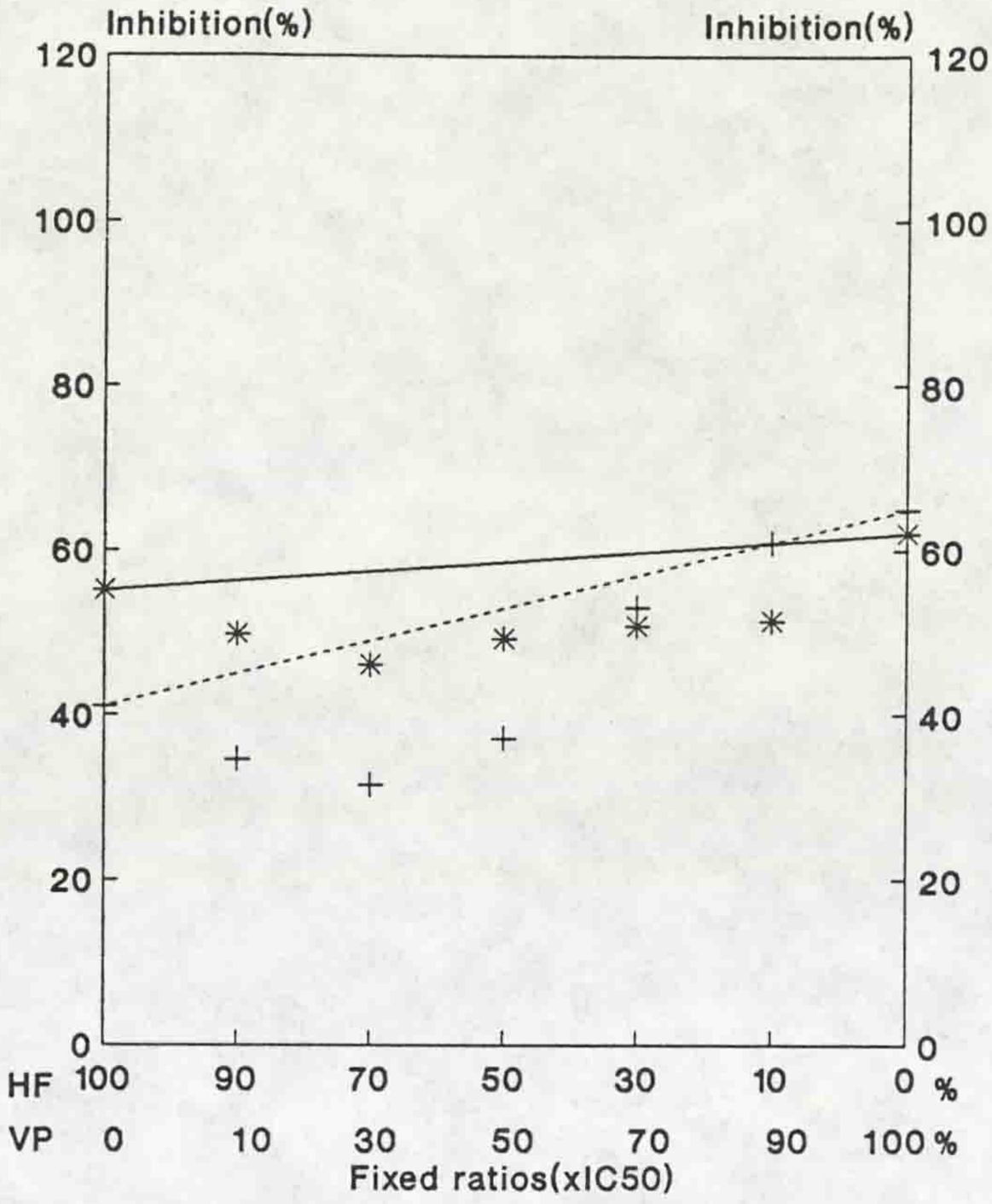


Fig.6.3.22 interaction between halofantrine(HF) and fluoxetine(FX) on T9.96(--+-) and T9.96HF(—*-).

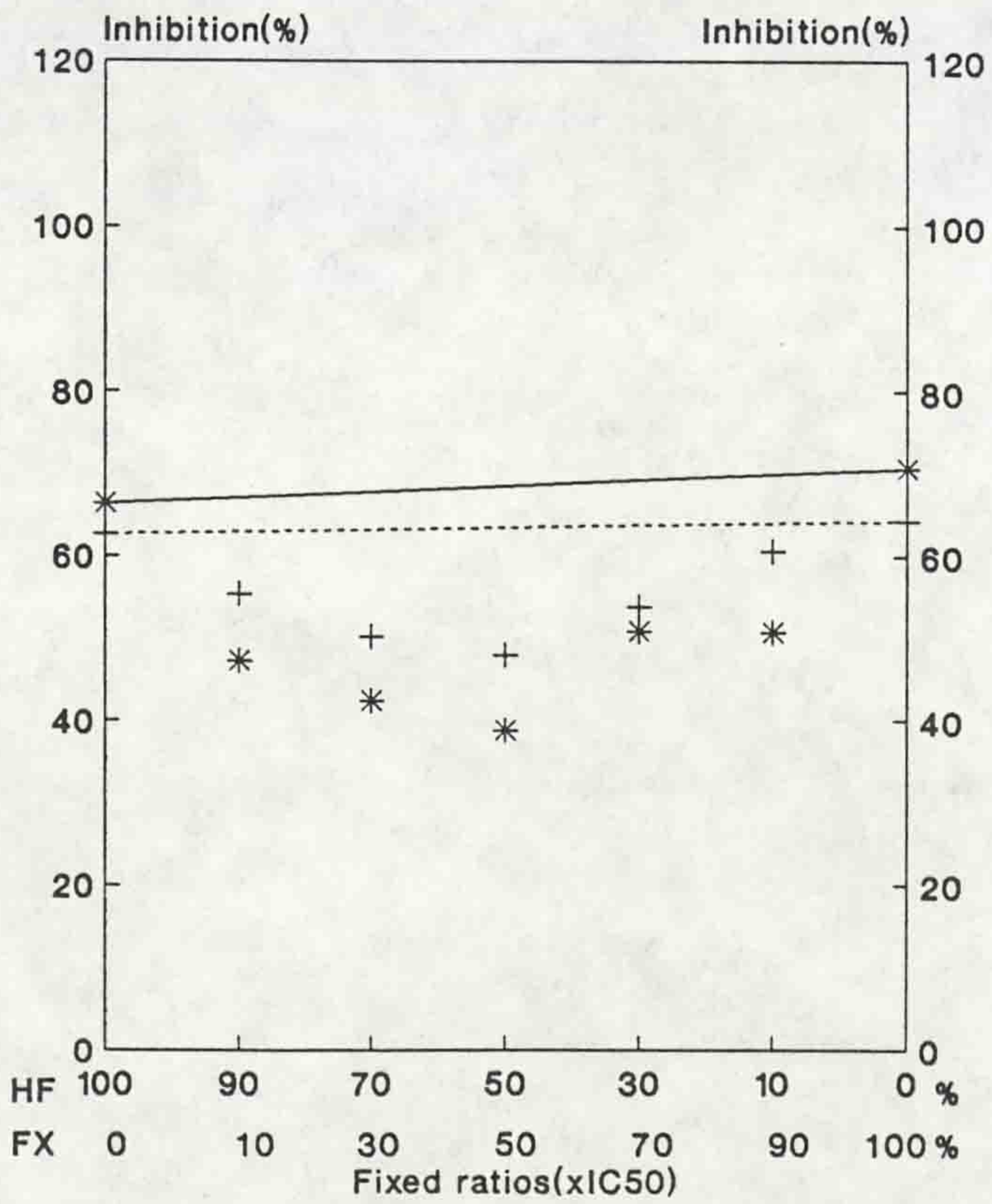
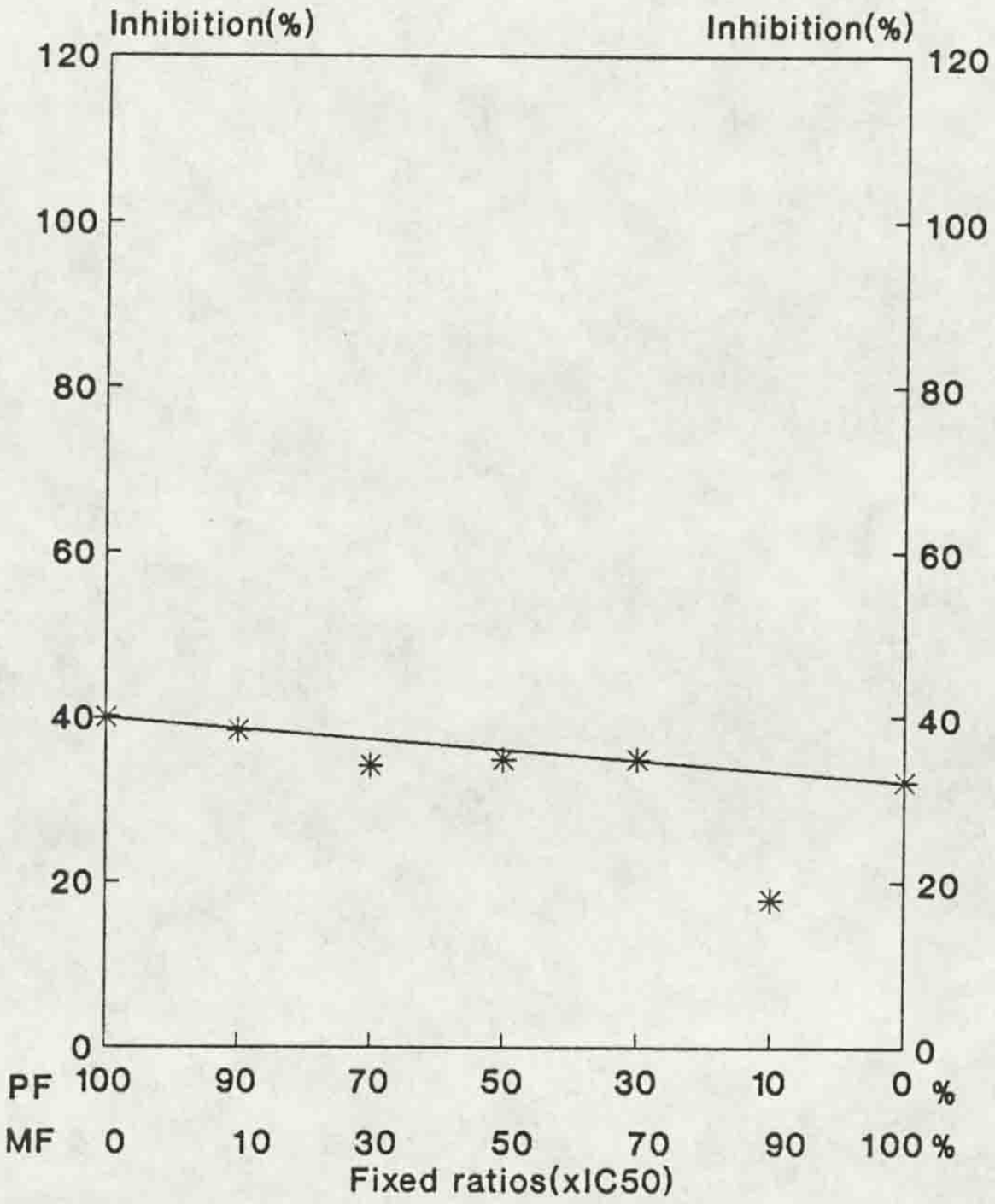


Fig.6.3.23 Interaction between penfluridol(PF) and mefloquine(MF) on W2-mef strain.



CHAPTER 7 : UPTAKE OF [14C] HALOFANTRINE BY HALOFANTRINE-RESISTANT AND HALOFANTRINE-SENSITIVE STRAINS OF *P. FALCIPARUM*

CHAPTER 7 Page

Uptake of [14C] halofantrine by halofantrine-resistant and halofantrine-sensitive strains of *P. falciparum*

7.1 Introduction

The extensive uptake of chloroquine by plasmodia-infected erythrocytes stimulated a number of workers to compare the accumulation of chloroquine in chloroquine-sensitive and chloroquine-resistant parasites as well as in uninfected erythrocytes. In 1966, Macomber and co-workers reported that *P. berghei*-infected erythrocytes accumulated more chloroquine than uninfected erythrocytes. Subsequently, Fitch (1969) demonstrated that chloroquine-resistance in *P. berghei* was associated with reduced uptake of the drug compared to a chloroquine-sensitive *P. berghei*. Fitch (1970) also reported that the accumulation of chloroquine was reduced in *Aotus trivirgatus* erythrocytes infected with chloroquine-resistant *P. falciparum*. These findings have been further studied by several investigators using *P. falciparum*-infected human erythrocytes (Yayon and Ginsburg, 1980; Verdier *et al*, 1985; Geary *et al*, 1986b; Krogstad *et al*, 1987). Krogstad and co-workers (1987) demonstrated that verapamil (a calcium channel blocker) increased the accumulation of chloroquine into the chloroquine-resistant parasites, but not into the chloroquine-sensitive parasites. They also showed that the efflux of chloroquine was more rapid from chloroquine-resistant parasites than from sensitive parasites in the absence of verapamil. The research which has been carried out to date is suggestive of altered chloroquine transport (ie. enhanced efflux) as a major resistance mechanism in *P. falciparum*.

In this study we have investigated the uptake of [14C] halofantrine by halofantrine-resistant and -sensitive parasites of *P. falciparum* either alone or in combination with penfluridol. These studies were undertaken to assess whether

the mechanism of resistance developed to halofantrine in the laboratory was related in any way to alterations in the transport of drug in the parasite.

7.2 Materials and methods

Two halofantrine-resistant K_1HF and $T_{9.96}$HF, and two sensitive K_1 and $T_{9.96}$ strains were employed in this study. The cultivation of parasites, as well as materials and methods for uptake of [14C] halofantrine are described in Chapters 2.1.2 and 2.3, respectively. The uptake of [14C] halofantrine into parasitised RBCs have been normalised for the uptake of [14C] halofantrine into uninfected RBCs, ie. reported [14C] halofantrine uptake = actual uptake - uptake into uninfected red blood cells.

7.3 Results

7.3.1 Uptake of [14C] halofantrine by infected and uninfected erythrocytes

The results of the uptake of [14C] halofantrine by K_1, K_1HF, $T_{9.96}$ and $T_{9.96}$HF-infected and uninfected erythrocytes are tabulated in Tables 7.3.5 and 7.3.6. Equilibrium was reached for halofantrine (HF) uptake into K_1 and K_1HF-infected and uninfected RBCs by 10 minutes. At this time the mean values for uptake of [14C]HF were 682.32 and 345.20 DPM for K_1 and K_1HF infected RBCs respectively (minus control RBC uptake). Uptake of [14C] HF by the halofantrine-sensitive K_1-infected erythrocytes was nearly 2-fold greater than in halofantrine-resistant K_1HF-infected erythrocytes (Table 7.3.1). Similar results were obtained for uptake of [14C] halofantrine by $T_{9.96}$ and $T_{9.96}$HF-infected

erythrocytes (equilibrium was attained by 60 minutes). The mean values for uptake of [14C] HF were 1476.84 and 827.23 DPM for $T_{9.96}$ and $T_{9.96}$HF-infected erythrocytes at 60 minutes respectively (minus control RBC uptake) (Table 7.3.6). The results are graphically presented in Fig. 7.3.1 and 7.3.2.

7.3.2 Uptake of [14C] halofantrine at the IC_{50} concentration for the sensitive parasites

The results obtained for the uptake of [14C] halofantrine at the IC_{50} for K_1 by K_1 and K_1HF-infected erythrocytes showed 2-fold greater uptake by K_1 than K_1HF-infected erythrocytes (Table 7.3.2). The mean values for uptake of 0.1μCi/ml [14C]HF + 2.2nmol/l HF were 494.40 and 210.77 DPM for K_1 and K_1HF-infected erythrocytes at 10 minutes respectively (minus control RBC uptake) (Tables 7.3.2. and 7.3.7). The results are graphically shown in Fig. 7.3.3.

Uptake of halofantrine at the IC_{50} concentration of $T_{9.96}$ by $T_{9.96}$ and $T_{9.96}$HF-infected erythrocytes indicated that the accumulation in the $T_{9.96}$HF parasites was nearly 1.5-fold less than in the $T_{9.96}$ parasites (Table 7.3.2). Additionally, the extent of drug uptake by the $T_{9.96}$-infected erythrocytes was slightly less than the K_1-infected erythrocytes. Equilibrium was attained by 10 minutes. The mean values for halofantrine radiolabel uptake were 329.72 and 207.05 DPM for $T_{9.96}$ and $T_{9.96}$HF at 10 minutes respectively (minus control RBC uptake) (Tables 7.3.2 and 7.3.8). The results are graphically presented in Fig. 7.3.4.

7.3.3 Uptake of [14C] halofantrine at the IC_{50} of $T_{9.96}$ in combination with penfluridol by $T_{9.96}$ and $T_{9.96}$HF-infected and uninfected erythrocytes

The results for the uptake of [14C] halofantrine at the IC_{50} value for $T_{9.96}$ in combination with penfluridol by $T_{9.96}$HF and $T_{9.96}$-infected erythrocytes are summarised in Tables 7.3.3, 7.3.4 and 7.3.9 to 7.3.11. The uptake of radiolabelled halofantrine was significantly increased in $T_{9.96}$HF-infected erythrocytes in combination with penfluridol compared to the $T_{9.96}$-infected erythrocytes, indicating that penfluridol facilitated the accumulation of halofantrine in the $T_{9.96}$HF parasites (Table 7.3.3). The equilibrium in this uptake was reached at 60 minutes. Although the ratio of uptake of [14C] HF by $T_{9.96}$HF-infected RBCs/$T_{9.96}$-infected RBCs was about 3-fold at 10 minutes, this ratio declined to less than 2-fold at 60 and 120 minutes (Table 7.3.3). The mean values for uptake were 262.89 and 500.89 DPM for $T_{9.96}$ and $T_{9.96}$HF-infected RBCs at 60 minutes respectively (minus control RBC uptake) (Tables 7.3.3 and 7.3.9). A comparative study between the uptake of [14C] halofantrine in the presence or absence of penfluridol by $T_{9.96}$ and $T_{9.96}$HF parasites has been summarised in Table 7.3.4. The results are graphically shown in Figs. 7.3.5 to 7.3.7.

7.4 Discussion

Radiolabelled drugs have been used extensively to study the uptake and steady state concentration of many drugs by plasmodia. Attempts have essentially focussed on the uptake of radiolabelled chloroquine and mefloquine

by *P. falciparum* and *P. berghei* (Fitch, 1970; Fitch *et al*, 1979; Verdier *et al*, 1985; Geary *et al*, 1986b). It is an approach which has been used to study the mode of action of chloroquine against *P. falciparum* and chloroquine-resistance mechanism. Recently this approach has shown that verapamil, diltiazem, vinblastine, daunomycin and desipramine alter the rate of chloroquine efflux in chloroquine-resistant parasites (Krogstad *et al*, 1987; Bitonti *et al*, 1988). They demonstrated that these agents enhanced the accumulation of chloroquine in the resistant parasites, but not in the susceptible parasites. These observations prompted us to study the uptake of radiolabelled halofantrine either alone or in the presence of penfluridol by halofantrine-resistant or sensitive parasites.

The results obtained for the uptake of [14C] halofantrine indicated that uptake was rapid and that equilibrium was achieved faster in K_1HF and K_1 parasites (10 minutes) than $T_{9.96}$HF and $T_{9.96}$ parasites (60 minutes). These results appear to be compatible with those suggestions that different strains of *P. falciparum* may inherently respond differentially to halofantrine (Ringwald *et al*, 1990). Additionally, the use of different isolates of *P. falciparum* to study the uptake of chloroquine by Verdier *et al* (1985) and Geary *et al*, (1986b) showed many differences in the time course of equilibrium in these isolates. However, a genetic or biochemical reason has been suggested for such discrepancies (Basco and Le Bras, 1990a). Furthermore, various parasite-dependent alterations in the erythrocyte membranes also may be involved in the rate of uptake of halofantrine by different isolates. Such alterations may facilitate the permeability of infected membranes to halofantrine in strains which are more sensitive to the drug. As such Verdier and co-workers (1985) suggested that because chloroquine uptake

in chloroquine-sensitive parasites is greater than in resistant parasites, the infected erythrocyte membranes are more permeable to chloroquine in sensitive parasites than in resistant ones. Indeed, differential alterations in the infected RBC membrane have been recently reported by a number of authors (Ginsburg *et al*, 1983; Tanabe *et al*, 1983; Sherman, 1984). However, further investigations would be required to verify the role of the RBC-membrane in halofantrine uptake.

The uptake and steady state levels of halofantrine are shown to be greater in sensitive parasites than the resistant ones. This would suggest that halofantrine resistance, at least in part, is related to the ability of the malaria parasite to accumulate toxic concentrations of the drug. However, the sub-cellular site for accumulation is unknown. In comparison to chloroquine accumulation in malaria parasites the ability of parasites to accumulate halofantrine is much reduced (ie. the magnitude of the concentration gradient is not nearly as great). Additionally, halofantrine accumulation into uninfected RBCs was significant accounting for approximately one-third of the total uptake into infected RBCs.

Penfluridol has been shown to reverse halofantrine resistance *in vitro* in $T_{9.96}$HF parasites (Chapter 6). If resistance is related to drug transport and steady state concentrations we would expect penfluridol to enhance halofantrine accumulation in these resistant parasites. Our results suggest that this is in fact the case with penfluridol enhancing halofantrine accumulation in resistant $T_{9.96}$HF to levels significantly greater than that seen in $T_{9.96}$.

Our studies with K_1HF parasites show that penfluridol has no effect on sensitivity of the parasites to halofantrine *in vitro*. This would suggest that the

resistance developed in the laboratory for K_1 and $T_{9.96}$ parasites, although produced by the same experimental approach and both resistance mechanisms being related to drug transport, show subtle differences. Our data suggest that the mechanisms by which halofantrine accumulation is reduced in our resistant strains are different, ie. the mechanism in $T_{9.96}$HF is penfluridol sensitive whereas in K_1HF it is not. This is an exciting observation which requires further detailed study of halofantrine transport processes in our strains of *P. falciparum*.

7.5 Conclusions

1. Halofantrine accumulation was greater in the halofantrine-sensitive parasites compared to resistant parasites.

2. Penfluridol which is capable of reversing halofantrine resistance *in vitro* also enhanced halofantrine steady state concentrations in these parasites.

3. The initial rates of halofantrine uptake by both strains (HF-resistant and sensitive) either in the presence or absence of penfluridol were similar.

4. The mechanism of halofantrine resistance in these parasites appears to be related in part to drug transport either into or out of the parasites.

5. There may be some analogy between resistance mechanisms developed against halofantrine and those reported for chloroquine.

Table 7.3.1 **The uptake of [14C] halofantrine by K_1-infected RBCs/K_1HF-infected RBCs and $T_{9.96}$-infected RBCs/$T_{9.96}$HF-infected RBCs**

Infected RBCs	Mean values of uptake (DPM)		K_1-infected	Mean values of uptake (DPM)		$T_{9.96}$-infected
Time (min)	K_1	K_1HF	K_1HF-infected	$T_{9.96}$	$T_{9.96}$HF	$T_{9.96}$HF-infected
10	682.32	345.20	1.97	1292.21	604.11	2.13
60	573.13	291.50	1.96	1476.84	827.23	1.78
120	595.17	235.47	2.52	1347.38	678.65	1.98

Table 7.3.2 **The uptake of [14C] halofantrine by K_1-infected RBCs/K_1HF-infected RBCs and $T_{9.96}$-infected RBCs/$T_{9.96}$HF-infected RBCs in the presence of unlabelled halofantrine**

Infected RBCS	Mean values of uptake (DPM)		K_1-infected	Mean values of uptake (DPM)		$T_{9.96}$-infected
Time (min)	K_1	K_1HF	K_1HF-infected	$T_{9.96}$	$T_{9.96}$HF	$T_{9.96}$HF-infected
10	494.40	210.77	2.34	329.72	207.05	1.59
60	574.51	302.76	1.89	322.64	232.71	1.38
120	601.86	335.41	1.79	351.46	239.84	1.46

Table 7.3.3 **The uptake of [14C] halofantrine + unlabelled halofantrine (6.6nmol.l) by $T_{9.96}$HF-infected RBCs/$T_{9.96}$-infected RBCs in the presence of penfluridol (493.3nmol/l)**

Infected RBCs	Mean value of uptake (DMP)		$T_{9.96}$HF-infected
Time (min)	$T_{9.96}$HF	$T_{9.96}$	$T_{9.96}$-infected
10	338.52	100.76	3.35
60	500.89	262.89	1.90
120	474.26	283.58	1.67

Table 7.3.4 **The uptake of [14C] halofantrine + unlabelled halofantrine in the presence of penfluridol/[14C] halofantrine + unlabelled halofantrine by $T_{9.96}$ and $T_{9.96}$HF-infected RBCs**

Infected RBCs and drug combinations	$T_{9.96}$HF		[14C] HF + HF + PF [14C] HF + HF	$T_{9.96}$		[14C] HF + HF + PF [14C] HF + HF
Time (min)	[14C] HF + HF + PF	[14C] HF + HF		[14C] HF + HF + PF	[14C] HF + HF	
10	608.15	561.81	1.08	563.34	649.07	0.86
60	904.86	742.03	1.21	743.17	862.08	0.86
120	804.80	668.36	1.20	750.75	800.37	0.93

Table 7.3.5 **Uptake of [14C] halofantrine by K_1 and K_1HF-infected and uninfected erythrocytes**

Infected and uninfected erythrocytes (+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
K_1	1725.2(*) 1919.63	1714.22 1879.04	1651.63 1584.73	589.44 775.20	523.12 623.14	657.27 533.08
K_1HF	1529.00 1441.60	1463.42 1566.58	1261.06 1255.90	393.24 297.17	272.32 310.68	266.70 204.25
RBCs	1135.76 1144.43	1191.10 1255.90	994.36 1051.65	- -	- -	- -

(*) All values are means of triplicate observations and presented as DPM

(+) All experiments were performed twice

Table 7.3.6 **Uptake of [14C] halofantrine by $T_{9.96}$ and $T_{9.96}$HF-infected and uninfected erythrocytes**

Infected and uninfected erythrocytes (+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
$T_{9.96}$	2937.86(*) 3588.58	3103.44 3662.29	3056.22 3639.90	835.96 1748.46	1145.30 1808.38	1243.19 1451.57
$T_{9.96}$HF	2719.90 2430.34	2674.13 2792.38	2573.50 2785.16	618.00 590.22	715.99 938.47	760.47 596.83
RBCs	2101.90 1840.12	1958.14 1853.91	1813.03 2188.33	- -	- -	- -

(*) All values are mean of triplicate observations and presented as DPM

(+) All experiments were performed twice

Table 7.3.7 Uptake of [14C] halofantrine in combination with 2.2nmol/l halofantrine by K_1 and K_1HF-infected and uninfected erythrocytes

Infected and uninfected erythrocytes(+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
K_1	2216.86(*) 2115.74	1922.06 2075.14	1758.40 1782.00	562.84 425.96	464.40 684.62	612.92 590.80
K_1HF	1886.36 1878.98	1809.06 1644.64	1445.84 1561.66	232.34 189.20	351.40 254.12	300.36 370.46
RBCs	1654.02 1689.78	1457.66 1390.52	1145.48 1191.20	- -	- -	- -

(*) All values are mean of triplicate observations and presented as DPM

(+) All experiments were performed twice

Table 7.3.8 Uptake of [14C] halofantrine in combination with 6.6nmol/l halofantrine by $T_{9.96}$ and $T_{9.96}$HF-infected and uninfected erythrocytes

Infected and uninfected erythrocytes(+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
$T_{9.96}$	1618.34(*) 1752.70	1369.30 1513.88	1205.34 1365.44	269.00 390.44	286.94 358.34	332.14 370.78
$T_{9.96}$HF	1491.92 1633.78	1274.62 1428.70	1189.40 1158.14	142.58 271.52	192.26 273.16	316.20 163.48
RBCs	1349.34 1362.26	1082.36 1155.54	873.20 994.66	- -	- -	- -

(*) All values are mean of triplicate observations and presented as DPM

(+) All experiments were performed twice

Table 7.3.9 Uptake of [14C] halofantrine in combination with 6.6nmol/l halofantrine and 493.3nmol/l penfluridol by $T_{9.96}$ and $T_{9.96}$HF-infected and uninfected erythrocytes

Infected and uninfected erythrocytes(+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
$T_{9.96}$	1376.56(*) 1336.98	1386.58 1351.84	1338.02 1362.24	86.76 114.76	289.34 236.44	268.96 298.20
$T_{9.96}$HF	1629.24 1559.82	1625.18 1589.24	1512.18 1569.44	339.44 337.60	527.94 473.84	443.12 505.40
RBCs	1289.80 1222.22	1097.24 1115.40	1069.06 1064.04	- -	- -	- -

(*) All values are mean of triplicate observations and presented as DPM

(+) All experiments were performed twice

Table 7.3.10 Uptake of [14C] halofantrine + 6.6nmol/l halofantrine and [14C] halofantrine + 6.6nmol/l halofantrine + 493.3nmol/l penfluridol by $T_{9.96}$HF-infected and uninfected erythrocytes

Drug combinations and relevant RBCs (+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
[14C]HF + HF (x)	2213.45(*) 2220.90	2114.15 2082.40	1963.82 1969.77	567.88 555.75	725.23 758.83	678.85 657.87
RBCs	1645.57 1665.15	1388.92 1323.57	1284.97 1311.90	- -	- -	- -
[14C]HF + HF + PO (o)	2368.65 2212.35	2392.42 2141.32	2009.85 2015.45	615.55 600.75	1007.82 801.90	826.35 783.25
RBCs	1753.10 1611.60	1384.60 1339.42	1183.50 1232.20	- -	- -	- -

(+) All experiments were performed twice

(*) All values are means of triplicate observations and presented as DPM

(x) HF = halofantrine

(o) PF = penfluridol

Table 7.3.11 Uptake of [14C] halofantrine + 6.6nmol/l halofantrine and [14C] halofantrine + 6.6nmol/l halofantrine + 493.3nmol/l penfluridol by $T_{9.96}$-infected and uninfected erythrocytes

Drug combinations and relevant RBCs (+)				Minus RBC control uptake		
	Time (min)			Time (min)		
	10	60	120	10	60	120
[14C]HF + HF (x)	2267.77(*) 2331.70	2349.24 2183.00	2050.02 2097.52	606.40 691.75	986.73 737.43	749.50 851.25
RBCs	1661.37 1639.95	1362.52 1445.57	1300.52 1246.27	- -	- -	- -
[14C]HF + HF + PF (o)	2112.95 2297.15	2067.90 2106.80	1932.14 2110.28	543.13 583.55	716.75 769.60	725.14 776.36
RBCs	1569.82 1713.60	1351.15 1337.20	1207.00 1333.92	- -	- -	- -

(+) All experiments were performed twice

(*) All values are means of triplicate observations and presented as DPM

(x) HF = halofantrine

(o) PF = penfluridol

Fig.7.3.1 Uptake of [14C]halofantrine
by K1HF and K1-infected RBCs.

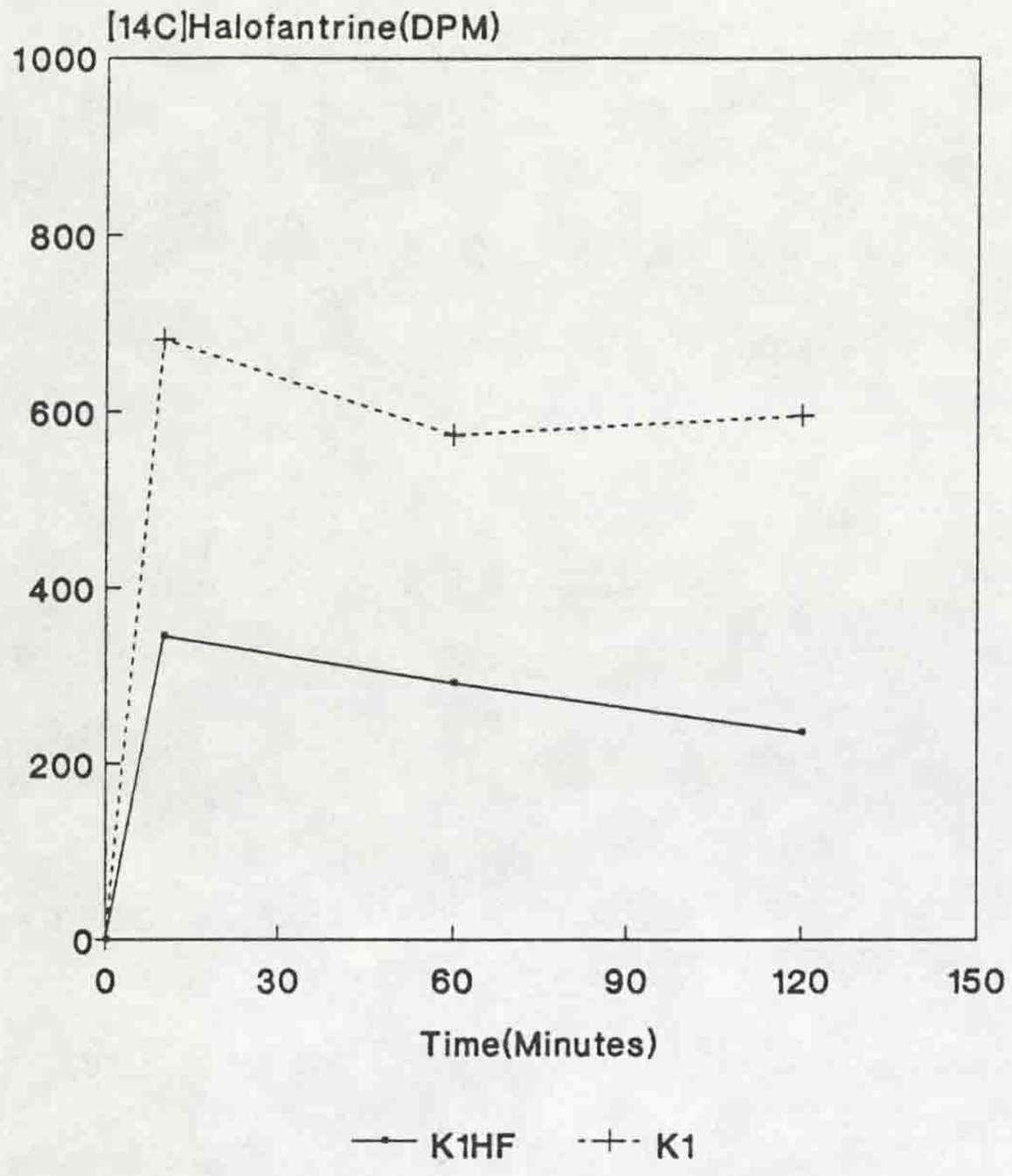


Fig.7.3.2 Uptake of [14C]halofantrine
by T9.96HF and T9.96-infected RBCs.

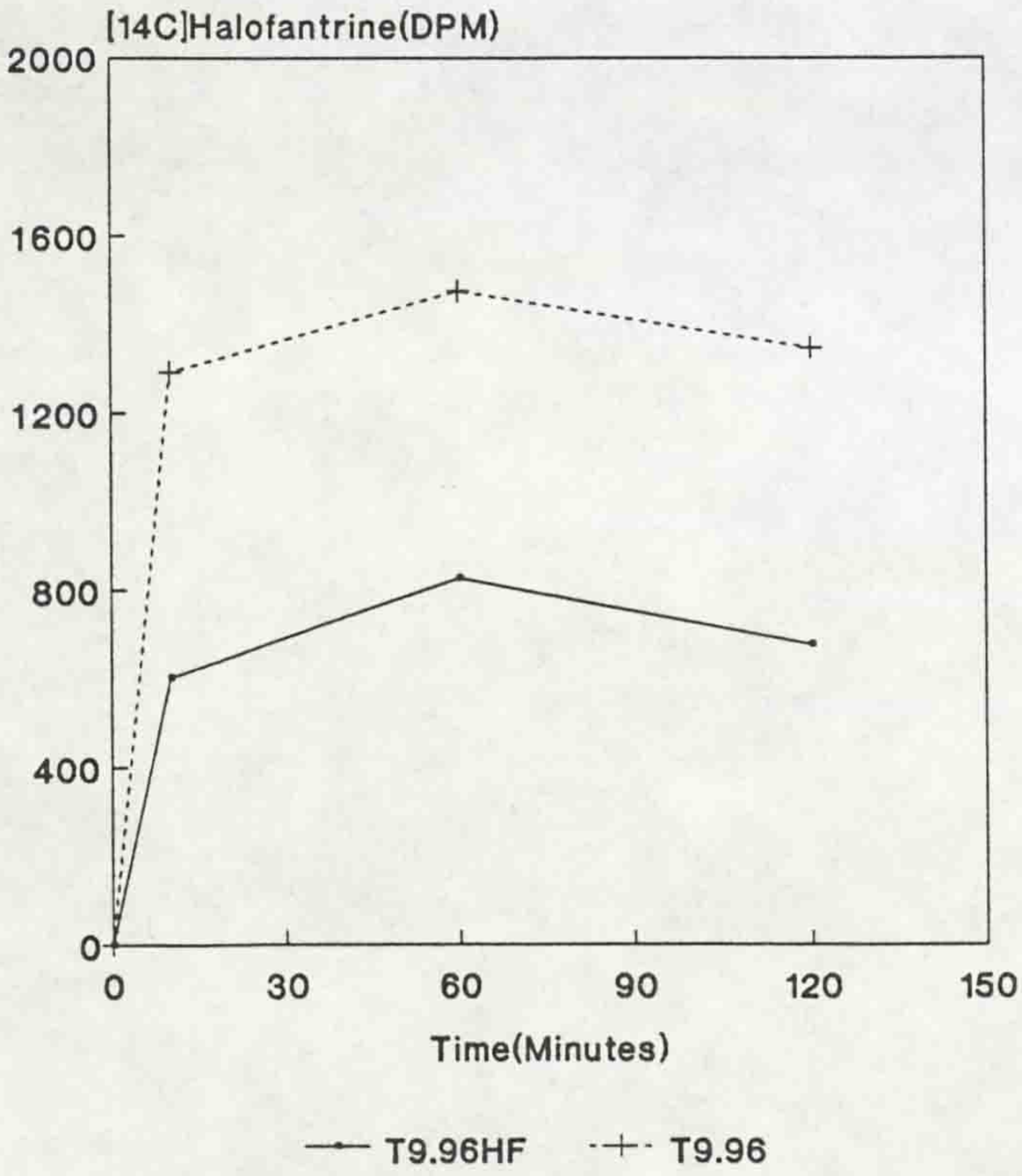


Fig.7.3.3 Uptake of [14C]halofantrine in combination with 2.2 nmol/l halofantrine by K1 and K1HF-infected RBCs.

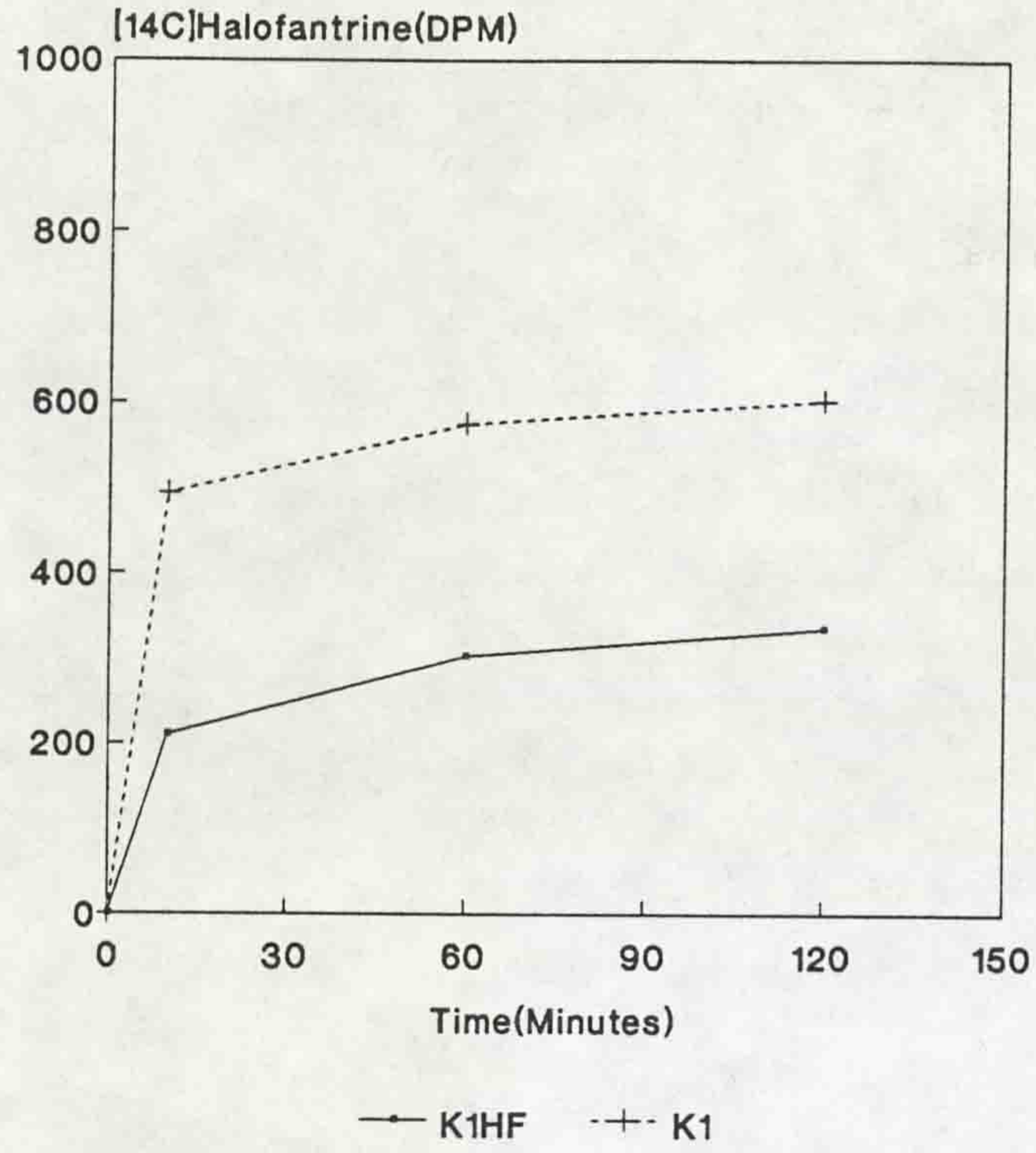


Fig.7.3.4 Uptake of [14C]halofantrine in combination with 6.6 nmol/l halofantrine by T9.96 and T9.96HF-infected RBCs

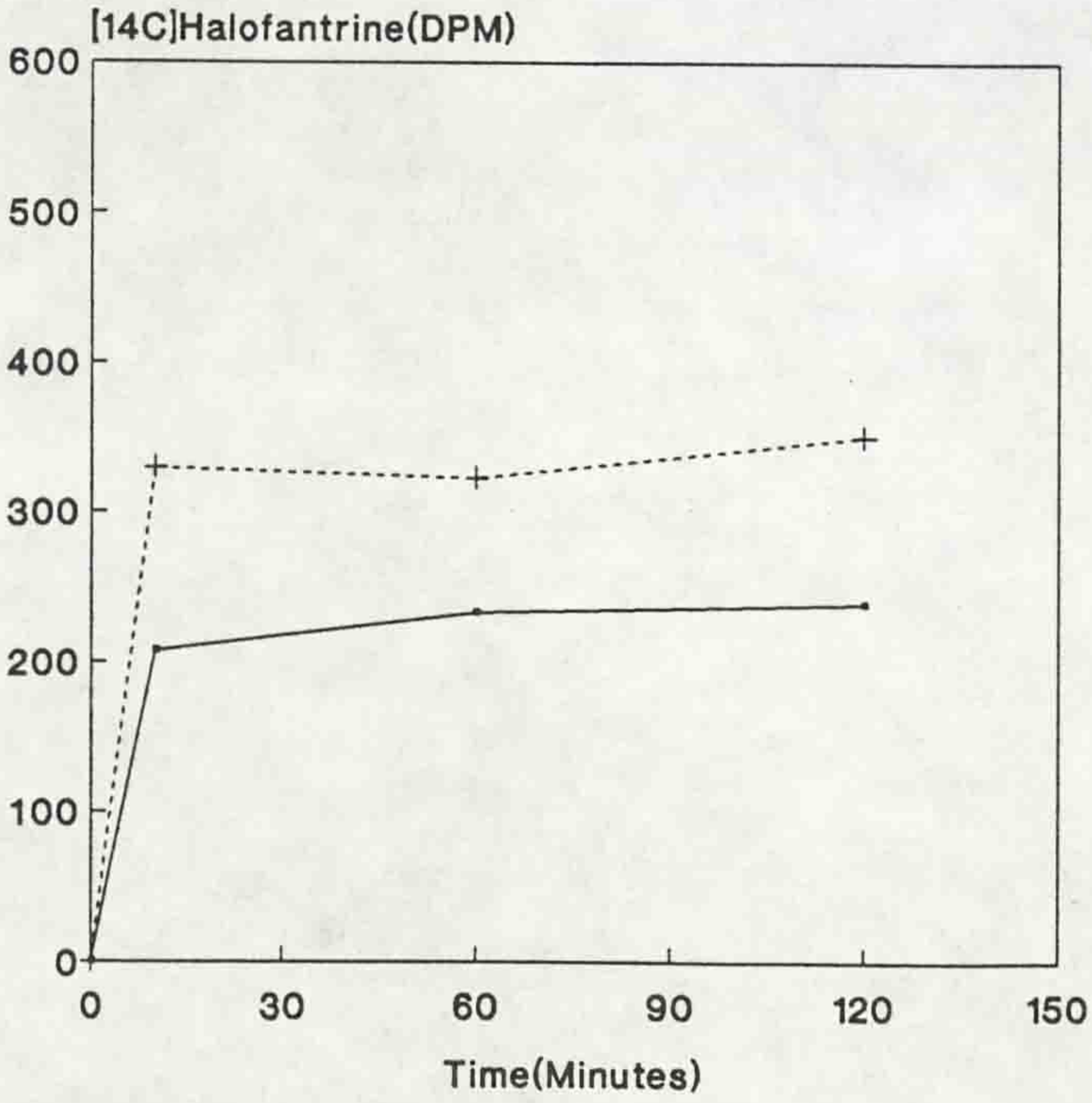
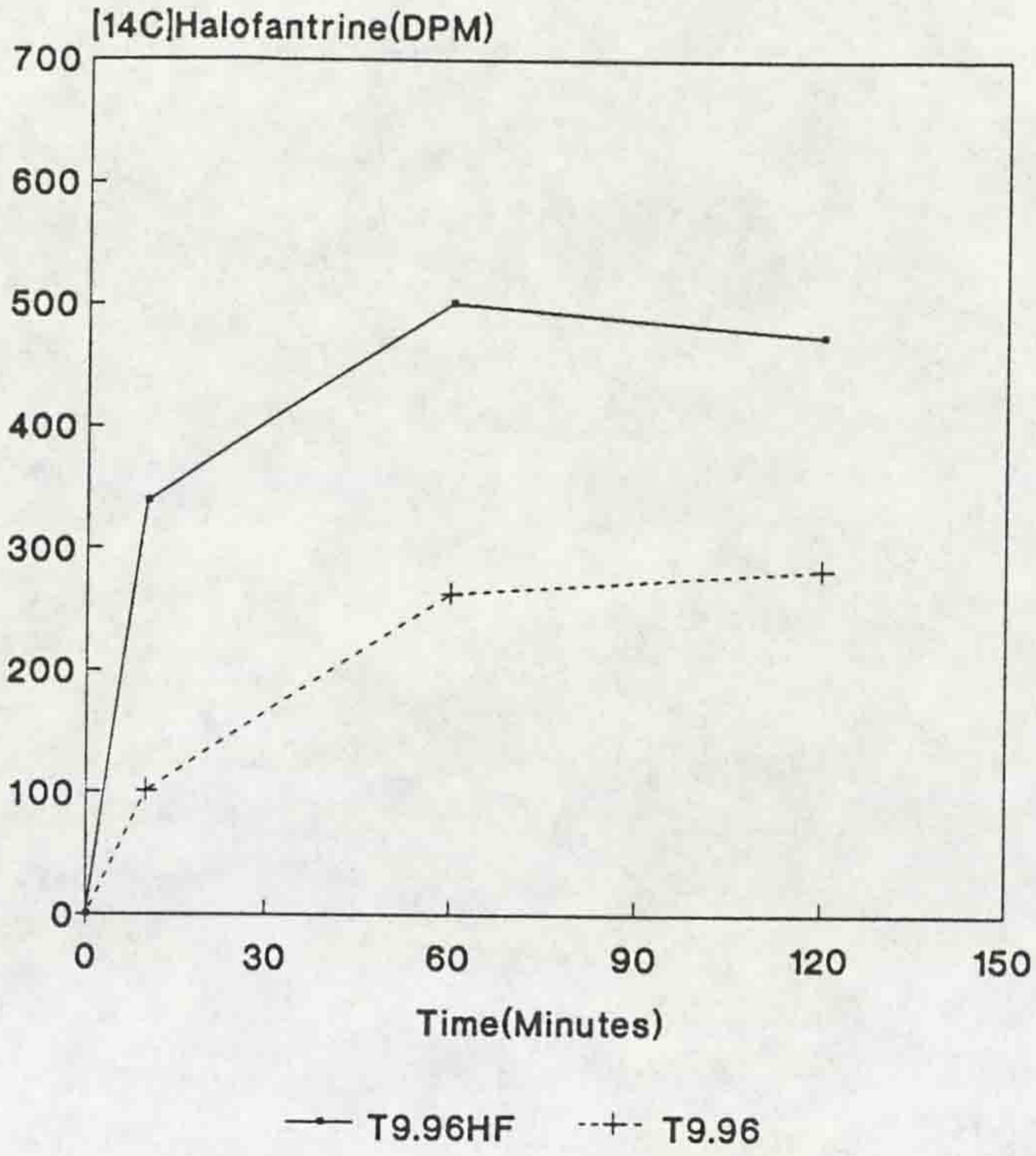
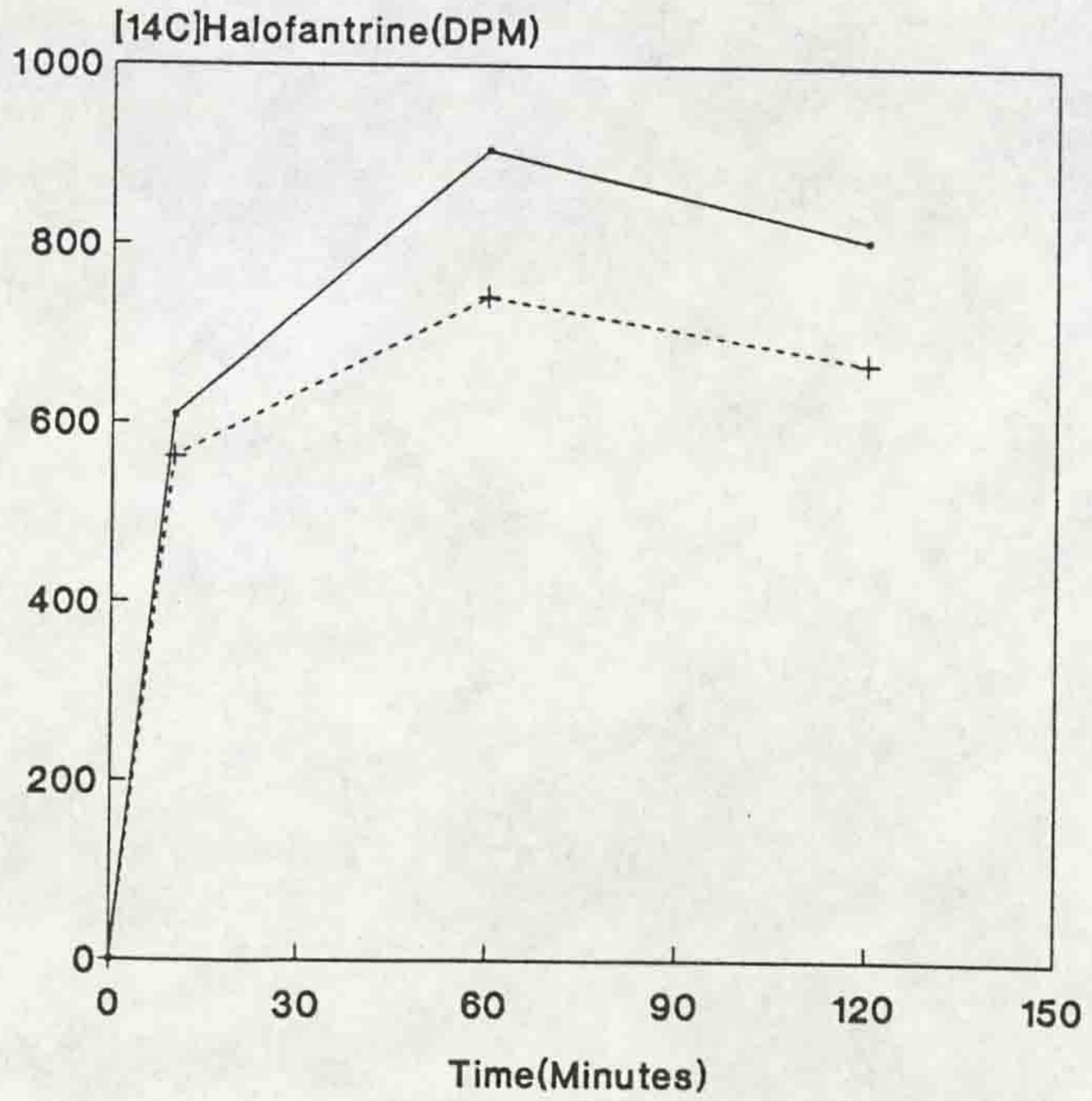


Fig.7.3.5 Uptake of [14C]halofantrine in combination with unlabelled HF and PF by T9.96HF and T9.96-infected RBCs.



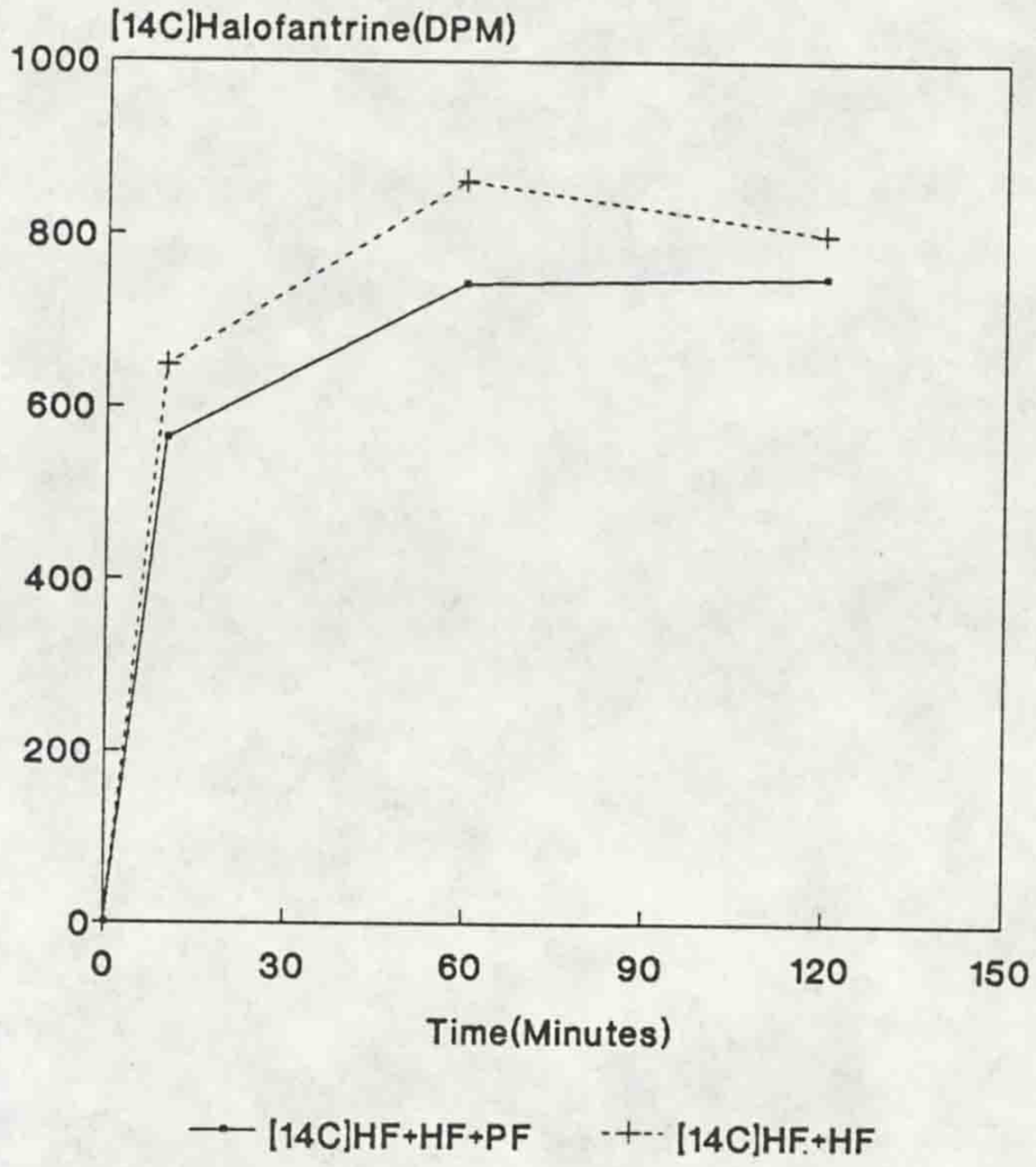
HF: halofantrine; PF: penfluridol

Fig.7.3.6 uptake of [14C]HF+unlabelled halofantrine or in combination with penfluridol by T9.96HF-infected RBCs.



HF: halofantrine ; PF: penfluridol

Fig.7.3.7 uptake of [14C]HF+unlabelled halofantrine or in combination with penfluridol by T9.96-infected RBCs.



CHAPTER 8 : GENERAL DISCUSSION AND CONCLUSIONS

The rapid spread of chloroquine-resistant *Plasmodium falciparum* infections in malarious areas which was followed by the emergence of multidrug-resistant parasites prompted a number of investigators to investigate new approaches to the chemotherapy of malaria. The report by Martin *et al* (1987) that chloroquine-resistance in *P. falciparum* was reversed by verapamil not only offered a new treatment strategy but also led to hope that the effectiveness of chloroquine may be retained. The use of these reversing agents in combination with chloroquine against chloroquine-resistant parasites was proved either with animal models or in continuous *in vitro* culture of *P. falciparum* (Bitonti *et al*, 1988; Bitonti and McCann, 1989; Peters *et al*, 1989; Watt and Shanks, 1990; Basco and Le Bras, 1990a; Basco and Le Bras, 1990b; Watt *et al*, 1990). Results obtained from these experiments generally indicated a reversal of chloroquine-resistance in *P. falciparum* although there are some discrepancies.

The interaction between a number of antimalarial drugs such as chloroquine, mefloquine, halofantrine and qinghaosu, and non-antimalarial agents, verapamil (a calcium channel blocker), chlorpromazine and penfluridol (psychotropic agents and calmodulin antagonists), fluoxetine and praziquantel (serotonin inhibitors) against drug-sensitive and -resistant parasites strains has been investigated (Chapter 4 and 6). The results of chloroquine plus verapamil against chloroquine-resistant K_1 and -sensitive $T_{9.96}$ parasites confirmed the results obtained by Martin *et al* (1987), indicating that chloroquine-resistant parasites remarkably lost their resistance to chloroquine when treated with chloroquine + verapamil. A mechanistic explanation of these results has been put forward by Krogstad *et al* (1987) who showed that verapamil decreased the

efflux of chloroquine from chloroquine-resistant parasites. Martin *et al* (1987) speculated that reversal of resistance in drug-resistant *P. falciparum* and carcinoma cells may share a common mechanism. In contrast, Ginsburg and Stein (1991) more recently suggested that chloroquine-resistance in *P. falciparum* parasites has no analogy in multidrug-resistant carcinoma cells. However, the reversal mechanism in chloroquine-resistant parasites remains still unclear.

Chlorpromazine decreased the IC_{50} value for chloroquine against chloroquine-resistant K_1 parasites, as well as potentiated the effect of chloroquine against the parasites. Additionally, use of a different method by Kyle *et al* (1990) to those described in this study culminated in the same results. In contrast, Scheibel and colleagues (1987) indicated antagonism between chloroquine and two calmodulin inhibitors cyclosporin A and R24571 against chloroquine-resistant FCB_{K+} and chloroquine-sensitive 7G8 strains of *P. falciparum*, but a low level potentiation between chloroquine and W-7 calmodulin inhibitor against the parasites. According to these results it can be suggested that interaction between chloroquine and different calmodulin antagonists has different effects on different strains of *P. falciparum*. Although the precise reason for such discrepancy is not clear, one can speculate that the variety of calcium-dependent sites may play an important role for the sensitivity of calmodulin to calmodulin inhibitors.

Combination of chloroquine with the schistosomicide fluoxetine showed a remarkable potentiation against chloroquine-resistant parasites, but not against chloroquine-sensitive parasites, whereas we have shown antagonism between chloroquine and schistosomicide praziquantel against the chloroquine-resistant parasites. The difference between fluoxetine and praziquantel in terms

of mode of action against *Schistosoma mansoni* was discussed by Pax *et al* (1979) and Harder *et al* (1987), but such differences may not apply to strains of *P. falciparum*. However, the opposing actions of the two schistosomicides tested with respect to calcium transport into cells (see Chapter 4) may have a role in explaining the results obtained from chloroquine + fluoxetine and chloroquine + praziquantel combinations against chloroquine-resistant parasites of *P. falciparum*.

Although resistance reversing agents are being widely used to reverse chloroquine-resistance in plasmodia experimentally *in vitro* or *in vivo*, the potential toxicity of such drug combinations has been forewarned by a number of investigators (Peters *et al*, 1989; Watt and Shanks, 1990). Watt and co-workers (1990) demonstrated that cultured human hepatocytes could not survive in medium containing a combination of chloroquine with verapamil, but survived in medium treated with the same concentration of either drug alone. Additionally, Houghton *et al* (1989) reported that mice given a combination of vincristine and verapamil suffered more mortality than those given vincristine alone. The fact is still unclear, but Watt *et al* (1990) speculated that the reversing agents potentiate the toxicity of chloroquine in the host cells by blocking p-glycoprotein (Pgp). However, further studies would be required to verify the consequences of reversing agent therapy.

The development of halofantrine-resistance in *P. falciparum* in this study culminated in the two stable halofantrine-resistant strains. Since small increases in drug concentrations resulted in complete elimination of parasites from cultures, attempts to produce halofantrine-resistance in chloroquine-resistant and -sensitive parasites by using continuous drug exposure remained unsuccessful.

Therefore, intermittent drug exposure was performed and two halofantrine-resistant strains were successfully selected. The results in this study indicated that resistance developed more rapidly in uncloned parasites than cloned parasites. In addition, Oduola *et al* (1988b) had previously shown that a long time was required to produce a significant increase in the IC_{50} for mefloquine against cloned W2 parasites. It can be speculated that genetic heterogeneity and range of individual parasite drug sensitivities in these parasites were responsible for such differences.

The development of halofantrine resistance was associated with a decrease in parasite drug sensitivity to other antimalarials containing the methanolic function, namely mefloquine and quinine. This phenomenon has an obvious implication in terms of cross-resistance between mefloquine and halofantrine in the field. Additionally, recent investigations indicate a related reduction in susceptibility to mefloquine and halofantrine in West African isolates of *P. falciparum* (Oduola *et al*, 1987; Ringwald *et al*, 1990). Another interesting point is that halofantrine resistance in parasites which were originally resistant to chloroquine showed significantly increased chloroquine sensitivity which was not apparent in chloroquine-sensitive parasites. If this phenomenon is displayed in the field, it may provide an alternative strategy for treating drug resistant parasites.

The combination of penfluridol with halofantrine and mefloquine resulted in potentiation against $T_{9.96}HF$ parasites, but not against $T_{9.96}HF4$, K_1HF and W2-mef parasites. As discussed (see Chapter 6) two speculations may be presented: firstly these strains have inherently different responses to

halofantrine + penfluridol and mefloquine + penfluridol combinations, secondly the quantity of interaction between halofantrine + penfluridol against halofantrine-resistant K_1HF and $T_{9.96}$HF and mefloquine + penfluridol against $T_{9.96}$HF, $T_{9.96}$HF4 and W2-mef strains depends on the degree of halofantrine and mefloquine resistance in the parasites. Remarkable increase of sensitivity of K_1HF parasites to halofantrine in combination with verapamil was another interesting result obtained in this study. Additionally, halofantrine-resistant strains exhibited remarkable cross-resistance between halofantrine and penfluridol compared to parent parasites.

Conclusion

A number of aims in this study were investigated which are summarised as follows:

1. The activities of a variety of standard antimalarial drugs and several non-antimalarial agents, ie. calcium channel blockers, calmodulin antagonists and serotonin inhibitors, were comparatively determined. Among the non-antimalarial agents, penfluridol exhibited reasonable antimalarial properties (see Chapter 3).

2. Interactions between standard antimalarial drugs and verapamil, chlorpromazine, fluoxetine, praziquantel and penfluridol against chloroquine-resistant, chloroquine-sensitive and halofantrine-resistant strains of *P. falciparum* were screened for synergy (see Chapter 4 and 6).

3. Two halofantrine-resistant strains were developed and stability of resistance was determined. These strains appear to be useful material for investigation of drug resistance mechanisms in *P. falciparum* (see Chapter 5), and the method used to develop resistance may be one which is of general use in

developing drug resistance and may be more relevant to the situation encountered in the field.

4. The uptake of [14C] halofantrine by halofantrine-resistant and -sensitive parasite-infected and uninfected erythrocytes in the presence or absence of penfluridol was performed to determine if halofantrine resistance is related to intra-parasite steady state concentrations of halofantrine and therefore transport (see Chapter 7).

As already discussed, halofantrine-resistant K_1HF parasites became considerably sensitive to halofantrine when treated with halofantrine + verapamil. Therefore uptake of radiolabelled halofantrine by K_1HF and $T_{9.96}$HF-infected erythrocytes in the presence or absence of verapamil, as well as cloning and characterisation of the halofantrine-resistant parasites can be suggested as a future study.

Finally, the use of intermittent drug exposure as a rapid and effective method to induce drug-resistance in sensitive strains of *P. falciparum* has been suggested in this study.

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APPENDIX

Publication

Nateghpour, M., Ward, S.A. and Howells, R.E. The development of halofantrine resistant and cross resistance patterns in *Plasmodium falciparum* (in preparation).

Abstracts

1. M. Nateghpour and R.E. Howells. The selection of halofantrine-resistance in *Plasmodium falciparum in vitro*. The British Society for Parasitology, Spring Meeting, University of Aberdeen, 4-6 April 1990, p.21.

Insignificant changes in the halofantrine sensitivity of *Plasmodium falciparum* were obtained by continuous exposure to sub-lethal drug concentrations. Intermittent exposure of both a chloroquine-sensitive and -resistant strain of *P. falciparum* resulted in a relatively rapid reduction in sensitivty to halofantrine. The response of the parent strains and the halofantrine-'resistant' strains to chloroquine, quinine, mefloquine, qinghaosu, amodiaquine and pyrimethamine has been determined. Halofantrine 'resistance' was associated with an enhanced chloroquine sensitivity in the strain derived from chloroquine-resistant parasites, but not in the strain derived from chloroqouine-sensitive parasites.

2. M. Nateghpour, S.A. Ward and R.E. Howells. Halofantrine transport in drug-sensitive and drug-resistant isolates of *Plasmodium falciparum*. The British Society for Parasitology, Spring Meeting, University of Liverpool, 3-5 April 1991, p38.

The transport of [14C] halofantrine has been studied in two halofantrine-resistant strains of *Plasmodium falciparum* and two sensitive strains. Steady-state concentrations of halofantrine were significantly higher in drug-sensitive parasites compared with the resistant isolates. We have shown previously that the psychotropic drug, penfluridol, but not verapamil, is capable of reversing halofantrine resistance in our isolates. Studies designed to examine this phenomenon in more detail have shown that the co-incubation of [14C] halofantrine with penfluridol resulted in steady-state halofantrine concentrations in resistant strains similar to those observed in sensitive parasites without penfluridol. The implications of these studies will be discussed.