THE NEUROTOXICITY OF ARTEMISININ AND ITS DERIVATIVES

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by

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Publications

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Abbreviations

The following abbreviations appear in the text of this thesis:-

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B_{max}	Maximum binding capacity of preparation
Bq	Becquerels
BSA	Bovine serum albumin
C_{max}	Maximum plasma concentration
CNS	Central nervous system
conc.	Concentrated
CRA	*P. falciparum* blood stage antigen 5.1
DABCO	1,4 diazoabicyclo[2,2,2]octane
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
ED	Electrochemical detection
ED_{50}	50% effective curative dose
ER	Endoplasmic reticulum
GABA	Gamma-aminobutyric acid
GAP-43	Growth-associated protein 43
GSH	Glutathione
IC_{50}	50% inhibitory concentration
i.m.	Intramuscular
i.p.	Interperitoneal
i.r.	Infra-red
i.v.	Intravenous
HPLC	High performance liquid chromatography
HRP	Histamine-rich protein
kBq	Kilobecquerel
K_D	Dissociation constant
kDa	Kilodalton
LD_{50}	Dose lethal to 50% of animals
MBq	Megabecquerel
MSA-1	Merozoite surface antigen 1
MSA-2	Merozoite surface antigen 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
N	Number of experiments performed
NF-H	Neurofilament-H
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline

PCT	Parasite clearance time
PIPES	Piperazine N, N`-bis (2-ethane sulphonic acid)
PMB-3	Parasite-modified band 3
p.r.	Rectal
RER	Rough endoplasmic reticulum
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TEMED	N,N,N',N'-tetramethyl ethylene diamine
$t_{½}$	Half-life
$t_{½a}$	Absorption half-life
$t_{½e}$	Elimination half-life
t_{max}	Time to C_{max}
WHO	World Health Organisation

The following abbreviations appear in the figures and tables of this thesis (abbreviations that also appear in the text are listed above):-

ATM	Artemisinin
AEE	Arteether
AEM	Artemether
ATS	Artesunate
bid	Twice daily
C	Cristae
Cl	Chloroquine
CC	Clinical cure
CON	Control
D	Doxycycline
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DHA	Dihydroartemisinin
FCT	Fever clearance time
H	Haemin
M	Mefloquine
MI	Mitochondria
MT	Microtubule
NU	Nucleus
NF	Neurofilament
od	Oral dose
p.o.	Oral
P-S	Pyrimethamine and sulphadoxine
Q	Quinine

qid	Four times daily
R	Radical
Ra	Randomised allocation of treatment
RC	Radical cure
SD	Standard deviation
SDo	Single dose
stat	Initial dose
TDo	Total dose
tid	Three times daily
V	Vesicle

Fishwick J The neurotoxicity of artemisinin and its derivatives.

Artemisinin is highly effective against the erythrocytic stages of *P. falciparum in vitro* and *in vivo*. Recent *in vivo* toxicity studies in dog and rat injected *i.m.* with artemether and arteether revealed a dose-dependent neurotoxic effect associated with movement disturbances and spasticity. Furthermore, children treated for cerebral malaria with artemether suffer more convulsions and prolonged coma compared with those treated with quinine and a case of acute cerebellar dysfunction in an adult following treatment with artesunate has been reported.

The effects of the artemisinin derivatives on cell growth and axonal maintainance were examined by testing their effects on Nb2a neuroblastoma and C6 glioma cell proliferation and neurite outgrowth from differentiating Nb2a cells. All the drugs significantly inhibited proliferation in a dose-dependent manner. They also caused a dose-related decrease in neurite outgrowth, at concentrations lower than those that significantly inhibited proliferation. The antimalarial activity of these compounds is thought to depend their endoperoxide linkage; desoxyartemisinin, an inactive artemisinin metabolite, lacks this moiety and did not inhibit cell proliferation or neurite outgrowth, therefore it is highly probable that it is also necesarry for artemisinin neurotoxicity.

The ultrastructural damage to Nb2a cells caused by the drugs was then investigated. Western blotting and immunocytochemistry failed to detect any specific changes in the cell cytoskeleton. Transmission electron microscopy revealed that dihydroartemisinin damaged mitochondria and endoplasmic reticulum. Scanning electron microscopy revealed that dihydroartemisinin severely damages the processes projecting from the surface of the cell body and neurites.

Much of the work in this thesis was performed using Nb2a cells as a model for artemisinin neurotoxicity. It was necesarry therefore to demonstate that the Nb2a cell was suitable for this purpose. Incubation of neurotoxic agents and compounds with non-specific toxic effects with both proliferating and differentiating Nb2a cells revealed that neurotoxic agents had a significantly greater effect on neurite outgrowth than on proliferation and that non-specific toxic compounds did not, suggesting that Nb2a neurite outgrowth is a valid model for the study of neurotoxicity, and confirming that the artemisinin derivatives have neurotoxic effects.

^{14}C-dihydroartemisinin binding to rat brain, Nb2a and C6 cells, and how this was influenced by a number of compounds, was studied to characterise drug-protein binding. Rosenthal plots demonstrated that there were two discrete sets of binding sites. Haemin, which catalyses the conversion of artemisinin derivatives into reactive products and increases the artemisinin toxicity to cultured cells, significantly increased protein binding. Co-incubation with equimolar arteether reduced ^{14}C-dihydroartemisinin binding by 50%, while desoxyartemisinin did not affect ^{14}C-dihydroartemisinin binding. Autoradiographs revealed that 24, 32 and 84 kDa rat brain proteins were labelled by ^{14}C-dihydroartemisinin These results suggest a link between pharmacological activity, toxicity and protein binding.

In summary, investigation of the neurotoxicity of artemisinin derivatives suggested that they have neurotoxic effects, that protein binding is important in the mediation of these effects, and the role of haemin and the endoperoxide moiety in this toxicity suggests that the mechanisms of neurotoxicity and antimalarial activity are closely related.

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CHAPTER ONE

INTRODUCTION

1.1. HISTORICAL OVERVIEW

1.1.1. Malaria and human history

Malaria is probably older than mankind. Palaeolithic studies have indicated that prehistoric man suffered from malarial chills and fevers. Malaria spread around the world with human migration from prehistoric times onward into all tropical and temperate zones. This widespread distribution is attributable to the adaptability of its vector, the *Anopheles* mosquito. In contrast sleeping sickness has been confined mainly to rainforest zones due to the fastidiousness of its vector the Tsetse fly.

The populations of temperate zones (including Britain) were regularly visited by summer epidemics of the disease until the advent of quinine. It has plagued travellers to the tropics, merchants, missionaries and of course soldiers, and so has had a considerable impact on the course of human history. Alexander the Great was probably killed by *falciparum* malaria; it decimated the Athenian army besieging Syracuse, leading to the downfall of Classical Greece; and in World War I the British army campaigns at Salonica and in East Africa were severely hampered by malaria outbreaks.

Malaria was studied by the early Chinese and Hindu writers and was common in ancient Greece and Rome. Hippocrates accurately described the disease in Epidemics I and III, though treatment was limited to extracts of hyocyamus. In contrast, the Romans most effective method of controlling the disease was by draining the Campagna marshes surrounding Rome. More effective methods of control and treatment of malaria were not available until the late seventeenth

century, when the Jesuits brought quinine back to Europe from South America in the form of the bark of the Chinchona tree. However, transmission of the disease was not understood until the late nineteenth century (Butler & Wu, 1992; Gilles & Warrell, 1993).

1.1.2. Discovery of malaria parasite and mosquito as vector

Malaria derived its name from Italian speculation that the disease came from the *mal aria* or bad air from marshes. The term was first used by British physician John McCulloch in 1629. Despite historical associations between swamps, mosquitoes and fever the precise method of malaria transmission was a mystery until the late nineteenth century, although the idea that mosquitoes transmit malaria had been suggested by Lancisin in 1717.

In 1880, French army surgeon Alphonse Laveran discovered black pigment in the blood cells and organs of people dying from malaria, this was iron pigment from the parasite-digested haemoglobin. Many were sceptical that these were actually parasites even though his findings were confirmed by Celli and Marchiafava in 1885. The parasite was given the generic name *Plasmodium.* Staining methods were developed by Romanovsky, and it was soon established that the patient's fevers corresponded to the release of parasite "spores" from the red blood cells. Sir Patrick Manson, who had demonstrated that filariasis was spread by mosquito, suggested to Ronald Ross, a surgeon in the Indian Army, that mosquitoes could also spread malaria. In 1897 Ross discovered larvae of *Anopheles* mosquitoes, hatched them and let them feed on malaria patients. When the mosquito's stomachs were dissected

cysts were discovered containing black pigment identical to that seen in human erythrocytes parasitised with malaria. Thus *Anopheles* mosquito was established as the malaria vector and subsequently Giovanni and Grassi conformed Ross's findings and described the parasite life cycle (Butler & Wu, 1992).

1.2. THE MALARIA PARASITE

1.2.1. Life cycle of *Plasmodium*

Malaria is caused by protozoal parasites of the family *Plasmodiiae,* within the order *C. occidiida,* sub-order *Haemosporidiidea.* There are nearly 100 species of *Plasmodia* infecting a wide variety of animals from reptiles through to man. The four species recognised to cause infection in man are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale.* Within each species of parasite are the morphological variations referred to as "strains" which are defined as populations of common stock descended from a single ancestor (Gilles & Warrell, 1993).

The *Plasmodium* life-cycle can be considered a sequence of four phases, one sexual without multiplication and three asexual with multiplication :-

Fertilisation. The sexual phase which takes place inside the mosquito's stomach.

Sporogony. The first asexual phase, in the stomach wall and body cavity of the mosquito.

Hepatic schizogony. The second asexual phase, it takes place in the human liver.

Erythrocytic schizogony. The third asexual phase which occurs in the erythrocytes. *P.(Plasmodium) vivax* and *P. ovale* infect only young erythrocytes, *P. malariae* infects mature cells and *P. falciparum* infects erythrocytes of all ages. Each asexual phase begins with feeding and growth (Knell, 1991).

Fertilisation. When a female *Anopheles* feeds on an infected human, gametocytes are released from red cells as they are digested. The female gametocyte matures into a single female gamete, while the male gametocyte releases four to eight flagella (male gametes) in a sudden violent exflagellation tearing themselves free. Flagella are really spermatozoa and swim away seeking a female gamete. Male and female gametes unite to form a zygote within the mosquito stomach which matures over the next few hours to become a mobile ookinete, an elongated invasive form, with an apical complex which it uses to penetrate (either through or between) epithelial cells of the insects stomach wall. Once it has reached the basement membrane of the stomach wall it loses the penetrative features of the ookinete and becomes a spherical cell known as an oocyst and the next phase begins.

Sporogony. The oocyst rapidly enlarges feeding on digested haemoglobin and can be up to 80 μm in diameter when mature. After approximately one week, depending on ambient temperature, the oocyst begins to differentiate into thousands of worm-like sporozoites. Seven days later the oocyst bursts and the sporozoites move through the haemocoel (body cavity) of the mosquito and enter the salivary glands. The mosquito is now infective, but not apparently disturbed by its parasite load. When

the mosquito feeds again sporozoites in the saliva are injected into the new hosts bloodstream and quickly invade cells of the liver.

Hepatic schizogony. The parasites enter the liver via its Kupffer cells which are usually part of the bodies defences against invading organisms, gaining entry to hepatocytes. Here they change into rounded hepatic trophozoites and absorb nutrients from the cell, growing rapidly in two days to about 40μm in diameter. It begins to divide internally, the nucleus of the parasite divides repeatedly and endoplasmic reticulum (ER) and mitochondria multiply. Nuclear division is accompanied by cytoplasmic division so that latterly the trophozoite is now known as a hepatic schizont and consists of 10-30,000 uninucleate invasive cells known as merozoites. This stage closely resembles oocyst division into sporozoites The maturation stage lasts from 5.5 to 16 days depending on the species of *Plasmodium.*

Rupture of the cell membrane releases the mature merozoites into circulation where many gain entry to erythrocytes within minutes. In *P. vivax* some sporozoites entering the liver do not develop immediately. They become dormant parasites known as hypnozoites. These persist for months or years to begin development and cause the delayed relapses typical of *vivax* malaria. *P. malariae* also cause relapses though the resting form is not known. *P. falciparum* does not cause such late relapses. At this stage the infected person has no symptoms and is unaware that the infection is progressing. This period between mosquito infection and symptom appearance is known as the pre-patient period.

Erythrocytic schizogony. Once inside the erythrocyte the merozoites lose their invasive form and becomes an erythrocytic trophozoite. Because there is a central vacuole within the parasite which displaces the nucleus to one side, this early erythrocytic stage is known as a ring form. Due to the restrictive size of the erythrocyte, this is the least productive phase of its life-cycle. The trophozoite digests the haemoglobin of the red blood cell as a source of amino acids and the haem molecules are incorporated into dark granules of malaria pigment or haemozoin. After 2 or 3 days maturation period the parasite nucleus and the cytoplasm has divided asexually and formed an erythrocytic schizont containing 8-16 new invasive merozoites. The erythrocyte membrane ruptures releasing the merozoites into the plasma where they will attempt to invade further erythrocytes. The process of erythrocytic schizogony is repeated during the course of infection leading to a rapid increase in parasite numbers, until the process is either controlled by the development of a host immunity, or results in the death of the host (as can occur in *P. falciparum* infections). Released along with the merozoites are pyrogens formed during schizont maturation, and these are responsible for the regular fevers that accompany each round of erythrocytic schizogony.

The duration of erythrocytic schizogony varies between species of *Plasmodia*, being 48 h in the cases of *P. falciparum, P. vivax* and *P. ovale* and 72 h in the case of *P. malariae*. Once several cycles of erythrocytic schizogony have occurred, some of the invading merozoites develop in an alternative way producing gametocytes. These take approximately 4 days to develop and can be dormant for long periods. These are the sex cells and will only develop further when ingested by a female *Anopheles* mosquito, becoming activated once in its stomach (Knell, 1991).

1.2.2. Clinical course of malaria

The typical symptom of malaria is a violent fever lasting 6-8 h, recurring every two to three days depending on the type of *Plasmodium* infection. A tertian fever has one day free of fever between attacks, a quartan fever has two. Anaemia and enlargement of the spleen develop as the disease progresses. These periodic attacks of prostrating fever distinguish malaria from other infections. Each attack is abrupt and severe and the patient feels very ill with headache or back ache. A feeling of unbearable coldness develops and causes violent uncontrollable shivering, but within an hour the body temperature rises to 41°C and the patient feels unbearably hot, this also lasts 1-2 h. Profuse sweating ends this attack cooling the body in 5-8 h. Each attack leaves the patient utterly exhausted.

Vivax, ovale and quartan malaria. The incubation period of *P. vivax* is usually between 12-17 days, that of *P. ovale* is between 16-18 days, and that of *P. malariae* is 18-40 days but in each case it can be much longer. The term "incubation period" refers to the delay between inoculation and appearance of clinical symptoms (*c.f.* pre-patient period). The clinical picture resulting from infection with these species of malaria is as described above. The periodicity of the fevers differ however. Typically they occur every 48 h (tertian) in the cases of *P. vivax* and *P. ovale* but every 72 h in the case of *P. malariae* (quartan malaria). In addition *P. malariae* infection in semi-immune children is associated with nephritic syndrome. Consequently although the risk of death from infection with these strains of *Plasmodia* is small, morbidity may be large; inadequately treated such infection may relapse repeatedly.

Falciparum malaria. *P. falciparum* is by far the most dangerous species infecting humans. It is poorly adapted to its human host, it causes severe or lethal illness in non-immune hosts and is especially dangerous to children and pregnant women. The incubation period for *falciparum* malaria varies between 9 and 14 days being most commonly about 12 days. The disease usually starts with non-specific symptoms such as myalgia, coughing, pain in the back and limbs, fatigue, anorexia, headache and diarrhoea, chills and vomiting. There may be few parasites in the blood at this stage, especially if the patient has been taking suppressive drugs, and the patient may not appear ill.

As the disease progresses the symptoms intensify and an irregular fever develops which does not usually comply to classical tertiary periodicity. Examination may reveal new symptoms developing including profuse sweating (even though the fever may be low), slight jaundice and mild enlargement of the spleen, fainting and mental disturbances may occur and pulse and respiratory rates increase. Dipstick urinalysis may reveal albuminuria, and granular casts may be seen on microscopic examination of urinary sediment. The full blood count may show slight anaemia, and there may be a degree of leucopenia with a relative monocytosis.

Recognition and treatment of the infection at this early stage may produce rapid improvement in the patients condition. However, if the infection is neglected life threatening complications may appear very suddenly, especially if the patient has no immunity to the organism. The World Health Organisation's (WHO) definition of severe *falciparum* malaria involves the demonstration of mature asexual forms of *P. falciparum* in a patient with a potentially fatal complication of malaria in whom other diagnoses have been excluded. The WHO recommends that such patients

require extensive management which should include parenteral chemotherapy. These complications include the presence of > 5% peripheral blood parasitaemia (up to 30% parasitaemia can occur compared to 1% with *vivax, ovale* and *malariae*, which is one of the reasons that *falciparum* malaria is so dangerous), a haemocrit of < 20%, renal failure, vomiting and diarrhoea, pulmonary oedema, shock, hyperpyrexia, clotting disturbances and complicating infections (*i.e.* 2^{o} bacterial infections) (Knell, 1991).

1.2.3. Sites of drug action in the parasites life-cycle

Antimalarial drugs have selective actions on the different phases of the parasite life-cycle which determines their therapeutic roles. Drugs such as amodiaquine, chloroquine, quinine, quinidine and mefloquine are "blood schizontoncides" and act within the erythrocytes to produce a rapid reduction in parasite number, and a corresponding improvement in the patients condition. This effect is seen in all four types of human malaria (Gilles & Warrell, 1993). The 8-aminoquinolines, quinine and mefloquine all have activity against the gametocyte stage of the life cycles of *P. vivax* and *P. ovale*, and primaquine is active against the gametocyte stage of *P. falciparum* as well, although its use is known primarily as a prophylactic. Pyrimethamine and proguanil act by inhibiting dihydrofolate reductase (DHFR) activity in all phases of parasite growth, and can therefore also be used to prevent sporogony growth phase in the mosquito. There is no drug active against sporozoites immediately after their inoculation into the host. However, the 8-aminoquinolones (and to a lesser extent, proguanil and pyrimethamine) are also

effective against the hepatic stage of the infection and are used to prevent relapses by destroying hypnozoites.

Artemisinin and its derivatives are also stage-specific. Like most antimalarial drugs they are schizonticidal; they are particularly effective against the late stage ring forms and trophozoites, more so than against small rings or schizonts (Caillard *et al.*, 1992; Geary *et al.*, 1989). They are also gametocytocidal (Dutta *et al.*, 1990; Kumar & Zheng, 1990; Chen *et al.*, 1994; Price *et al.*, 1996; Tripathi *et al.*, 1996). However, the hepatic stages of *P. falciparum* and *P. vivax*, *i.e.* sporozoites and hypnozoites, are not affected (China Cooperative Research Group On Qinghaosu and Its Derivatives as Antimalarials, 1982c) and so these drugs are not normally used alone for radical cure or as a prophylactic treatment.

1.2.4. Drug resistance

Malaria was thought to be near eradication in the late 1950s. A combination of new drugs and insecticides, to destroy both the parasites and the vectors, meant that the disease was being slowly eradicated from many areas with a corresponding drop in the number of infections. Yet at the same time that the disease seemed to be disappearing, the malaria parasites began to develop resistance to the modern drugs used against them. Resistance of *P. falciparum* to the DHFR inhibitors proguanil and pyrimethamine was first noted forty years ago. This was followed by reports of resistance to chloroquine from South America in the early 1960s. Since then chloroquine resistance has spread to most parts of the world and it seems inevitable that eventually resistance will be found throughout the range of *P falciparum*. This is

an alarming development as chloroquine was for many years the main drug used in malaria treatment. Resistance to quinine has not developed in most parts of the world and it is often used to treat patients with chloroquine-resistant strains of malaria despite its toxicity. Resistance to other drugs and drug combinations has also continued to spread. Mefloquine resistance is found wherever the drug has been used (Palmer *et al.*, 1993) and resistance to the pyrimethamine-sulphadoxine combination, Fansidar, is found in Southeast Asia and East Africa. Although multidrug resistant malaria is not generally a problem in Africa, some Southeast Asian strains of *P. falciparum* may be resistant to almost all classical antimalarial drugs. Burgeoning drug resistance has led to a large increase in the number of malaria cases and has come at the same time that international travel has increased massively, greatly increasing the risk to non-immune travellers from the temperate zones (Knell, 1991).

Two factors are largely responsible for the growing problem of drug resistance:-

The adaptability of P. falciparum. Pyrimethamine and proguanil resistance is caused by point mutations in the active site of DHFR that prevents the drug from binding to its active site. Chloroquine resistance appears to be the result of reduced accumulation of the drug in the parasite food vacuole, due to decreased permeability of the vacuole membrane.

The improper use of antimalarials in prophylaxis and as a routine treatment for various undiagnosed fevers. The presence of the drug at sub-therapeutic levels has

led to the introduction of selection pressure among the malaria parasites and selected out those parasites with mutations that enable them to survive chemotherapy.

The ever increasing problem of drug resistance has resulted in enormous efforts to find new antimalarial drugs, and led to the development of alternatives including mefloquine, halofantrine and the artemisinin derivatives. However, the introduction of mefloquine, halofantrine and other new drugs into areas of drug-resistant malaria has been swiftly followed by the appearance of parasite resistance to these drugs (Zucker & Campbell, 1995).

It is because of these problems that the artemisinin derivatives are the most important of the new antimalarial drugs. Even in Southeast Asian countries such as Thailand where multidrug resistant malaria is at its most problematic there is, as yet, no significant parasite resistance to the artemisinin derivatives and these drugs are now well established as the most effective treatment for multidrug resistant *falciparum* malaria. In addition, they are the most rapidly acting of all antimalarial drugs, they have proven to be potent treatments for severe and cerebral malaria, and are effective whether given orally, parenterally, or by suppository (White, 1994a). This unique combination of qualities makes the artemisinin derivatives the most promising development in malaria chemotherapy since World War II.

1.3. ARTEMISININ AND ITS DERIVATIVES

Artemisia annua Linn is a weedlike plant that is found throughout the temperate zones of the world. It is commonly known by the names annual wormwood, sweet wormwood and qing hao. It is member of the *Compositae* family which contains over 300 species of *Artemisia,* many of which have been used as spices, insect repellents, oils (Klayman, 1993) in the neurotoxic narcotic drink, absinthe (*Artemisia absinthium*) (Arnold, 1989).

1.3.1. Treatment of malaria in traditional Chinese medicine

Qing hao has been used for centuries in China as a treatment for various fevers and chills. Its earliest mention occurs in the *Recipes For 52 Kinds of Diseases* found in the Mawangdui Han dynasty tomb dating back to 168 BC, though in this text, the herb is recommended for use in haemorrhoids. The plant is also mentioned in *Ji Zhou Hou Bei Fang* (*Handbook of Prescriptions For Emergency Treatments*) written in 340 AD. The author Ge Hong, advised that to reduce fevers one should soak one handful of qing hao in 1 sheng (about one litre) of water, strain the liquor and drink it. The first mention of qing hao as a specific treatment for malaria occurred in 1596 AD when Li Shizen wrote in his *Bencao Gangmu (Systematic Materia Medica)* that qing hao "can cure malaria, fevers and cold". A concoction of *A. (Artemisia) annua* and *Carapax trionycis* was also suggested in the *Wenbing Tiaoban,* written in 1798, as a treatment for malaria and the use of qing hao as a cure for malaria was once again recommended in *The Barefoot Doctors Manual* (Trigg,

1989; Klayman, 1993). The use of qing hao was widespread and has continued in various folk medicines in rural China up to the present day.

1.3.2. Early development of artemisinin

In the late sixties there was a resurgence of malaria and an increase in parasite resistance to drug therapy, and so the Chinese government implemented a program to examine indigenous plants used in traditional remedies as a potential source of drugs. *A.. annua* was one such herb, though hot water extracts of its leaves proved to have little antimalarial (or antipyretic) activity. In 1971 a low temperature extraction of the dry plant leaves, an ethyl ether extraction, was attempted. Crude ether extractions of the plant proved an effective treatment in mice infected with the malaria parasite *P. berghei.* Further research led to the isolation of a plant constituent that had not previously been reported in the chemical literature (Qinghaosu Antimalaria Coordinating Research Group, 1979), though it has recently come to light that Jeremić and co-workers (1973) may have isolated the compound first, though they reported an incorrect ozonide structure. The fine colourless crystalline compound has the systematic name 3,6,9-trimethyl-3,12a-epidioxyperhydropyrano[8,9,10-*jk*]benzoxepin-10-one (see figure 1.3 for numbering system). This is rather unwieldy, so it was named "qinghaosu", meaning "active principle of qing hao" and also the more Western sounding artemisinine, though this was altered to artemisinin to avoid the suggestion that it was an amine or alkaloid.

1.3.3. Extraction of artemisinin from *A. annua*

Many derivatives of artemisinin have been synthesised over the past twenty years and although *de novo* synthesis are possible they are very complex and not very practicable or affordable sources of artemisinin or its derivatives (Schmid & Hofheinz, 1983; Xu *et al.*, 1986; Avery *et al.*, 1992; Ravindranathan *et al.*, 1990). Consequently, most are synthesised from artemisinin which is extracted from cultivated *A. annua*. Like most naturally occurring compounds, artemisinin exists in the plant in very small amounts with the content varying in different parts of the plant, with approximately 89% in the leaves and 11% in the lateral shoots (Laughlin, 1994), and the highest concentrations being 0.95% (w/w) of the dried plant (Trigg, 1989). Crude artemisinin is extracted from air-dried leaves in petroleum ether and removed *in vacuo*. The residue is redissolved in chloroform, and acetonitrile added to precipitate out plant matter such as sugars and waxes. This extract is chromatographed on a silica gel column. The fractions containing a high artemisinin content crystallise readily, and this recrystallisation is performed using 50% ethanol or cyclohexane. This method has subsequently been modified and developed into a large scale extraction technique for possible commercial use (Klayman, 1993).

1.3.4. Chemical properties of artemisinin

Artemisinin is a white crystalline compound that is poorly soluble in oil and water and decomposes in other protic solvents. It is soluble in most aprotic solvents and is stable up to 150°C. Indeed artemisinin shows a remarkable thermal stability,

and does not explode at its melting point, 156-157°C. Mass spectrometry analysis identified its molecular weight as 282 and suggested an empirical formula of $C_{15}H_{22}O_5$. Its *i.r.* spectrum shows absorption at 1745 cm^{-1} for a δ-lactone and at 831 cm^{-1}, 881 cm^{-1}, and 1115 cm^{-1} for a peroxide group (Trigg, 1989). An ^{1}H-NMR (nuclear magnetic resonance) spectrum of artemisinin indicates the presence of one tertiary and two secondary methyl groups and one acetal proton. ^{13}C-NMR analysis reveals three methine carbons, four methylene carbons, two quaternary carbons and one carbonyl carbon (China Cooperative Research Group On Qinghaosu and Its Derivatives as Antimalarials, 1982a; Blaskó *et al.*, 1988).

The structure of artemisinin as well as its absolute configuration was determined by x-ray diffraction. The structures of artemisinin, and its commonly used derivatives are given below on figure 1.1.

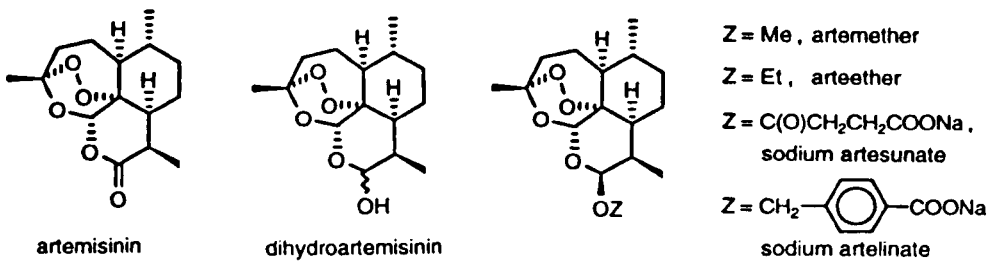


Figure 1.1. The structures of artemisinin, dihydroartemisinin, and the commonly used derivatives of dihydroartemisinin.

The artemisinin molecule is a tetracyclic structure with a trioxane ring and a lactone ring. The trioxane ring contains an endoperoxide moiety which is the

pharmacophore of the molecule. The lactone ring has a trans-configuration and is reduced to a lactol by sodium borohydride, to give dihydroartemisinin, without affecting the peroxide moiety. Hydrogenation with palladium and calcium carbonate removes one of the peroxide oxygens from the active moiety to give the inactive desoxyartemisinin (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982b).

1.3.5. Postulated mode of action

The artemisinin derivatives are selectively toxic to malaria parasites. This is partially due to the fact that the parasites actively take up the drugs. Erythrocytes infected with *P. falciparum* concentrate ^{14}C-artemisinin and ^{3}H-dihydroartemisinin up to 150 times the concentrations measured in uninfected erythrocytes (Gu *et al.*, 1984; Meshnick *et al.*, 1991). The lipophilicity of the compounds means that they readily partition into biological membranes and have been demonstrated to interact with isolated erythrocyte membranes (Asawamahasakda *et al.*, 1994a). Electron microscopy of *P. berghei* and *P. falciparum* parasites incubated with ^{3}H-dihydroartemisinin reveals that they also associate with parasite membranes, particularly limiting membranes, mitochondria, and digestive vacuole membranes. This interaction of the drugs with parasite membranes seems central to its antimalarial activity. *In vitro* electron microscopy studies reveal that the drugs induce a variety of morphological changes in parasites at concentrations as low as 300 nM. There are slight differences in the sequence of events in the different studies that have been performed but they are in general agreement about the damage

caused to the parasite. The onset of drug effects is extremely rapid and changes in parasite membranes occur within thirty minutes of commencing the incubation. Distension of the mitochondria, swelling of the outer mitochondrial membranes and the limiting membranes occur, followed by whorling of the food vacuole membranes and nuclear membranes, dissociation of ribosomes with endoplasmic reticulum, altered endoplasmic reticulum leading to cytoplasmic vacuolisation and finally the formation of autophagic vacuoles which cause degeneration of the inner structures and eventually death (Ellis *et al.*, 1985; Maeno *et al.*, 1993). The morphological changes in ribosomes and endoplasmic reticulum correlate with the depression of protein synthesis and the inhibition of nucleic acid synthesis, though changes in protein synthesis and nucleic acid synthesis are probably not the drugs main targets. As the drugs are localised in the membranes, changes in endoplasmic reticulum and perinuclear membrane integrity could precede the early depressions of nucleic acid and protein synthesis (Ellis *et al.*, 1985). Changes in phosphorus, potassium and sodium contents of the infected red blood cells occur on treatment with artemisinin are also consistent with drug alterations to parasite membranes (Lee *et al.*, 1988). The effects are most prominent in early and late trophozoites. This is consistent with *in vitro* studies showing that inhibition of growth and maturation is maximal in the trophozoite stage and the early schizont stage (Gu *et al.*, 1984).

How do the artemisinin derivatives cause this damage to the parasite ? As mentioned above, malaria parasites actively take up and concentrate the artemisinin derivatives. During erythrocytic infection the parasites consume ~25% of the total haemoglobin in the host cell and the concentration of haemoglobin in an erythrocyte is equivalent to 20 mM haem. Consequently, the levels of iron both surrounding and

within the parasite are extremely high, and this is thought to partially explain the selectivity of the drugs for the parasite and lack toxicity for the host. In the parasite food vacuole haemoglobin is broken down. The amino acids are digested, releasing haem which is then polymerised into an insoluble material called haemozoin that is excreted by the parasite. Free iron and haem are also released into the vacuole during the digestion of haemoglobin.

Chloroquine-resistant *P. berghei* lack haemozoin and are resistant to artemisinin, which suggests that presence or synthesis of haemozoin is important for artemisinin activity. However, *in vitro* haemoglobin and haemozoin do not react significantly with artemisinin but do react and bind with haem and haemin (Meshnick *et al.*, 1991; Hong *et al.*, 1994). Furthermore, iron chelators and chloroquine (which binds haem (Chou *et al.*, 1980)) antagonise the antimalarial activity of artemisinin (Stahel *et al.*, 1988) suggesting that haem and / or iron may be responsible for the activation of artemisinin rather than haemoglobin or haemozoin. This suggests that the artemisinin derivatives react with iron / haem after the digestion of haemoglobin but before its incorporation into haemozoin.

There is strong indirect evidence that the antimalarial activity of these drugs depends on the generation of free radicals. An endoperoxide moiety is a well known source of free radicals and derivatives of artemisinin that lack this group, *e.g.* desoxyartemisinin, also lack antimalarial activity (Klayman, 1985). Free radical scavengers such as ascorbic acid and vitamin E have been demonstrated to antagonise the antimalarial activities if the drugs *in vitro* (Krungkrai & Yuthavong, 1987; Meshnick *et al.*, 1989) and *in vivo* (Levander *et al.*, 1989). Moreover, high oxygen tension and co-incubation with free radical generating compounds, *e.g.*

doxorubicin or miconazole, enhance the *in vitro* antimalarial activities of the drug (Krungkrai & Yuthavong, 1987). Consequently, it was no surprise when haem and iron were demonstrated to catalyse the conversion of artemisinin derivatives into free radicals (Posner & Oh, 1992; Zhang *et al.*, 1992; Meshnick *et al.*, 1993). This reliance on iron / haem for the catalytic production of toxic products is another factor, along with parasite uptake of the drugs, that confers on the artemisinin derivatives their selectivity for the parasite over the host.

A free radical is a highly reactive molecule containing an unpaired electron and many biologically important free radicals are partially reduced forms of oxygen. Furthermore, many drugs are known to generate oxygen free radicals that cause widespread damage to the cells (Halliwell & Gutteridge, 1989). Artemisinin derivatives cause lipid peroxidation in infected and uninfected red blood cells and thiol oxidation in isolated erythrocyte membranes (Meshnick *et al.*, 1989, 1991). This oxidation of cell components may be responsible for some effects of the drugs such as decreased red cell deformability, premature lysis of infected erythrocytes and haemolysis (Gu & Inselburg, 1989; Scott *et al.*, 1989). However, these oxidative effects are only observed at concentrations of the drugs that are much higher than those that kill the parasites, *e.g.* 100 μM+, and are therefore unlikely to be responsible for the antimalarial activity of the drugs.

Although this protein and lipid oxidation does occur, the action of the artemisinin derivatives is thought to be mediated through the generation of carbon-centred free radicals. Both haem and free iron catalyse the decomposition of organic peroxides into free radicals *in vitro* (Halliwell & Gutteridge, 1989). They readily

catalyse the reductive cleavage of artemisinin's endoperoxide bridge (Zhang *et al.*, 1992; Meshnick *et al.*, 1993). This cleavage of the oxygen-oxygen endoperoxide bridge by iron (II) or haem, which is thought to involve the transfer of an electron from ferrous iron (Fe(II)) and the formation of an Fe(IV)=O (Posner & Oh, 1992; Posner *et al.*, 1995a), leads to an intermediate oxygen radical which then abstracts a hydrogen atom from the C-4 carbon (see figure 1.3 for the numbering system) of the trioxane ring. The result of this catalytic interaction with iron / haem are reactive carbon-centred radicals and powerful alkylating agents such as a 1,5 diketone and an electrophilic epoxide (Posner & Oh, 1992; Posner *et al.*, 1994, 1995a, 1995b, 1996) that are potentially capable of alkylating, crosslinking and oxidising macromolecules vital to parasite survival. An outline of the proposed mechanism of action of the artemisinin derivatives is given in figure 1.2 below.

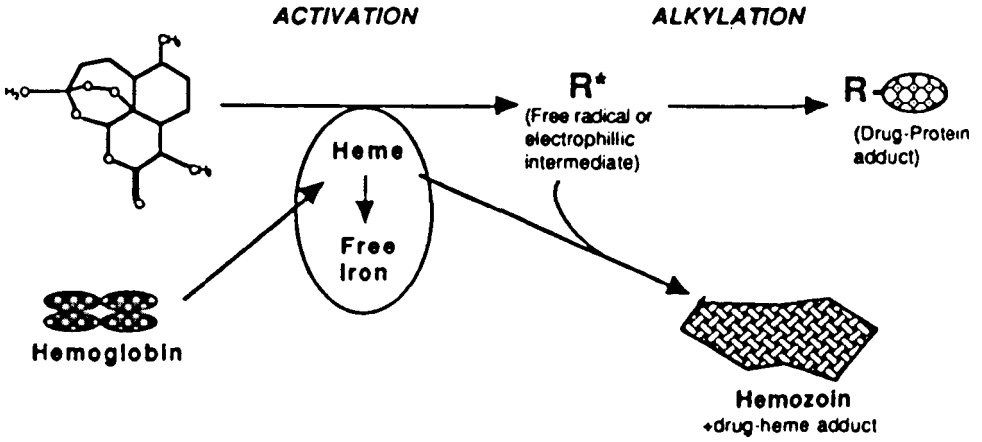


Figure 1.2. Proposed mechanism of action of the artemisinin derivatives (from Meshnick, 1994).

Incubation of *P. falciparum* with several different radiolabelled artemisinin derivatives (Asawamahasakda *et al.*, 1994b; Kamchonwongpaisan *et al.*, 1997) revealed that all the drugs covalently bind to six proteins; four major bands of 25, 50, 65 and 200+ kilodaltons (kDa) and two minor bands of 32 kDa and 42 kDa. These were not the most abundant parasite proteins implying that the binding may be specific and that one or more of these proteins is the key target for the antimalarial activity of these drugs.

The identity of these target proteins are not known. There are quite a few proteins specific to *P. falciparum* and parasite-infected red blood cells that are of a similar size to these proteins, *e.g.* histamine-rich protein (HRP), parasite-modified band 3 (PMB-3), merozoite surface antigen 1 & 2 (MSA-1 & MSA-2) and *P. falciparum* blood stage antigen 5.1 (CRA) (Cumming *et al.*, 1997). The function of these proteins is varied, they are all important in the induction of human immunological response to the parasites, and PMB-3 and HRP are present on the surface of the infected erythrocyte and are involved in erythrocyte adhesion and accumulation in microvessels that damage internal organs, especially the brain. The artemisinin derivatives could alkylate one of these but equally the target could be a previously obscure protein. Whatever the target or precise mechanism of action of the drugs, protein alkylation is clearly of great importance in this process.

1.3.6. Structure-activity relationships of artemisinin and its derivatives

By far the most important structural feature of the artemisinin molecule is the endoperoxide bridge (figure 1.3). It is this moiety that is largely responsible for

antimalarial activity. Other structural features of the molecule may modulate the precise degree of activity, but the endoperoxide is indispensable for activity (Tani *et al.*, 1985; Imakura *et al.*, 1988; Butler & Wu, 1992). Removal of the endoperoxide

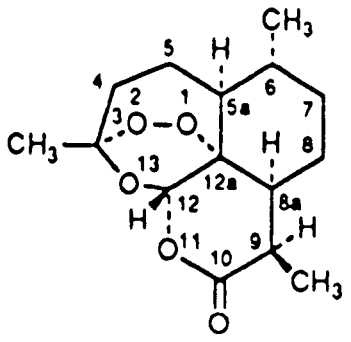


Figure 1.3. The numbering system for the artemisinin molecule. The numbering system of the carbon atoms is a common one used in many chemistry and structure-activity studies, although other numbering systems are used by some groups.

bridge leads to a loss of antimalarial activity *e.g.* desoxyartemisinin and other non-peroxy metabolites are devoid of antimalarial activity (China Cooperative Research Group On Qinghaosu and Its Derivatives as Antimalarials, 1982c; Jung *et al.*, 1986) and replacement of an oxygen atom in the endoperoxide bridge with a carbon atom also leads to a loss of activity (Imakura *et al.*, 1988). Most of the other structural features of artemisinin can be removed and some antimalarial activity will be retained. Yingzhaosu A, derived from a Chinese vine, and arteflene, a synthetic derivative of this compound, possess a peroxide moiety but are otherwise structurally unrelated to the artemisinin derivatives and are highly active against malaria parasites *in vitro* and *in vivo*, though inferior to the artemisinin derivatives (Zucker & Campbell 1995). The recent development of simplified trioxanes eliminate some of the complex ring structure of artemisinin but preserve the trioxane

ring and are active against malaria parasites *in vitro* and *in vivo* (Posner *et al.*, 1994, 1995b). Replacement of O-13 in the trioxane ring with a carbon atom leads to a dramatic loss of activity (Avery *et al.*, 1996a), therefore it may be that the whole trioxane ring contributes to the high potency of the artemisinin derivatives rather than just the endoperoxide linkage. Although most of the three ring structure of artemisinin appears to be unnecessary, more than just an endoperoxide moiety / trioxane ring may be required for legitimate activity. Unlike arteflene, many compounds that contain an endoperoxide moiety but are not otherwise structurally related to artemisinin possess no antimalarial activity *in vivo* but some do have activity *in vitro* (Vennerstrom *et al.*, 1989). This suggests that some other structural features are important in delivering the artemisinin molecule in an active form to its site of action (Butler & Wu, 1992).

All the commonly used artemisinin derivatives are analogues of artemisinin, identical in every respect except that they have different moieties on the C-10 of the lactone ring in place of the carbonyl group. Dihydroartemisinin, the most potent of the artemisinin derivatives, has a lactol ring with the C-10 carbonyl reduced and is 4-5 times more active *in vitro* than its parent compound. Various attempts have been made to increase the efficacy solubility and prolong the half-life ($t_{½}$) of artemisinin by synthesising derivatives with various moieties added onto the lactol group of dihydroartemisinin. Many ether, ester, carbonate and carboxylic ester derivatives have been synthesised, some of which were more potent than artemisinin or dihydroartemisinin *in vivo* against *P. berghei.* The overall order of potency being carbonates > esters > ethers > artemisinin (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982c; Luo & Shen, 1987). The

carbonates were the most potent but have not been further developed as they are very difficult to produce. An ester derivative, the hemisuccinyl salt, sodium artesunate (Barradell & Fitton, 1995) and a more stable ether derivative sodium artelinate (Lin *et al.*, 1987) have been investigated extensively because the side-chain groups greatly improve their water solubility, increasing their rapidity of action, making them potentially useful for *i.v.* treatment of severe malaria and are approximately 4-5 times more active than artemisinin *in vitro*. The carboxylic ester derivatives are less efficacious than the ester and ether derivatives, perhaps due to this groups lower lipid solubility reducing distribution and concentration at the site of action. The ether derivatives have the advantage of being more oil soluble than artemisinin. Artemether (Li *et al.*, 1982), the methyl derivative of dihydroartemisinin, and arteether (Brossi *et al.*, 1988; Davidson, 1994), the ethyl derivative, whose development has been sponsored by the WHO, are being used in the field in south-east Asia with great success being more efficacious than either artemisinin (approximately 2-3 times as active *in vitro*) or chloroquine (de Vries & Dien, 1996).

The carbonyl group of the lactone ring cannot be an important moiety as its loss when the derivatives are formed does not correspond with a loss of antimalarial activity (Jung *et al.*, 1990). Indeed. all the common derivatives with different C-10 substituents have greater activity than artemisinin. Furthermore, *in vitro* studies (Acton *et al.*, 1993; Avery *et al.*, 1996a, 1996b) suggest that it is the loss of the carbonyl as much as the addition of the new substituents that improves activity. 10-deoxoartemisinin, artemisinin with the C-10 carbonyl group entirely removed, is stable and active with an *in vitro* potency six times greater than that of artemisinin (Avery *et al.*, 1996b). The addition of bulky groups at the C-9 position of artemisinin

greatly reduces *in vitro* activity, however if the C-10 carbonyl is removed, or converted to another group, then derivatives with these bulky groups at C-9 possess greater *in vitro* activity than artemisinin (Acton *et al.*, 1993; Avery *et al.*, 1996b). Artemisinin derivatives with bulky hydrocarbon substitutions at C-3 on the trioxane ring have poor *in vitro* activity because this substituent physically hinders access to the peroxide moiety (Haynes & Vonwiller, 1996), however, 10-deoxoartemisinin derivatives with very bulky substituents at C-3 mostly have much greater activity than artemisinin (Avery *et al.*, 1996b). The presence of the carbonyl group appears to have a negative impact on the activity of artemisinin analogues. It has been suggested that these differences between artemisinin and 10-deoxoartemisinin are due to altered pharmacokinetics (Avery *et al.*, 1993).

As mentioned above, the addition of large substituents to the C-3 and C-9 positions of artemisinin reduce activity. The C-3 substituents do this by hindering access to the endoperoxide bridge (and C-4 (Posner *et al.*, 1995c)), this is confirmed by the observation that analogues with branched substituents at C-3, that are bulkier, have a lower potency *in vitro* than compounds with unbranched substituents of the same mass. A similar effect was noted with C-9 substituents (Avery *et al.*, 1996b).

The O-11 oxygen of the lactone ring can be replaced by a nitrogen atom and this provides a site by which many alkyl and aryl substituents may be attached. Many 11-substituted compounds with short alkyl or aryl chains are more potent *in vitro* than artemisinin, however, long side-chains lead to a loss of activity (Avery *et al.*, 1995). Most of the methyl groups on the artemisinin molecule can be removed without losing the compounds pharmacological activity (Avery *et al.*, 1989). Removal of the methyl groups at C-6 and C-9 does not greatly alter the activity of

the compounds (Avery *et al.*, 1993), although removal of the C-3 methyl group on the trioxane ring reduces activity (Avery *et al.*, 1996b).

Given the importance of the trioxane ring for activity it is perhaps not surprising that it should be most sensitive to structural changes. The replacement of O-13 with a carbon atom greatly reduces activity as O-13 stabilises the radicals formed at C-4 when the endoperoxide moiety is broken (Posner *et al.*, 1994; Avery *et al.*, 1996a; section 1.3.5). Stereochemistry at the C-4 position also affects the potency of these compounds. Groups which stabilise radicals improve the potency of the compounds if they are placed at C-4β but not if they are placed at C-4α as this prevents hydrogen atom transfer and generation of the carbon-centred radical crucial for antimalarial activity (Posner *et al.*, 1994).

The stereochemistry of the C-10 methyl and ethyl substitutions of artemether and arteether also affects potency. Against *P. falciparum in vitro* the α-anomer of arteether is less potent than the β-anomer (Brossi *et al.*, 1988; Shmuklarsky *et al.*, 1993). However, the differences are not great which suggests that stereoisomerism at this position does not greatly affect drug potency.

1.4. ARTEMISININ PHARMACOLOGY STUDIES *IN VITRO* AND *IN VIVO*

Little is known about artemisinin metabolism in man (section 1.5.2). However, extensive metabolic studies have been carried out using animal models and these may provide useful information for the evaluation of the potential benefits and hazards of these drugs.

1.4.1. Metabolism and pharmacokinetics of artemisinin derivatives *in vivo* and in *vitro*

Artemisinin. Studies using mice given ^{3}H-artemisinin and desoxyartemisinin orally reveal that the compounds are rapidly absorbed from the gastrointestinal tract. The maximum blood concentration was recorded 1 h later and the $t_{\frac{1}{2}}$ of the compounds is ~4 h. The drug is widely distributed in the body, and 80% of the oral dose is excreted within 24 h (Qinghaosu Antimalaria Coordinating Research Group, 1979). Intramuscular injection of artemisinin leads to a longer duration in the blood and an increased $t_{\frac{1}{2}}$. Intramuscular (*i.m.*) injection of artemisinin suspended in oil to rats also gives a $t_{\frac{1}{2}}$ of 4-5 h, longer than when the drug is given orally, as is also the case with dogs where *i.m.* injection of artemisinin in sesame oil give peak plasma levels in 2-3 h.

In *in vitro* studies in which rat liver slices are incubated with artemisinin, only 8.3% of the compound is recovered unchanged after 1 h. This high degree of conversion suggests that the liver is the main site of metabolism of artemisinin (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982d). Similar studies reveal that kidney and lung are less active sites of metabolism and gut and blood totally inactive. This information is broadly in line with clinical studies which suggest that the liver is the major organ of metabolism and that a first pass effect occurs. Little information on artemisinin metabolites is available but it is known that artemisinin is converted primarily into a number of inactive metabolites such as desoxyartemisinin and dihydrodeoxyartemisinin (Lee & Hufford, 1990).

Arteether. Arteether is converted by rat liver microsomes to dihydroartemisinin, deoxydihydroartemisinin, 3α-hydroxydeoxyarteether, 14-hydroxyarteether, 3α-hydroxydeoxydihydroartemisinin, 9β-hydroxyarteether (Lee & Hufford, 1990) and several other monohydroxylated arteethers (Baker *et al.*, 1988). A different rat liver microsome study identified two major arteether metabolites, dihydroartemisinin and 9α-hydoxyarteether and a minor metabolite 2α-hydroxyarteether (Leskovac & Theoharides, 1991a). In contrast to artemisinin where most of the metabolites are inactive, these hydroxy metabolites possess an endoperoxide bridge and so presumably retain antimalarial activity. Incubation of arteether with rat liver cytosol produces two metabolites : deoxydihydroartemisinin and artemether 1 (a methyl metabolite with an altered ring structure) (Baker *et al.*, 1988; Leskovac & Theoharides, 1991b). Acton & Roth (1995; and Baker *et al.*, 1993) reacted arteether with aqueous ethanolic hydrochloric acid, to simulate the conditions inside the stomach after oral administration and isolated four metabolites some of which possess potentially reactive ketone groups.

An *in vivo* study in which dogs were given a 25 mg/kg *i.m.* dose of arteether in oil, revealed that the drug is rapidly absorbed, widely distributed, has an absorption half-life ($t_{½a}$) of 0.3 h and has an elimination half-life ($t_{½e}$) of 27.9 h, much longer than other artemisinin derivatives, which could be useful therapeutically. However, dihydroartemisinin, thought to be a major metabolite, was not detected (Benakis *et al.*, 1991).

Artemether. Artemether has a high affinity for brain tissues. Five minutes after intravenous (*i.v.*) injection of ^{14}C-artemether into rats the highest levels are found in the brain (Jiang *et al.*, 1989). Moderate levels of ^{14}C-artemether are also found in the lungs, skeletal muscle and heart, whereas levels in the liver and kidneys at this time are low. This concurs with toxicity studies (Brewer *et al.*, 1994a, 1994b) that demonstrate that this drug causes extensive damage in the rat brain. Artemether seems to be rapidly metabolised in all tissues. In mice, 25% of artemether injected *i.v.* or *i.m.* undergoes demethylation within 24 h of administration and in rabbits and monkeys injected *i.m.* with artemether, 30% of the drug is metabolised to dihydroartemisinin within 24 h (Trigg, 1989). *In vitro* studies of artemether metabolism reveal that artemether perfused through rat liver or incubated with liver microsomes for 2 h produces four metabolites: dihydroartemisinin, deoxydihydroartemisinin, 9α- and 9β- hydroxyartemether. As with arteether these hydroxyartemethers retain the endoperoxide bridge and so retain antimalarial activity (Leskovac & Theoharides, 1991a, 1991b).

Artesunate and artelinate. The pharmacokinetics of 20 mg/kg artelinate in rabbits following administration by different routes has been studied. Intravenous administration gives the highest plasma concentrations, 76 ng/ml after 6 min, and the drug is rapidly eliminated from the body with an $t_{½e}$ of 15 min. There are large variations in absorption following *i.m.* and rectal (*p.r.*) administration and bioavailability is very poor (4.6%) following oral administration

compared to *i.m* and *p.r.* administration (44% & 47% respectively). This is probably due to a large first pass effect (Titulaer *et al.*, 1993).

In vivo studies with dogs and rats have demonstrated that following *i.v.* administration of the drugs there is rapid hydrolysis to dihydroartemisinin, as there is in human subjects (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982d; Barradell & Fitton, 1995; section 1.5). Approximately 80% of artesunate and artelinate are converted to dihydroartemisinin *in vitro* and the 2° metabolites are not easy to measure. One of the metabolites of artelinate produced by rat liver microsomes has been identified as a 4-carboxybenzaldehyde (Leskovac & Theoharides, 1991a). However, with both artesunate and artelinate, dihydroartemisinin is considered to be the active antimalarial agent as hydrolysis is extremely rapid, and so they should really be considered prodrugs.

Dihydroartemisinin. All the artemisinin derivatives yield dihydroartemisinin as the major metabolite, except artemisinin itself, and it contributes significantly to the antimalarial activity of all these drugs, especially in the case of artelinate and artesunate. Dihydroartemisinin metabolism was studied in rat liver microsomes (Leskovac & Theoharides, 1991a) and was found to be more slowly metabolised than the parent drugs into seven major metabolites, including four monohydroxylated derivatives of dihydroartemisinin and one monohydroxylated derivative of deoxydihydroartemisinin.

1.4.2. *In vitro* antimalarial studies

The antimalarial activity of the artemisinin derivatives has been studied extensively in a number of malaria parasite cultures. It has been demonstrated the artemisinin derivatives are highly effective against chloroquine, mefloquine and multidrug resistant strains. There is general agreement in the results of these studies about the relative toxicity's of the various artemisinin derivatives. Initial studies revealed that artemisinin, dihydroartemisinin, artemether and artesunate are highly effective *in vitro* against the erythrocytic stages of two chloroquine-resistant Hainan strains of *P. falciparum* with artesunate and dihydroartemisinin the most potent, being 4 or 5 times more active against the parasites as artemisinin (Li *et al.*, 1983; Trigg, 1989). Subsequently it has been demonstrated that the *in vitro* activity of the artemisinin derivatives against chloroquine susceptible and resistant clones and *falciparum* isolates is comparable to that of mefloquine (Klayman *et al.*, 1984). Trigg (1989) reported that the activity of artemisinin and several of its derivatives against a variety of drug resistant clones and isolates of *P. falciparum in vitro.* This study confirmed that all the compounds are highly effective, with both artemether and arteether being 2-3 times more active than artemisinin against the W2 and D6 clones; again dihydroartemisinin was the most effective of all the derivatives tested. Both α- and β-arteether are 2-3 times as potent as artemisinin against the D6 and W2 clones of *P. falciparum* in agreement with earlier work (Shmuklarsky *et al.*, 1993). Dihydroartemisinin is also 6 times more active than artemisinin *in vitro* against *P. berghei* parasites (Janse *et al.*, 1994). More recent studies using *P. falciparum*

isolates from Cambodian and Vietnamese patients confirm earlier work (Basco & Le Bras, 1994; Wongsrichanalai *et al.*, 1997).

Overall, the most potent artemisinin derivatives *in vitro* are the water soluble artesunate and dihydroartemisinin. These compounds are 4 to 5 times more effective than artemisinin, with IC_{50} values (concentration required for 50% inhibition of *P. falciparum* growth) ranging from 0.36 to 7 nM for dihydroartemisinin and 1.66 to 2.18 nM for artesunate. Artemether and arteether are 2 to 3 times more potent than artemisinin. Their IC_{50} values range from 0.57 to 6.1 nM for artemether and 1.74 to 3.44 nM for arteether. Artemisinin itself has an IC_{50} value of 10 to 100 nM (Li *et al.*, 1983; Lin *et al.*, 1987; Brossi *et al.*, 1988; Hassan Alin *et al.*, 1990, 1992; Shmuklarsky *et al.*, 1993; Basco & Le Bras, 1994).

Interactions of other antimalarial compounds with the artemisinin derivatives have also been examined. It appears that chloroquine-resistant strains of parasite are more sensitive to artemisinin than chloroquine-sensitive strains. Parasites that are sensitive to artemisinin also appear to be sensitive to mefloquine and halofantrine (Basco & Le Bras, 1994). There may be a synergistic relationship between the artemisinin derivatives and other antimalarials *e.g.* mefloquine, primaquine quinine and tetracycline (Chawira *et al.*, 1986a, 1986b). The development of resistance has been studied in parallel with chloroquine by repeated transfer of parasite cultures in the presence of increasing concentrations of the drugs. It was discovered that there was a 52-fold increase in the resistance to chloroquine in 10 transfers and only a 13-fold increase in artemisinin resistance in 17 transfers, so *in vitro* artemisinin is less prone to induce resistance in *Plasmodia* and has low resistance compared with the classical antimalarials (Qinghaosu Antimalaria Coordinating Research Group, 1979).

1.4.3. *In vivo* antimalarial studies

The results of the *in vivo* studies, like those of the *in vitro* studies, demonstrate that the artemisinin derivatives are extremely effective in the treatment of malaria. In *P. berghei*-infected mice, parasites are cleared following 3 days of treatment with a total dose of 150 mg/kg and the 50% effective curative dose (ED_{50}) was found to be 138.8 mg/kg (Qinghaosu Antimalaria Coordinating Research Group, 1979). The ED_{50} for artemether and arteether in *P. berghei*-infected mice is 55 mg/kg (Lin *et al.*, 1987, 1989). Artemether and arteether appear to be 2 to 3 times as potent as artemisinin *in vivo* as well as *in vitro*. Artelinic acid has been also been demonstrated to be a more effective treatment for *P. berghei* in infected mice than artemisinin (Titulaer *et al.*, 1993). Other studies, in which mice were given artemisinin *i.m.* as a suspension in water or oil, demonstrated that oil suspension is a more effective approach with potency comparable to chloroquine (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982d).

Aotus monkeys infected with *P. falciparum* were effectively treated with artemether and arteether, the ED_{50}'s being 7.1 mg/kg for artemether and 11.8 mg/kg for arteether (Shmuklarsky *et al.*, 1993). Monkeys infected with *P. cynamolgi* sporozoites and administered artemisinin at 2000 mg/kg through a gastric tube for 3 days were aparasitaemic on the third day. However, parasitaemia recurred in 5 of the 7 animals, despite artemisinin administration being continued after parasitaemia had apparently cleared (Qinghaosu Antimalaria Coordinating Research Group, 1979). Similar results were seen in chickens infected with *P. gallinaceum* and

treated with 200 mg/kg artemisinin daily for 6 days, where parasitaemia redeveloped on days 8 and 9. Recrudescence of infection is also a problem in a clinical setting (section 1.5).

Recent studies in mice have demonstrated the efficacy of artemisinin against chloroquine, primaquine, cycloguanil, mefloquine, menoctone, pyrimethamine and sulphonamide resistant strains of *P. berghei* (Peters *et al.*, 1986). In addition, there is potentiation between artemisinin and pyronaridine when used in combination in mice infected with *P. yoelii* (Peters & Robinson, 1997). However, resistance to artemisinin has been observed experimentally. Curing mice infected with the chloroquine-resistant RC *P. berghei* strain, which does not synthesise haemozoin, requires fifty times the dose of artemisinin needed to cure mice infected with the haemozoin producing N-strain (Peters *et al.*, 1986). Artemisinin-resistance in strains of *P. yoelii* has also been observed in mice. Nevertheless, in over 20 years of clinical use of the drugs, there has only been one report of any resistance to the drugs in man. A *P. falciparum* strain isolated from a patient in Mali showed a slight decrease in sensitivity to the drugs (Gay *et al.*, 1994). There have been no reports of resistance that could be considered *clinically* significant.

1.4.4. Gametocytocidal activity

The activity of the artemisinin derivatives against the erythrocytic schizont stage of malaria is well known but they also possess significant gametocytocidal activity. This gametocytocidal activity was first demonstrated in Rhesus monkeys infected with *P. cynomolgi* and treated with artemisinin, sodium β-artelinate and

arteether (Bajpai *et al.*, 1989; Dutta *et al.*, 1990; Tripathi *et al.*, 1996). It was also reported that the artemisinin derivatives lack sporontocidal action (Dutta *et al.*, 1990). The findings of these studies have been confirmed both *in vitro*, against *P. falciparum* (Kumar & Zheng, 1990), and in clinical studies using artemisinin which reported that artemisinin reduced the gametocyte count of patients suffering from *P. falciparum* and reduced the infectivity of the gametocytes to mosquitoes (Chen *et al.*, 1994). In a large prospective study on the Thai-Burmese border, involving 5200 patients over 5 years, it was demonstrated that *P. falciparum* gametocyte carriage from man to mosquito was significantly reduced by artemether and artesunate (Price *et al.*, 1996). Both derivatives are up to eight times more effective at preventing gametocyte transmission than mefloquine, halofantrine or quinine. The artemisinin derivatives do not kill mature gametocytes. Instead they prevent gametocyte development by attacking immature gametocytes and the young and more mature asexual stage parasites. Remarkably, since the introduction of the standard artesunate / mefloquine combination treatment (section 1.5) into the study area the incidence of *P. falciparum* malaria has dropped by 50%. The implications of this for control of malaria are profound. Indeed, it has subsequently been suggested that the artemisinin derivatives should be introduced into areas where multidrug resistance is emerging rather than wait for existing therapies to lose their efficacy.

1.5. CLINICAL STUDIES

1.5.1. Clinical efficacy of the artemisinin derivatives

The first clinical trials of artemisinin took place in China over 5 years in the mid-1970s and demonstrated that artemisinin was a very efficacious treatment for both *P. falciparum* and *P. vivax* malaria (Qinghaosu Antimalaria Coordinating Research Group, 1979; China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982f). Since then artemisinin and its derivatives, most commonly artemether and artesunate, have been successfully used in a variety of formulations to treat both uncomplicated and severe malaria around the globe. In China they are now the main treatment for *falciparum* malaria and their use is widespread throughout southeast Asia where multidrug resistant *P. falciparum* infections have become common. The results of the published clinical studies are summarised on table 1.1.

Parasite clearance is the measure of efficacy of an antimalarial drug in cases of uncomplicated malaria. Taken as a whole, it can be seen from the results of these studies that the artemisinin derivatives induce an immediate decrease in parasitaemia and cure malaria more rapidly any of the other antimalarial drugs. This decrease in parasite clearance time, approximately 20 h or 30% total parasite clearance time (PCT) compared with other drugs, is the most impressive feature of the artemisinin derivatives and is probably a result of higher clearance of ringform-infected erythrocytes. In cases of uncomplicated malaria *i.v.* and oral artesunate are equally effective, and reduce parasitaemia extremely rapidly. However, oral artesunate or

Table 1.1. Summary of treatment studies in *P. falciparum*

Country, study	Patient group	No.	Drug Regimen	FCT (h)	PCT (h)	CC rate (%)	RC rate (%)
Uncomplicated *P. falciparum* malaria							
Artemisinin, Oral							
China, a		10	M 1000 mg	31	103		
		10	ATM 1000 mg at 0 & 24 h	42	104		
		10	ATM *im* 600 mg at 0 h, 300 mg at 24 & 48 h	34	79		
		10	Q 500 mg tid x 3 d	22	68		
China, b	9-57 y	20R	ATM 1000 mg + M 750 mg SD	24	82	100	100(90)[a]
		20	ATM 1000 mg + P-S 75 mg-1500 mg	22	60	100	100
		20	M 750 mg + P-S 75 mg-1500 mg	14	55	100	100
		20	ATM 1000 mg at 0 h, 500 mg at 24 & 48 h	16	45	100	65 (50)[a]
Tanzania		20R	ATM 500 mg day 1, 250 mg bid 4 d, 500 mg day 6	22*	31*	100	65
		20	ATS 100 mg day 1, 50 mg bid 5 d	19	26	100	80
Vietnam		59R	Q 10 mg/kg tid 7 d	41	66	98	84
		45	ATM 20 mg/kg + Q 10 mg/kg tid 3 d	34	43	100	72
		53	ATM 20 mg/kg + D 4 mg/kg od 3 d	31	41	94	33

Artemisinin, Rectal

Vietnam, a		32Ra	ATM 600 mg at 0 & 4 h,400 mg at 24, 32, 48, 56 h	30	42	100	40
		30	Q 500 mg tid 14 d	39	68	90	63
Vietnam, b	Children	30Ra	ATM adapted to age	54	26	100	2/6
		30	Q 10 mg/kg tid 10 d	54	43	100	2/6

Artesunate

Tanzania	25Ra	ATS *iv* 1.6 mg/kg stat, 0.8mg/kg bid 4 d	21	24	100	84
	25	ATS *po* 100 mg stat, 50 mg bid 4 d	19	23	100	80
Thailand, a	6	ATS *po* 200 mg stat & at 12 h, 100 mg bid 4 d	42	31	100	100[b]
	10	ATS *po* 100 mg stat & at 12 h, 50 mg bid 4 d	24	27	100	90
	6	AEM *im* 160 mg stat, 80 mg od 4 d	33	29	100	100
	5	ATS *iv* 120 mg stat, 60 mg at 4, 24, 48 h	26	34	100	20
	5	ATS *po* 100 mg tid 2 d	27	28	100	0
	5	ATS *po* 200 mg at 0, 12, 24 h	39	29	100	0
	21	ATS *po* 200 mg stat, 50 mg tid 5 d	24	28	100	95
	5	ATS *po* 200 mg + Cl 1500 mg over 48 h	21	31	100	0
	5	ATS *po* 200 mg + P-S	24	31	100	0
Thailand, b	43Ra	ATS *po* 200 mg stat, 100 mg od days 1, 4, 5, 6	20	40	100	93[b]
	46	ATS *po* 200 mg stat, 100 mg od 4 d	29	40	100	85
Thailand, c	25Ra	ATS *po* 200 mg stat, 100 mg od 4 d	20	40	100	72[b]
	25	ATS *po* 100 mg stat & 12 h, 50 mg bid 4 d	28	40	100	76

Thailand, d		42Ra	ATS po 100 mg stat, 50 mg bid 5 d	35	36	100	88[b]
		43	M 750 mg stat, 500 mg at 6 h	70	64	100	81
		42	ATS 100 mg stat, 50 mg bid 5 d + M 1250 mg	38	38	100	100
Thailand, e	Recrud-escence	24	ATS 100 mg stat, 50 mg bid 2 d + M 1250 mg	33	40	100	100[b]
Thailand, f		54Ra	M 1250 mg + D 200 mg od 7 d	64	69	100	96[b]
		55	ATS 100 mg stat, 50 mg bid 2 d + D 200 mg od 7 d	39	41	100	80
Thailand, g		32Ra	ATS 200 mg stat, 100 mg at 12 h, 100 mg od 4 d			100	94
		34	ATS 200 mg stat, 100 mg at 12 h, M 750 mg SDo at 24 h			100	79
Thailand, h	Children & adults	146Ra	M 25 mg/kg TDo	3.3 d*	1.5 d*	94	78
		151	ATS *po* 4 mg/kg + M 25 mg/kg TDo	2 d	1.1 d	99	83
		169	M 25 mg/kg TDo	3.3 d	2.6 d*	83[c], 96[d]	61[c], 83[d]
		179	ATS *po* 4 mg/kg stat, 2 mg/kg od or 1 mg/kg bid 2 d + M 25 mg/kg TDo	1.6 d	1.2 d	100	98[c,d]
Thailand, i		182Ra	ATS 4 mg/kg od 3 d + M 25 mg/kg SDo	1.6 d*	1.9 d*	100*	86(91)[e]*
		180	AEM 4 mg/kg od 3 d + M 25 mg/kg SDo	1.6 d	1.9 d	100	88(92)[e]
		178	M 25 mg/kg SDo	1.7 d	2.6 d	86	51(61)[e]

Artemether							
Gambia	Children	15	AEM *im* 4 mg/kg stat, 2 mg/kg od ≤24 h until Cl *po* 25 mg/kg TDo	19.3 (8.5)[i]	37*		
		15	Cl *im* 3.5 mg/kg qid ≤24 h until Cl *po* to 25 mg/kg	10.7 (8.4)[i]	48		
Thailand, a		34Ra	AEM *im* 200 mg stat, 100 mg at 6 h, 100 mg od 4 d M 1250 mg TDo	34*	30	100	97
		12	M 1250 mg TDo	64	27	100	73
Thailand, b		30Ra	AEM *po* 300 mg + M 1250 mg TDo	40	31	100	96
		27	AEM *po* 300 mg + M 750 mg SDo	37	34	100	75
Arteether							
India		51	α,β-AEE *im* 150 mg od 3 d	52	2d	100	86
Dihydroartemisinin							
Thailand		53	DHA *po* 120 mg stat, 60 mg od 6 d	37	40	100	90

Severe / complicated malaria

Gambia, a	Severe; children	21Ra	AEM *im* 4 mg stat, 2 mg/kg until Cl PO 25 mg/kg TDo	30(9)[i] (16)[f]	16	90[g]	
		22	Cl *im* 3.5 mg/kg qid until Cl *po* to 25 mg/kg TDo	30(10)[I] (18)[f]	18	73	
	Cerebral; children < 10 y	288Ra	AEM *im* 3.2 mg/kg stat, 1.6 mg/kg od 4 d (P-S 1.25 mg/kg - 25 mg/kg) SDo	30(26)[f]*	48	79[g]	97[j]
Gambia, b		288	Q *im* 20 mg/kg stat, 10 mg/kg bid 5 d + P-S 1.25 mg/kg - 25 mg/kg SDo	33(20)[f]	60	78	95
Malawi	Cerebral; children	28Ra	AEM *im* 3.2 mg/kg stat, 1.6 mg/kg until *po* P-S 1.25 mg/kg - 25 mg/kg SDo	(8)[f]	28	89[g]	80[h]
		37	Q *iv* 20 mg/kg tid until *po* P-S 1.25 mg/kg-25 mg/kg	(14)	40	89	93
Myanmar, a	Severe	30	AEM *im* 200 mg stat, 100 mg bid 2 d + M 750 mg SDo	40*	40*	100	
		25	Q *iv* 10 mg/kg tid 7 d	63	65	92	
Myanmar, b	Severe	31	AEM *im* 200 mg stat, 100 mg bid 2 d	31	38	100	60
		31	Q *iv* 10 mg/kg tid 7 d	47	79	94	91

Myanmar, c	Severe	50	AEM *im* 200 mg stat, 100 mg bid 2 d + M 1000 mg SDo	42*(37)[f]	27	86[g]	100
		24	ATS *iv* 120 mg stat, 60 mg bid 2 d + M 1000 mg SDo	31(34)[f]	42	92	100
		67	Q *iv* then *po* 600 mg tid 10 d + T 250 mg qid 7 d	42(43)[f]	47	77	88
Myanmar, d	Severe	30	ATS *pr* 200 mg 0, 4, 8 & 12 h then bid 2 d + M 1250 mg TDo	71	50	100	92
Nigeria, a	Moderate; children	54Ra	AEM *im* 1.6 mg/kg bid 3 d	27	40	100	15
		51	P-S *im* 33 mg/kg - 1.65 mg/kg	34	53	98	0
	Cerebral; children	25	AEM *im* 3.2 mg/kg stat, 1.6 mg/kg od 4 d	39(40)[f]	47	88	4
		29	Q *iv* 20 mg/kg stat, 10 mg/kg tid 7 d	37(37)[f]	58	79	0
Nigeria, b	Cerebral; children	25Ra	AEM *im* 3.2 mg/kg stat, 1.6 mg/kg od 4 d	47(40)[f]	39	40[g]	86[h]
		29	Q *iv* 20 mg/kg stat, 10 mg/kg tid 7 d	58(37)[f]	37	37	91
Nigeria, c	Severe; noncerebral; children		AEM *im* 3.2 mg/kg stat, 1.6 mg/kg od 4 d	19	35	100	
	Cl resistant		AEM *im* 3.2 mg/kg stat, 1.6 mg/kg od 4 d	16	36	100	
Kenya	Cerebral; children	89R	AEM *im* 3.2 mg/kg stat, 1.6 mg/kg od ≤2 d + P-S 1.25 mg/kg - 25 mg/kg SDo	32(12)[f]	40	80[g]	
		71	Q *iv* 20 mg/kg stat, 10 mg/kg tid ≥1 d + P-S 1.25 mg/kg - 25 mg/kg SDo	32(13)	48	89	

Thailand, a	Uncompli-cated; hyperpar-asitaemia	30Ra	ATS *po* 4 mg/kg od 3 d + M 25 mg/kg SDo	19*	36*	100	70*
		30	Q *iv* 20 mg/kg stat, 10 mg/kg 8 & 16 h + M 25 mg/kg SDo	47	82	100	39
Thailand, b	Severe	13Ra	AEM *im* 300 mg stat, 100 mg od 4 d	85	52	100	38
		15	AEM *im* 160 mg stat, 80 mg od 6 d	68	60	100	73
Thailand, c	Severe	47Ra	AEM *im* 160 mg stat, 80 mg od 6 d	79(40)[f]	54*	87[g]*	
		50	Q *iv* 20 mg/kg stat, 10 mg/kg tid 7 d	84(48)[f]	78	63	
Vietnam, a	Cerebral	31Ra	ATS *iv* 60mg stat, & at 4, 24 &48h + 500mg SDo	39(69)[f]	28	84[g]*	
		18	ATM *pr* 600 mg stat & at 4 h, 400 mg at 24, 32 48 & 56 h + M 500 mg SDo	77(56)[f]	38	72	
		30	Q *iv* 500 mg tid 14 d	78(58)[f]	51	73	
Vietnam, b	Severe	14Ra	ATS *iv* 2 mg/kg stat, 1 mg/kg at 12 & 24 h + M 500 mg SDo	33	29	93[g]	
		14	ATS *iv* 2 mg/kg stat, 1 mg/kg at 12 & 24 h + M 500 mg SDo				
Vietnam, c	Severe	284Ra	AEM *im* 4 mg/kg stat, 2 mg/kg tid > 72 h until *po*	126(77)[f]*	72*	87	
		276	Q *im* 20 mg/kg, 10 mg/kg tid > 72 h until *po*, *po*(both regimens) = M 15 mg/kg or Q 10 mg/kg tid 7 d	90(48)	90	83	

(from de Vries & Dien, 1996)

a: *P. vivax* included; **b:** no reinfection possible; **c:** adults; **d:** children; **e:** adjusted for reinfection; **f:** coma recovery time; **g:** survival; **h:** survival without neurological sequelae; **i:** time to first temperature below 37.5°C; **j:** proportion of patients afebrile at 24/48 h or with negative bloodsmear at 24 h.

Abbreviations and symbols: ATM = artemisinin; AEE = arteether; AEM = artemether; ATS = artesunate; bid = twice daily; Cl = chloroquine; CC = clinical cure, defined as initial recovery with complete initial disappearance of parasites; D = doxycycline; DHA = dihydroartemisinin; FCT = fever clearance time, usually defined as normalisation of temperature for 24 consecutive hours; *im* = intramuscular; *iv* = intravenous; M = mefloquine; od = once daily; PCT = parasite clearance time usually defined as time to first consecutive blood smears; *po* = oral; *pr* = rectal; P-S = pyrimethamine + sulphadoxine; Q = quinine; qid = four times daily; Ra = randomised allocation of treatment; RC = radical cure defined as clinical cure without recrudescence by day 28; SDo = single dose; stat = initial dose; TDo = total dose; tid = three times daily; * = significant difference.

oral artemether used in combination with mefloquine are as effective as artesunate monotherapy and are also associated with much lower recrudescence rates (Hassan Alin *et al.*, 1995; Bunnag *et al.*, 1996).

A patient is regarded as cured of malaria if they become aparasitaemic within 7 days of commencing treatment and remain so for 28 days. In most cases treated with artemisinin derivatives there is complete clearance within 48 h of beginning treatment. However, recrudescence rates for patients treated with artemisinin derivative monotherapy are high compared to other antimalarial drugs. The length of treatment, rather than total dose, seems to determine the recrudescence rate. The longer treatment is continued after initial clearance of parasites and clinical recovery, the lower the recrudescence rates are: the rate of recrudescence after 3 days of treatment with artemisinin derivatives can be up to 50%; if treatment is continued for 5 days then the recrudescence rate is approximately 10%. This is presumably due to the rapid elimination of the drugs from the body as the recrudescence rate for a 3 day treatment with arteether, which is eliminated much more slowly from the body than the other derivatives (section 1.5.2 below), is only 14% (Mishra *et al.*, 1995).

This problem has been overcome by using the artemisinin derivatives with other antimalarial drugs. A short course of treatment with an artemisinin derivative is followed by, or combined with, treatment with a second agent. Artemisinin derivatives have been combined with mefloquine, tetracycline, doxocycline, quinine and pyrimethamine-sulphadoxine. An artemisinin derivative plus mefloquine is the most widely used combination.

Recrudescence is not the primary concern in the treatment of severe / complicated malaria. The main aim of treatment in these cases is survival of the

patient rather than clearance of parasites. Again, artemisinin and its derivatives have proven to be highly effective against severe and complicated malaria. Recently published studies suggest that they are at least as effective as. quinine (Karbwang *et al.*, 1995; van Hensbroek *et al.*, 1996; Murphy *et al.*, 1996; Hien *et al.*, 1996; table 1.1). In each study the artemisinin derivatives reduced parasitaemia more rapidly than quinine. However, this was not always associated with a lower mortality; indeed in one study *i.v.* quinine was a more effective treatment than artemether (Murphy *et al.*, 1996). The clinical significance of this is not clear. One explanation is that in cases of complicated malaria the pathological effects are thought to be caused by sequestration of parasitised erythrocytes into the microvasculature of vital organs. Consequently it is not the parasites in circulation, whose removal is measured and used to calculated PCT's, that are responsible for the organ damage and high mortality associated with complicated malaria.

As a result of these clinical studies (table 1.1) some generalised guidelines for the treatment of malaria with the artemisinin derivatives have been suggested. Uncomplicated malaria should be treated with a three day course of an artemisinin derivative followed by mefloquine to prevent recrudescence. A commonly used dosing regimen for adults and children over 6 months is 4 mg/kg oral artesunate for 3 days combined with 25 mg/kg mefloquine split into two doses (15 mg/kg and 10 mg/kg) (White, 1994c). Artemisinin derivatives should be taken for at least 5 days when not combined with a second agent to ensure radical cure. In cases of complicated or severe malaria 2 mg/kg *i.v.* or *i.m.* artesunate followed by 1 mg/kg daily until oral treatment can begin is recommended (Looareesuwan *et al.*, 1994);

i.m. artemether, 3.2 mg/kg initially followed with 1.6 mg/kg until oral treatment can begin has also been used successfully (White, 1994c).

1.5.2. Human pharmacokinetics

The pharmacokinetics (and pharmacodynamics) of these drugs are not well understood, as reliable and reproducible methods for measuring artemisinin, its derivatives and metabolites in the body have proved difficult to develop. The difficulties in measurement of these compounds arises because they are all thermally labile and lack ultraviolet absorbent or fluorescent chromophores (Edwards, 1994). Derivatisation of the molecules to make them amenable to ultraviolet high performance liquid chromatography (HPLC) reduces the specificity (Titulaer *et al.*, 1990; Thomas *et al.*, 1992). Post-column alkali or acid decomposition of products to ultraviolet light absorbing compounds allows selective determination of products but reduces sensitivity. This reduction in sensitivity is not serious as plasma concentrations of the drugs are considerably higher than the detection limit (Hassan Alin *et al.*, 1996). However, HPLC-electrochemical detection (ED) is both a sensitive and specific assay for the detection of artemisinin derivatives and is instead widely regarded as the best technique for pharmacokinetic studies (Duc *et al.*, 1994; Teja-Isavadharm *et al.*, 1996a), unfortunately it is both difficult and expensive (Edwards, 1994). A bioassay has been used in conjunction with HPLC-ED (Teja-Isavadharm *et al.*, 1996a, 1996b); it lacks specificity but is extremely sensitive and is useful in pharmacodynamic studies.

The difficulties in development of easily reproducible assay methods has meant that there has been a lack of published pharmacokinetic data. Consequently, all the dosing regimens used have been derived from pharmacodynamic measurement of fever and parasite clearance, and this means that they may not be optimal ones.

Artemisinin. Several studies have been performed in healthy volunteers and patients suffering from malaria. The usual route of administration was oral with a total dose of 400-500 mg. The drug was also administered by *i.m.* injection and in suppository form. Orally artemisinin is poorly absorbed, the bioavailability following oral administration is only 32% compared to that following *i.m.* injection of artemisinin suspended in oil (Titulaer *et al.*, 1990), consequently it undergoes extensive first pass metabolism. Overall, following oral administration, the maximum plasma concentrations (C_{max}), reached range from 116 μg/L to 588 μg/L, and reached this level (t_{max}) in 1 to 3.1 h, and the $t_{½e}$ ranged from 1.9 to 4.3 h. Elimination is rapid and can be described by a first order, one compartment model (Duc *et al.*, 1994). The amount of unmetabolised drug excreted is negligible (de Vries & Dien, 1996). Suppositories administered to healthy volunteers and patients, doses of 10 to 15 mg/kg, produced peak concentrations which were lower than with oral administration and which took longer to attain C_{max}= 40-180 μg/L, t_{max}= 4.6-11.3 h, and $t_{½e}$= 4.0-4.8 h. There was little difference between the pharmacokinetic parameters of volunteers and patients regardless of route of administration (Titulaer

et al., 1990; Duc *et al.*, 1994; Hassan Alin *et al.*, 1996; de Vries & Dien, 1996; Benakis *et al.*, 1997).

Artemether. Oral artemether, administered to healthy volunteers and patients suffering from uncomplicated malaria (Thomas *et al.*, 1992; Na Bangchang *et al.*, 1994) is, as with artemisinin and other derivatives, poorly absorbed and rapidly eliminated. Malaria has no effect on the pharmacokinetics (Na Bangchang *et al.*, 1994) of an dose of 200-300 mg the drug, with C_{max}= 118-231 μg/L, t_{max}= 1.7-3.0 h, and $t_{½e}$ = 2.6-4.2 h. 5 mg/kg artemether administered to volunteers *i.m.* and *p.r.* in arachis oil reaches C_{max}= 175 μg/L with t_{max}= 8.7 h for *i.m.* dosage, and C_{max}= 75μg/L and t_{max}= 3.1 h for *i.r.* administration (Teja-Isavadharm *et al.*, 1996a). Absorption was too slow and erratic to obtain precise $t_{½e}$ values.

Artemether demethylation is slower than artesunate hydrolysis and so artemether as well as the metabolite dihydroartemisinin contribute significantly to the pharmacological effect (White, 1994b). A recent study (Teja-Isavadharm *et al.*, 1996a) assayed the levels of both artemether and dihydroartemisinin in healthy volunteers following a 5 mg/kg dose of the parent drug, administered orally, *i.m.* and *p.r.* It was demonstrated that the C_{max} of dihydroartemisinin was much greater than that of artemether following oral administration but considerably lower than the C_{max} of artemether following *i.m.* and *p.r.* administration, indicating that artemether undergoes extensive first pass metabolism.

Arteether. Although the structures and activities of artemether and arteether are very similar, studies on healthy volunteers have revealed that there are important pharmacokinetic differences. When arteether was administered by *i.m.* injection, suspended in oil, $t_{½e}$= 23 h for a single dose, and $t_{½e}$= 69 h for a multiple dose regimen, longer than that of artemether ($t_{½e}$ = 2.6 h for single oral administration). Elimination can be described as a two-compartment, first order elimination model. The $t_{½e}$ of arteether is the longest of all the artemisinin derivatives and can lead to a marked accumulation in multiple dose studies (Kager *et al.*, 1994). This could be useful for treatment of cerebral malaria as its lipophilicity also means that high levels may accumulate in the brain.

Artesunate. Artesunate is essentially a prodrug for dihydroartemisinin. Artesunate was administered to Vietnamese patients with severe malaria *i.v.* and *i.m.*, 2 mg/kg, and the levels of both artesunate and dihydroartemisinin were assayed. The C_{max} following *i.v.* administration is 2640 μg/L and 2020 μg/L for artesunate and dihydroartemisinin, which are reached very rapidly as $t_{½e}$= 23 min and 95 min respectively. Following *i.m.* administration C_{max}= 510 μg/L and 390 μg/L for artesunate and dihydroartemisinin respectively. Oral administration of 200 mg artesunate to healthy volunteers has a C_{max}= 120 μg/L for artesunate after 15 min and C_{max}= 570 μg/L after 45 min for dihydroartemisinin (Benakis *et al.*, 1997); oral administration of artesunate so reduces the availability of the parent drug that further pharmacokinetic data is not available. Pharmacokinetic data is extremely scarce with no data concerning the extent or rate of absorption. Hydrolysis proceeds so quickly

that when the drug is given orally it is hydrolysed to dihydroartemisinin almost entirely before it enters circulation and so may be considered a prodrug (Benakis *et al.*, 1993; Barradell & Fitton, 1995; de Vries & Dien, 1996; Benakis *et al.*, 1997).

Dihydroartemisinin. Despite being the active metabolite of most of the artemisinin derivatives and therefore making a considerable contribution to the activity of these drugs, there has only been one study performed to determine the pharmacokinetics of dihydroartemisinin itself. A dose of 2.2 mg/kg was administered orally to healthy volunteers and assaying plasma samples revealed that C_{max}= 710 μg/L, t_{max}= 1.33 h and $t_{½e}$= 1.57 h. Dihydroartemisinin has not been used clinically and is unlikely to be so in the future (de Vries & Dien, 1996).

Generally, the artemisinin-related compounds are erratically absorbed, quickly metabolised, widely distributed (passing readily through the blood / brain and blood / placenta barriers), and eliminated rapidly. In each case the active metabolites of the drugs contribute significantly to the antimalarial effects. However there is little information on interaction with other drugs in a clinical setting, changes in the plasma concentrations in children, or patients with vital organ dysfunction (except a solitary report stating that cirrhosis of the liver does not alter artemisinin pharmacokinetics). Dihydroartemisinin seems to be the main metabolite of the artemisinin derivatives, and one study also identified desoxyartemisinin, deoxydihydroartemisinin, and dihydrodihydroartemisinin as metabolites also, all of which were inactive. There are probably many more hydroxylated and methylated

metabolites also being produced but how much each of these metabolic pathways contribute to metabolism in humans is not known at present.

1.6. TOXICITY

1.6.1. Toxicity of sesquiterpene lactones

Artemisinin and its derivatives are part of a large family of compounds called sesquiterpene lactones that are derived from plants, particularly from the Compositae genus (Rodriguez *et al.*, 1976). Sesquiterpene lactones are colourless, bitter constituents of plants that are synthesised from *trans, trans* farnesyl pyrophosphate (Geissman, 1973). All contain a basic terpene skeleton and a lactone group (Rodriguez *et al.*, 1976), many also possess an epoxide ring or hydroxyl groups. Terpenes are synthesised from isoprene fragments and are categorised on the number of double isoprene units they contain. A sesquiterpene (sesqui = one and a half) therefore is a molecule that contains 15 carbon atoms. Artemisinin is not the only medicinally useful compound to have been discovered in this family of compounds. A sesquiterpene endoperoxide with antimalarial activity has also been isolated from the tubers of *Cyperus rotundus* (Thebtaranonth *et al.*, 1995). Both parthenin, derived from *Parthenium hysterphorus* and eupatoriopicrin possess strong anti-tumour activity against mouse tumour cells *in vitro* (Kupchan *et al.*, 1971). Dehydroleucodin, derived from *Artemisia douglasiana* and ludortin, helenalin and mexicanin I all possess anti-ulcer activity in rats and helenalin also exert a positive inotropic effect on strips of guinea-pig heart (Ivie *et al.*, 1983; Takeya *et al.*, 1983).

Finally, parthenolide, the active constituent of *Tanacetum parthenium*, is widely used to treat migraines prophylactically in England and other areas of northern Europe under the name "feverfew". Their mechanisms of action are not clear though it seems that alteration of prostaglandin synthesis is important in some cases (Johnson *et al.*, 1985).

However, several studies have shown that some sesquiterpene lactones have neurotoxic effects in animals and humans. Ingestion of a constituent of *Cycas circinalis,* β-N-oxalloylamino-L-alanine, causes severe neurological disorders in the basal ganglia, cerebral cortex and spinal cord in monkeys and humans and is linked with the neurological syndrome Guam's Disease (Butterfield *et al.*, 1993). The highly active anti-feedant *Germacranolide angelates*, derived from the sunflower, when injected into adult rootworms caused symptoms similar to that of the gamma-aminobutyric acid (GABA)-gated chloride ion channel antagonist picrotoxin, with which it also shares certain structural similarities, suggesting a link between sesquiterpene toxicity and GABA (Mullin *et al.*, 1991). Chewing disease, a fatal progressive neurological disorder that afflicts horses and manifests itself initially as idle chewing and lack of facial muscular mobility has been linked with ingesting large amounts of *Centaurea repens* (Stevens *et al.*, 1990). The sequiterpene lactones of this plant were isolated and incubated with chick dorsal root ganglia and found to inhibit all cell neurite outgrowth significantly. The neurotoxic effects of solstitalin and cyanopicrin, derived from *Centaurea solstitalis*, the yellow star thistle have also been examined (Wang *et al.*, 1991). Both exhibit toxicity to cultured rat foetal brain

cells in a concentration-dependent manner, and may be implicated in neurological disorders.

Finally, Robles and co-workers (1995), also working on repin toxicity, suggested that these neurotoxic effects were mediated by the active moiety interacting with enzymes containing a sulphydryl group, inhibiting enzyme activities, metabolism, and glutathione (GSH). They demonstrated that repin was toxic to different cell lines and specifically to brain cells. Repin also depletes the levels of GSH in mice *in vivo* and *in vitro*. Depletion of GSH reduces protection from oxidative stress and so repin toxicity may be mediated by the depletion of intracellular GSH. Theories abound as to how these compounds act (GABA, GSH, *etc.*) but there is little hard evidence to support any of them and it may be that they do not act by the same or similar mechanisms. Consequently, the mechanism of artemisinin's pharmacological activity and toxicity may be unconnected to the mechanism of action of other sesquiterpene lactones.

1.6.2. Neurotoxicity of antimalarials

Chloroquine. Chloroquine is a 4-aminoquinoline and has a small therapeutic window. It is very toxic when the dose is high, indeed when it was developed in the 1930's, it was considered too toxic for human use (Coatney, 1963). There is a rapid onset of neurological effects and also respiratory and cardiovascular effects (Oakley, 1973). Neurological symptoms associated with chloroquine begin with dizziness, vomiting and headache and is followed by general CNS depression and visual disturbances. Furthermore, there are incidents of hyperexcitability and seizures

which are followed by circulation arrest and death in 35% of cases of serious toxicity. Less seriously, in those patients given the drug prophylactically, there have been a few reports of tonic-clonic seizures (Fish & Espir, 1988). It has been estimated that the incidence of serious chloroquine-induced neurological adverse events is about 1/5000 in patients treated with therapeutic levels of the drug. In most cases the symptoms are much milder and usually manifest themselves as headaches, sensory disturbances and psychiatric symptoms such as confusion, apathy and anxiety (Steffen *et al.*, 1993). The incidence of serious central nervous system (CNS) adverse effects following chloroquine prophylaxis is about 1/13600.

Amodiaquine. Amodiaquine is also a 4-aminoquinoline. Dizziness, headaches and visual impairments were reported in 22%, 6% and 20% respectively of amodiaquine users, compared with 5%, 0% and 75% of chloroquine users. Strangely, in patients with rheumatoid arthritis amodiaquine has a significantly higher incidence of CNS effects than chloroquine (Kersley & Palin, 1959). Another unusual symptom often seen in patients treated with amodiaquine is a protruding tongue (Akindele & Odejide, 1976). No adverse CNS effects have been reported in patients given amodiaquine prophylactically.

Mefloquine. Mefloquine, a 4-aminoquinoline, is also associated with adverse CNS effects. These are primarily headaches, dizziness and insomnia but in more severe cases, psychosis, toxic encephalography and acute brain syndrome are seen (Stuiver *et al.*, 1989). The incidence of these adverse effects ranges from 47% to 90% in adults and 57% to 61% in children taking the drug (Palmer *et al.*, 1993). 1/200

Westerners suffer adverse CNS drug reactions (Phillips-Howard & ter Kuile, 1995) and women seem more susceptible to these reactions (Bem *et al.*, 1992).

Quinine and Quinidine. Quinine and quinidine are alkaloids derived from the chinchona tree. The adverse effects of these drugs, "cinchonism", are well-defined: vomiting, nausea, tinnitus, deafness, headache, disturbed vision and vasodilation, though all of these symptoms are fairly mild at standard therapeutic doses (Boland *et al.*, 1985). High doses can lead to a permanent loss of hearing and sometimes seizure and death (Roche *et al.*, 1990). Quinidine is a more potent, and more toxic, drug than quinine and its toxicity is associated with psychiatric disorders (White, 1988; Deleu & Schmedding, 1987).

Mepacrine. Mepacrine has an acridine nucleus instead of a quinoline nucleus, and was originally synthesised when it was thought that chloroquine and quinine were too toxic for clinical use. However, it caused severe headaches and an increase seizures in those of that suffer from epilepsy and sometimes major psychiatric disorders (Hoops, 1935).

Artemisinin. The artemisinin derivatives dihydroartemisinin, artemether, arteether, sodium artelinate and sodium artesunate are also associated with clinical neurological defects and death in animals studies (Brewer *et al.*, 1993, 1994a, 1994b) and these will be discussed shortly.

Other regularly used antimalarials such as the sulphonamides, primaquine, halofantrine, proguanil and pyrimethamine are rarely associated with CNS adverse effects, and those that do occur at therapeutic levels are likely to be idiosyncratic reactions (Akinyanju *et al.*, 1973; Bloland *et al.*, 1991; Haas, 1985).

Adverse drug reactions in the CNS are dramatic and their repeated occurrence has influenced clinical opinion on the use of antimalarials. Mild CNS events such as headaches and dizziness and insomnia are commonly reported with all antimalarials at therapeutic doses, but as malaria also causes these symptoms it is difficult to assess the relative contribution of the drugs and malaria. Serious CNS events are usually documented following severe malaria (Sowunmi *et al.*, 1993). However in severe malaria drugs are used in higher doses, given via a more rapidly acting parenteral route, so the problem of establishing causality is still difficult. Even so the continued evolution of resistance of *P. falciparum* parasites necessitates the use of currently available drugs. Reports of toxicity therefore need to be interpreted with caution, and a greater understanding of the mechanism of this CNS toxicity gained so that informed decisions can be made on the continued use of these drugs.

1.6.3. Artemisinin toxicity

In vivo artemisinin toxicity studies. The toxicity of artemisinin, artemether and sodium artesunate have been tested on a number of different animal species and by several different routes of administration. In each case the artemisinin derivative has a considerably higher LD_{50} (dose lethal to 50% of the animals) value and therapeutic index than chloroquine. Artemisinin when administered orally to mice had an LD_{50}

of 4228 mg/kg and a therapeutic index of 384. These are higher than the therapeutic index and LD_{50} of chloroquine orally administered to mice which are 216 and 400 mg/kg. Furthermore, artemisinin suspended in oil and injected *i.m.* into mice gave an LD_{50} of 3840 mg/kg and a therapeutic index of 4987 (*i.m.* chloroquine: 63 mg/kg and 95 respectively); interperitoneal (*i.p.*) injection gave an LD_{50} of 1558 mg/kg, while subcutaneous injection of an aqueous suspension of the drug gave an LD_{50} of > 9000 mg/kg. In rats, oral administration of artemisinin had an LD_{50} of 5576 mg/kg whilst *i.m.* injection of artemisinin gave an LD_{50} of 2571 mg/kg (Qinghao Antimalaria Coordinating Research Group, 1979; China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e; Trigg, 1989). So regardless of route of administration, artemisinin seemed to be a remarkably non-toxic drug.

Having determined the lethality of the drugs, more detailed studies of the toxicity of artemisinin and its derivatives were performed. Investigations were carried out on groups of cats, dogs, rabbits, rats and mice, administered orally, *i.m.* and *i.p.* with artemisinin with doses ranging from 100-1600 mg/kg for 1-7 days. Dogs given a single dose of 400 or 800 mg/kg, administered *i.m.*, showed reduction in the reticulocyte count, liver function abnormalities and also immediate but short-lived CNS disturbances (*e.g.* tonic-clonic convulsions). These were caused by transient mild fatty acid degeneration in the liver and mild oedema of the brain tissue (Qinghaosu Antimalaria Coordinating Research Group, 1979). The symptoms and toxicity seen were entirely reversible and in addition there were no noticeable abnormalities of urine, renal function or electrocardiogram (ECG).

Symptoms of acute toxicity in animals were: tremor, incoordination, slow respiration and disappearance of the righting reflex. The toxic effects on the CNS in this study were not observed in smaller animals, but in large animals, *e.g.* rabbit, cat dog, pigeon, tonic-clonic seizures were observed. In those animals that died during the study respiration always preceded cardiac arrest. In rats given 600 mg/kg for 7 days there were slight degenerative changes in the heart, liver, lung, kidney and spleen (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e). Generally, rats were the species that were most tolerant of the drug while pigeons were the least.

Monkeys administered *i.m.* artemisinin for 14 days with doses up to 192 mg/kg/day developed profound inhibition of the haemopoietic cells of the bone marrow, more wide ranging than just a reduction in reticulocyte count, and vacuolar degeneration of the liver parenchyma at doses as low as 96 mg/kg/day. Most seriously, cardiac toxicity resulting in the death of three quarters of the monkeys occurred. Extensive degeneration of myocardial cells was noted (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e; Wang & Liu, 1983).

The toxicity of the artemisinin analogues artemether and sodium artesunate were subsequently examined by Chinese researchers. The LD_{50} for *i.m.* and *i.v.* sodium artesunate in mice are 475 mg/kg and 699 mg/kg respectively, the chemotherapeutic index for *i.v.* administration of sodium artesunate is 1733. Artemether was the most toxic artemisinin derivative tested by this group with an *i.m.* LD_{50} in mice of 263 mg/kg, although it should be noted that the chemotherapeutic index is 447. Following *i.v.* administration of high doses of

artesunate to rabbits, toxic effects were seen in the cardiovascular system, but these were less than those seen with comparable doses of chloroquine, indeed no toxicity was seen until the accumulated dose of artesunate reached 300 mg/kg and cardiac arrest did not occur until it reached 1200 mg/kg (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e). Other toxicity studies of artesunate demonstrated that even at 50 times the clinical dose (100 mg/kg, either *i.p.* or *i.v.*) there was no toxic effects on rodents or rabbits (Trigg, 1989). At higher doses some adverse effects did appear: sedation, reduced tolerance to a lack oxygen, analgesia, tremor, convulsions, muscle relaxation and disturbance of gastrointestinal activity and some fatty acid degeneration of liver. Artemether toxicity testing revealed only mild degeneration of liver cells. Artemether and artesunate effects on the haemopoietic system were not reported.

Murine bone marrow polychromatic erythrocyte micronucleus and Ames Tests failed to reveal any mutagenic activity of artemisinin and it has also been demonstrated to lack carcinogenicity (Trigg, 1989). However, when 25 mg/kg/day artemisinin was administered to pregnant mice and rats in the middle and late stages of gestation it was found to be embryotoxic, particularly in the middle and late stages of gestation, and at doses 1/200 of the LD_{50} value all embryos were reabsorbed (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e).

Although a wide range of toxic effects induced by artemisinin and its derivatives were demonstrated in animals it should be stressed that virtually all of the toxicity reported in animal toxicity testing occurred at doses far in excess of those that would be used therapeutically and with longer periods of administration,

and in most cases where toxicity was not fatal the damage was reversible (Luo & Shen, 1987).

Recent in vivo neurotoxicity studies. The Chinese animal toxicity tests and years of widespread clinical use in southeast Asia without any apparent toxicity suggested that these drugs were extremely safe. However, in 1993, studies were performed by a research group at the Walter Reed Army Institute of Research (Brewer *et al.*, 1993, 1994a, 1994b) uncovering severe neurotoxicity in dogs and rats caused by the artemisinin derivatives.

In this study dogs were administered 20 mg/kg/day arteether *i.m.* for 8 days. A progressive syndrome of neurological defects and cardiorespiratory collapse occurred, and 5 out of 6 dogs died after total doses as low as 120 mg/kg. Dogs administered 15 mg/kg/day arteether *i.m.* for 28 days also developed neurological symptoms, neuropathological lesions and ECG abnormalities. In rats that were dosed with *i.m.* arteether and artemether at 12.5 to 50 mg/kg/day for 28 days neurological abnormalities were noted in the rats after 6-14 days at doses as low as 25 mg/kg/day. Neurological defects and abnormalities included loss of pain response reflexes, loss of spinal reflexes and loss of brainstem and eye reflexes, along with spasticity, gait disturbances. Animals that died during the study displayed shallow respiration, which became progressively depressed, until respiratory and cardiac arrest intervened. Pathological examination of dog and rat brains revealed similar dose-related patterns of damage. There was scattered neuronal degeneration and necrosis, increased eosinophilia, vacuolisation of cytoplasm with the loss of Nissl substance, swelling of nerve cell bodies and swelling and fading of nuclei and general damage

to the motor, vestibular and auditory systems. Neuropathological lesions were apparent in rats given 5 mg/kg/day for 28 days, but these did not obviously manifest themselves behaviourally. The neuropathological lesions were dose-related and sharply confined to the pons and medulla, which largely consists of myelinated axons and their associated glial cells. There was no significant difference in the type or extent of damage caused by arteether and artemether. Similar damage was also caused by dihydroartemisinin, sodium artesunate and sodium artelinate (Brewer *et al.*, 1993).

There have subsequently been other reports of *in vivo* toxicity. In a separate study, a similar pattern of lesions was observed in rats although at the higher dose of 50 mg/kg/day arteether, total dose 250 mg/kg (Kamchonwongpaisan *et al.*, 1997).

Neurological effects have also been demonstrated in *Macaca mulatta* monkeys (Petras *et al.*, 1994). The monkeys were given 6-24 mg/kg/day arteether for 14 days. Fine motor tremors with nystagmus and diffuse piloerection were observed in those receiving 24 mg/kg/day. However, neuropathological damage was also observed in the lower dose groups without any of the accompanying behavioural changes noted in the higher dosage groups. The dose-related neuropathological lesions seen in the brain conformed to the pattern observed in the dogs and rats studied by Brewer and colleagues.

Behavioural studies were performed on rats to develop a screening technique for early warning of artemisinin toxicity, and 12.5 mg/kg/day arteether was administered for 17 days. However, no behavioural deficits occurred during this period but neuropathological lesions were noted on examination of the brain (Genovese *et al.*, 1995).

The implications of these findings with respect to possible neurotoxicity in man are discussed below.

Artemisinin toxicity in humans. Very little toxicity has been reported in humans (Hien & White, 1993). Data on the effects of the drugs on pregnant women is scarce, but the documented cases of pregnant women receiving the drugs reported no abnormalities in the pregnancy or the children. There have been reports of local pain and tenderness around the injection site when the drugs are injected intramuscularly (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e), but less than quinine. Cardiotoxic effects have also been noted *e.g.* transient first degree in a small number of patients receiving artesunate and a prolongation of the QT interval in 25% of patients with severe malaria receiving high doses of artemether (Hien *et al.*, 1996). In healthy volunteers administered 12 mg/kg artesunate or artemether there are dose-related decreases in reticulocyte and neutrophil counts (Hien & White, 1993). Transient episodes of fever occurred in up to 25% of volunteers receiving the artemisinin derivatives in some studies, but none at all were reported in others.

In the earliest clinical studies there were reports of single episodes of CNS events with artemether but these were in severely ill patients, making it difficult to distinguish whether these CNS events were related to treatment or malaria. General clinical experience over a number of years suggested that the artemisinin derivatives did not have neurotoxic effects in man. However, some recent reports have cast doubt on this belief. In a recent study involving Gambian children with cerebral malarial convulsions occurred more frequently, and recovery from coma took longer,

following treatment with artemether compared to treatment with quinine (van Hensbroek *et al.*, 1996). This longer recovery period from coma was also noted in a Vietnamese study (Hien *et al.*, 1996). It is possible that the enlargement of these effects is a manifestation of artemether neurotoxicity. Acute cerebellar dysfunction has been reported following oral artesunate treatment of *P. falciparum* malaria (Miller & Panosian, 1997). Two days after completing treatment the patient began to develop persistent neurological symptoms such as ataxic gait, impaired shin-to-heel, rapid alternating movements and slurred speech. There was moderate recovery after four months. Unlike convulsions and prolonged coma, cerebellar dysfunction would be an extremely unusual complication, especially as there were no neurological symptoms associated with the malaria in this case. Furthermore, ataxia was one of the main symptoms of artemisinin neurotoxicity in dogs and rats (Brewer *et al.*, 1994a, 1994b).

Although these are the only reports suggesting that the artemisinin derivatives are toxic in humans they are still a cause for concern. In several of the *in vivo* neurotoxicity studies (Brewer *et al.*, 1994a, 1994b; Petras *et al.*, 1994; Genovese *et al.*, 1995) it has been demonstrated that neuropathological damage occurs at doses as low as 5 mg/kg/day, even though there were no accompanying behavioural changes. The behavioural tests on rats (Genovese *et al.*, 1995) were performed as although overt behavioural symptoms developed during *in vivo* studies (Brewer *et al.*, 1994b), they only did so shortly before death and an early indicator of neurotoxicity was desirable. Consequently, for much of the period in which neurotoxicity was developing in the test animals there were obvious outward signs.

These "silent" lesions observed with the lower dosing regimens indicate that neuropathological damage can occur without any obvious external signs.

The recommended parenteral clinical dose for artemether is 10 mg/kg/day (Malaria Unit, WHO, 1996). This is only slightly higher than the doses which produced silent neuropathological lesions in the behavioural studies *viz.* 12.5 mg/kg/day; moreover, 6 mg/kg/day produced neuropathological lesions in monkeys, and Brewer and colleagues demonstrated considerable neurotoxicity in dogs and rats following daily doses of 15 mg/kg/day and 25 mg/kg/day respectively. This raises the possibility that the neurotoxic effects of artemisinin derivatives have been occurring in human subjects but have gone unrecorded.

There are several reasons why artemisinin neurotoxicity may have gone unnoticed in the past. Patients that have been given the drugs are not monitored for drug-related neurotoxicity either during or after treatment. However, even if neurological problems are observed, these drugs are frequently used on severely ill or comatose patients who often suffer malaria-induced neurological damage and it may be difficult to recognise any damage caused by the artemisinin derivatives under these conditions, indeed, neurological sequelae to malaria are quite common. Another obstacle to identifying any neurotoxicity associated with artemisinin use is that it is often used in conjunction with other antimalarials, *e.g.* mefloquine, to prevent recrudescence and many these drugs have also been associated with neurological side-effects (Phillips-Howard & ter Kuile, 1995). Finally, as neuropathological lesions to the brain can occur without obvious neurological signs, how is this damage to be identified ? The lesions produced by low drug doses were distributed in the auditory system nuclei of the brainstem which is a clinically silent

area of the brain. Therefore the significance of damage to this area, either in animals or man, is uncertain. It is possible that the onset and progression of artemisinin neurotoxicity is highly specific and did not reach a degree sufficient to manifest itself behaviourally at low dosage.

Artemisinin neurotoxicity has been demonstrated in dogs, rats, and monkeys (Brewer *et al.*, 1994a, 1994b; Petras *et al.*, 1994) at daily doses only slightly higher than those used clinically. The difference between *in vivo* toxicity studies and clinical use is the length of treatment time: 8-28 days in the toxicity studies and 3-5 days clinically. This relatively short period of administration, coupled with the fact that the drugs are so rapidly eliminated from the body, could be responsible for the apparent lack of toxicity in man; long-term exposure or accumulation of a long-lived toxic metabolite may be necessary for toxicity. Unfortunately, individuals may be regularly exposed to doses of artemisinin derivatives much higher, and for longer periods, than recommended. People who live in regions in which malaria is endemic may be reinfected several times during a transmission season, and consequently receive a large amount of drug in a relatively short period of time. Anti-malarial drugs are freely available and people often administer the drugs to themselves, or sick relatives, and do so without being diagnosed as actually suffering from malaria. Recrudescence is a problem with the artemisinin derivatives and inadequate treatment may lead to repeated courses of therapy in an effort to completely clear the malaria. Finally, the Chinese have began to use the drugs prophylactically. Under these circumstances the dosing regimens more closely resemble those that produce neurotoxicity in animals. Unfortunately, there is limited knowledge of the

pharmacokinetics and metabolism of the artemisinin derivatives in man (section 1.5), and no data on the effects of long-term or cumulative administration.

Previous studies have not examined the mechanisms of artemisinin neurotoxicity. They provide little insight into the early signs of CNS toxicity or the reversibility of the damage. Moreover, it is not known if the antimalarial effects of the drugs and the toxicity are in any way connected, or if they are, if it is possible to separate them. Therefore there is an urgent need to investigate and characterise the mechanism of this neurotoxicity.

1.7. AIMS OF THIS STUDY

The work in this thesis is split into four parts :-

Part 1: The effects of the artemisinin derivatives on cultured neural cells.

Part 2: The morphological and immunocytochemical effects of dihydroartemisinin on differentiating Nb2a neuroblastoma cells.

Part 3: Validation of Nb2a cells as a model suitable for the study of the neurotoxicity of the artemisinin derivatives.

Part 4: Characterising dihydroartemisinin-protein binding.

The purpose of the work in each part is discussed in detail at the beginning of that section of the thesis.

CHAPTER TWO

MATERIALS & METHODS

PART ONE

2.1. THE EFFECTS OF THE ARTEMISININ DERIVATIVES ON CULTURED NEURAL CELLS

The mechanisms by which the artemisinin derivatives induce damage to the central nervous systems of dogs and rats (Brewer *et al.*, 1994a, 1994b) are unclear. Morphological examination of the animal brain tissues reveal neuropathological lesions such as necrosis in myelinated axons, swelling of axonal processes and spheroid formation with axonal degeneration. These findings suggest that axons and their associated glial cells are the main targets for this neurotoxicity. Cultured neuronal cells are widely used as a model to study normal cell functions, they are also routinely used to screen compounds and quantify any potential toxicity and pharmacological activity they may possess.

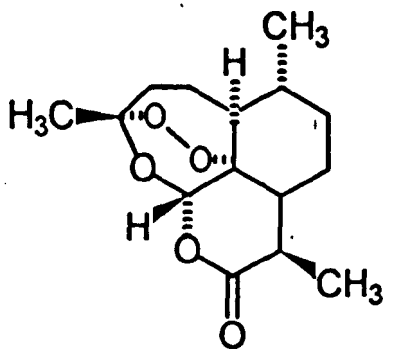
In these cell culture studies the Nb2a mouse neuroblastoma and the C6 rat glioma cell lines were used to examine the effects of the artemisinin derivatives on cell growth and maintenance of axons. The toxicity of the artemisinin derivatives to cell growth was assessed by measuring their inhibition of ^{3}H-thymidine incorporation into the deoxyribonucleic acid (DNA) of proliferating cells. The effects of the artemisinin derivatives on axonal maintenance were examined by measuring their influence on neurite outgrowth from differentiating Nb2a cells. The purpose of this work was to determine which, if any, artemisinin derivatives are

toxic to the cells, to compare the toxicity of artemisinin derivatives between neuronal cells and glial cells and to compare toxicity between cell growth and axonal maintenance.

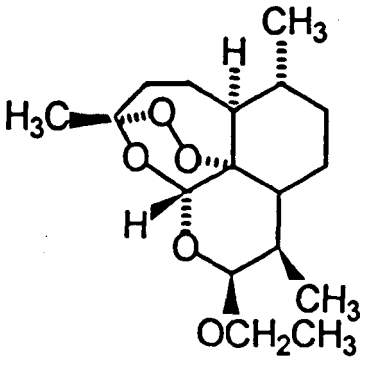
All chemicals and other materials used in these assays were obtained from the Sigma Chemical Co., Poole, UK unless otherwise stated.

2.1.1. Artemisinin derivatives

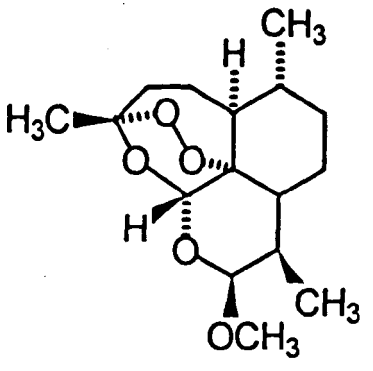
Seven artemisinin-related compounds were assessed for toxicity in Nb2a and C6 cells *in vitro*. These are shown in figure 2.1: artemisinin, which is the sesquiterpene lactone that was originally isolated from *A. annua*; dihydroartemisinin is artemisinin with the carbonyl reduced; artemether, the β-methyl ether of dihydroartemisinin; arteether, the β-ethyl ether of dihydroartemisinin; sodium artelinate, an ether of dihydroartemisinin with a carboxybenzyl fragment; desoxyartemisinin, an artemisinin metabolite with an epoxide linkage rather than an endoperoxide linkage and desoxyarteether, an arteether metabolite with an epoxide linkage. Artemisinin, dihydroartemisinin, artemether, arteether, sodium artelinate, desoxyartemisinin and desoxyarteether had their effects on proliferation of Nb2a cells assessed, dihydroartemisinin, arteether, artemether and desoxyartemisinin were tested for their effects on proliferation of C6 cells and artemisinin, dihydroartemisinin, artemether, arteether and desoxyartemisinin for their ability to affect the growth of neurites from differentiated Nb2a cells. Artemisinin, dihydroartemisinin, artemether, arteether, sodium artelinate,



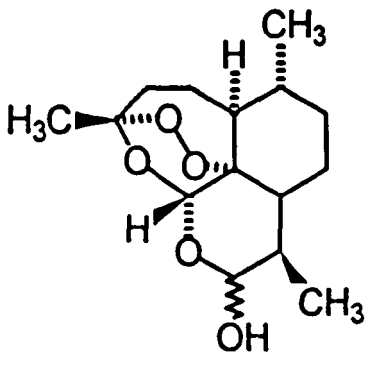
ARTEMISININ



ARTEETHER



ARTEMETHER



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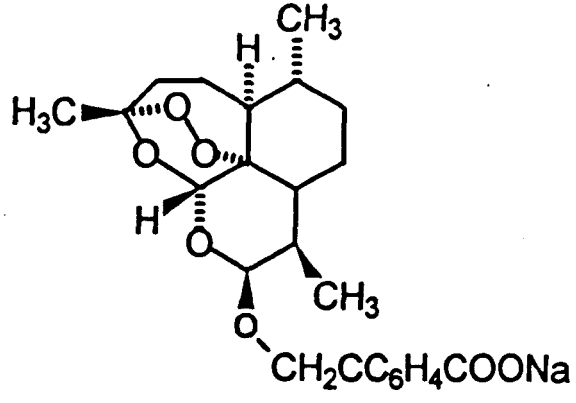
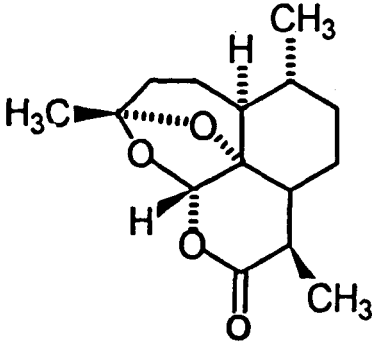
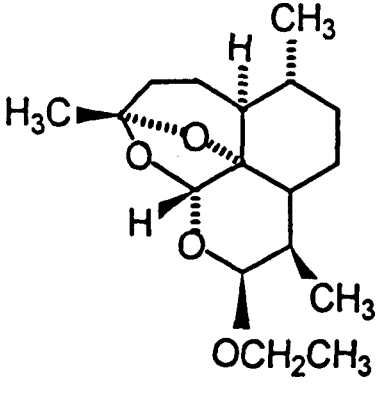


SODIUM ARTELINATE



DESOXYARTEMISININ



DESOXYARTEETHER

Figure 2.1. Structures of the artemisinin derivatives used in the Nb2a neuroblastoma and C6 glioma cell culture studies.

desoxyartemisinin and desoxyarteether used in cell proliferation and neurite outgrowth assays were kindly supplied through the World Health Organisation by Dr. J.D. McChesney (University of Mississippi, USA) and Dr. Peter Buchs (SAPEC, Lugano, Switzerland). Dihydroartemisinin and desoxyartemisinin used in protein binding assays and electron microscopy studies were also supplied by Dr. Peter Buchs. ^{14}C-labelled dihydroartemisinin (C-16 labelled, specific activity 447.7 megabecquerels (MBq)/mmol) was obtained from Triangle Research Park, North Carolina, USA.

2.1.2. Cell culture

Nb2a neuroblastoma cells and C6 glial cells were cultured in 25 cm^3 vented plastic culture flasks (obtained from Falcon/Fred Baker Scientific, Runcorn, UK) at a concentration of 10^6 cells/ml in a humidified Heraeus Ins. incubator with a 5% CO_2 atmosphere at 37 °C. The cell culture medium consisted of high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing Glutamax-1 (Gibco BRL, Life Technologies Limited, Uxbridge, UK), 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamicin with 5% (v/v) horse serum and 5% (v/v) foetal calf serum (Gibco BRL, Life Technologies Limited, Uxbridge, UK), for Nb2a cells or 10% (v/v) foetal calf serum for C6 cells. Cells took approximately one week to reach confluency.

2.1.3. Determination of appropriate cell densities for the cell proliferation assays

Nb2a and C6 cells proliferate by absorbing nutrients present in the growth medium to grow and divide. If, during the course of an assay, the cells proliferate to such an extent that the supply of nutrients from the growth medium becomes insufficient to sustain further growth the cells will cease proliferating and possibly die. Such an occurrence during a proliferation assay, in which cells are being incubated with a potentially toxic compound, would cause a serious error in the result. In order to ensure that any inhibition of cell proliferation that occurred in these assays was solely the result of incubating the cells with artemisinin derivatives and not in part due to an adverse increase in cell density, preliminary assays were carried out to determine starting Nb2a and C6 cell densities which would allow linear cell proliferation throughout the assay. Nb2a cells were added to Microtest 96-well plastic culture plates (Falcon/Fred Baker Scientific, Runcorn, UK) in 200 µl of culture medium at cell densities from 1000 cells/ml to 300000 cells/ml, and C6 cells were plated in 200 µl of culture medium at densities ranging from 1000 cells/ml to 200000 cells/ml. Nb2a cells were then incubated for 3 days, and C6 cells for 6 days. Twenty-four h before the end of the incubation 740 Bq of ^{3}H-thymidine (Amersham, Aylesbury, UK; specific activity 1.1 x 10^{6} MBq/mmol) (in medium) was added to each well of Nb2a cells and 7.4 kBq to each well of C6 cells. At the end of the incubation the cells were harvested by vacuum filtration onto glass microfibre filter paper discs (Whatman Lab. Division, Maidstone, UK) with a

Dynatech Minimash 2000 cell harvester and the incorporated radioactivity was measured by liquid scintillation counting with a Beckman LS 1801 scintillation counter. ^{3}H-thymidine was taken up by dividing cells and incorporated into their DNA; the higher the incorporated radioactivity, the greater the rate of DNA synthesis and the greater the extent of cell proliferation. For each cell density a mean value was obtained from replicates of 5 wells.

2.1.4. Effects of the artemisinin derivatives on Nb2a and C6 cell proliferation

The effects of the artemisinin derivatives on neuronal cell growth were assessed by measuring their inhibition of the incorporation of ^{3}H-thymidine into proliferating Nb2a cells. Having determined the appropriate cell densities (above), Nb2a cells were plated into Microtest 96-well plastic culture plates at a density of 6000 cells/ml and C6 cells at 4000 cells/ml in 200 µl/well culture medium and allowed to adhere to the plate for 24 h. Artemisinin derivatives were then added to the wells to produce final concentrations ranging from 0.1 to 100 µM. The solubility of the artemisinin derivatives in culture medium is poor; consequently, they were initially dissolved in methanol (Fisons, Loughborough, Leics., UK). As methanol was found to be toxic to cultured cells at a concentration > 0.5% of the total volume, so 20 mM stock solutions (x 200 highest drug concentration) of each drug were used to make the appropriate drug concentrations by serial dilution. The Nb2a cells were then incubated with the drugs for a further 2 days and the C6 cells for a further 5 days. Twenty-four h before the end of the experiment, 7.4 kBq of ^{3}H-thymidine (in

medium) was added to each well. Cells were then harvested by vacuum filtration onto filter paper discs and the incorporated radioactivity was measured by liquid scintillation counting as above. For each experiment, a mean value was obtained from replicates of 5 wells. In addition to wells of drug-treated and control cells, each plate also had five additional control wells with half the number of cells that the other wells contained. ^{3}H-thymidine incorporation in these wells was compared with that of normal control wells to ensure that cell proliferation on the plate was within the linear proliferation range of the cell density curves. Any plate where the ^{3}H-thymidine incorporation in the half-control wells was not approximately 50% that in normal control wells was not used.

2.1.5. Effects of the artemisinin derivatives on Nb2a neurite outgrowth

The effects of the artemisinin derivatives on axonal maintenance were assessed by measuring their inhibition of neurite outgrowth from differentiating Nb2a cells. Nb2a cells were plated into Multiwell 24-well plates (Falcon/Fred Baker Scientific, Runcorn, UK) at 50,000 cells/ml (this cell density was arrived at by trial and error as that which gave adequate numbers of cells whose neurites could easily be counted) in 400 µl/well culture medium. After 24 h, the medium was replaced with serum-free medium containing 500 µM dibutyryl cyclic AMP and the various artemisinin derivatives at concentrations from 1 nM to 100 µM. The medium of control cells was replaced with serum-free medium containing 500 µM dibutyryl cyclic AMP alone. The cells were incubated with the drugs for a further 24 h and

were then fixed at room temperature with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, at pH 7.4) for 10 min, followed by staining of the fixed cells with Coomassie Blue stain (0.25% (w/v) in aqueous solution containing 45% (v/v) methanol and 9% (v/v) glacial acetic acid (Fisons, Loughborough, UK) for 3 min, washing twice with PBS and once with distilled water. The plates were then examined by light microscopy using a Willovert Wetzlar 21 light microscope; in the absence of serum and with dibutyryl cyclic AMP present, Nb2a cells underwent differentiation and showed a marked increase in the number and length of neurites or neurite-like extensions projecting from the cell body. For each experiment, a mean value was obtained of the number of extensions / neurites from 50 cells selected at random.

2.1.6. Calculation of data and statistical analysis

IC_{50} values were calculated as geometric means from the number of experiments as indicated, with 95% confidence limits in brackets. The Shapiro-Wilk test was used to assess the normality of the distribution of data. Where data were normally distributed, a two-way analysis of variance (ANOVA) with a Bonferroni modified t-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Where data was not normally distributed a two-way Mann Whitney U-test was used to establish statistical significance, rejecting the null

hypothesis when $p \leq 0.05$. Statistical analysis was performed using Arcus Pro-Stat 3.12 computer software (Medical Computing, Aughton, UK).

PART TWO

2.2. MORPHOLOGICAL AND IMMUNOCYTOCHEMICAL EFFECTS OF DIHYDROARTEMISININ ON DIFFERENTIATING NB2A NEUROBLASTOMA CELLS

Measurement of the effects of the artemisinin derivatives on Nb2a cell proliferation and neurite outgrowth allows comparison of the relative toxicity of the drugs. However, quantifying their toxic effects does not significantly further our understanding of their mechanism of action. The purpose of these studies was to determine which cell structures are damaged and which processes are disrupted by the artemisinin derivatives and to locate possible targets for the drugs. To accomplish this the effects of dihydroartemisinin on differentiated Nb2a cells were studied by transmission and scanning electron microscopy, Western blotting, immunocytochemistry and autoradiography.

Artemisinin binds extensively to proteins and many neurotoxic agents act on proteins of the cytoskeleton (Clarkson, 1986), consequently, Western blots of dihydroartemisinin-treated cell proteins and fixed Nb2a cells were incubated with antibodies directed common cytoskeletal proteins *e.g.* neurofilaments and tubulin. Polyacrylamide gels of the proteins of cells and rat cortex incubated with dihydroartemisinin were stained with silver and Coomassie Blue cell stain, to determine if aggregation or degradation of proteins had taken place. Autoradiography of polyacrylamide gels of cells and rat cortex proteins treated with

^{14}C-dihydroartemisinin was also performed to identify possible target proteins of the artemisinin derivatives. Damage to cell membrane and organelles and disruption of the cytoskeleton are difficult to observe by light microscopy. However, drug-induced damage such as this can readily discerned by electron microscopy, which allows close examination of cell components. Therefore the effects of dihydroartemisinin on the structure of differentiated Nb2a cells were studied by scanning and transmission electron microscopy. Dihydroartemisinin is a metabolite common to many of the artemisinin derivatives (Lee & Hufford, 1990), it is also the most toxic of the derivatives and this makes it the most useful artemisinin derivative with which to study the neurotoxicity of these compounds. Therefore dihydroartemisinin was used in all the subsequent experiments to study the mechanism of action of the artemisinin derivatives.

2.2.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate Nb2a cell and rat cortex proteins for Western blotting, autoradiography and Coomassie Blue- and silver-staining of proteins. The acrylamide content of the resolving gels used in these experiments varied, see individual sections for details. Drug-treated and control cell / cortex protein (12.5 μg, determined by the method of Lowry *et al.* (1951), below) were boiled for 3 min in sample buffer containing 8 M urea, 10% (v/v) 2-mercaptoethanol (National Diagnostics, Aylesbury, UK), 2% (w/v) SDS and 1% Bromophenol Blue

dye (BDH, Poole, UK). After boiling the protein samples were clarified by centrifugation at 12000 g in a Jouan MR14.11 centrifuge for 5 min. The proteins were then separated on 8 cm, 0.75 mm thick one-dimensional SDS / polyacrylamide minigels using Hoefer Sci. Ins. Mighty Small II Model No. SE250 minigel apparatus under a constant current of ~ 20 mA/gel from a Hoefer Sci. Ins. Power supply PS500X DC with water cooling. Polyacrylamide gels were a composite of a 6%, 7.5% or 15% resolving gel overlaid with a 4% stacking gel, into which wells were created for the addition of samples to the gel. The 6%, 7.5% and 15% resolving gels contained mixtures of 6% acrylamide (6% (w/v) acrylamide (BDH, Poole, UK) and 0.52% (w/v) N,N'-methylenebisacrylamide (BDH, Poole, UK)), 7.5% acrylamide (7.5% (w/v) acrylamide and 0.65% (w/v) N,N'-methylenebisacrylamide) and 15% acrylamide (15% (w/v) acrylamide and 1.3% (w/v) N,N'-methylenebisacrylamide) respectively with 0.38 M Tris (hydroxymethyl) aminomethane (Tris), 0.1% (w/v) SDS, 0.035% (w/v) ammonium persulphate, and 0.14 % (v/v) N,N,N',N'-tetramethyl ethylene diamine (TEMED), pH 8.8. Stacking gels consisted of 4% acrylamide (4% (w/v) acrylamide and 0.35% (w/v) N,N'-methylenebisacrylamide) with 0.126 M Tris, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.2% (v/v) TEMED, pH 6.8.

Sample protein was measured using the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) and sample protein were dissolved in PBS. The BSA solution was diluted to produce standards containing 10-50 μg protein in 500 μl PBS. Similarly, 10-60 μl of sample protein solution were diluted to 500 μl. Solutions of 1% (w/v) copper sulphate and 1% (w/v) sodium potassium tartrate were

both diluted 1/100 in 2% (w/v) sodium carbonate (Fisons, Loughborough, UK) in 0.1 M sodium hydroxide. Two ml of this mixture was then added to each dilution of BSA and sample protein and allowed to stand for 10 min, after which 200 µl Folin and Ciocalteau's phenol reagent (BDH, Poole, UK) was added. After 30 min the absorbance of the BSA standards and samples was read at 570 nm on a Dynatech MR600 microplate reader. A standard curve of absorbance against protein concentration was plotted and protein concentration of samples determined.

2.2.2. Silver-staining of polyacrylamide gels

Neurotoxic agents often cause the aggregation or degradation of cytoskeletal proteins and these changes in their molecular weights can be observed by separating the proteins on polyacrylamide gels and staining with ammoniacal silver nitrate solution. Consequently, in this study the effects of dihydroartemisinin and haemin on the proteins of differentiating Nb2a cells were examined by silver-staining polyacrylamide gels of their resolved proteins. Nb2a cells (approximately $1x10^6$ cells in 5 ml culture medium in 25 cm^3 vented plastic flasks) were induced to differentiate by removing serum from the flask and replacing it with serum-free medium containing 0.5 mM dibutyryl cyclic AMP with 2 µM dihydroartemisinin or 2 µM dihydroartemisinin and 2 µM haemin, in serum-free medium. 2 µM dihydroartemisinin was used as this concentration had been demonstrated (section 3.1.4) to inhibit the majority neurite outgrowth and consequently any drug-induced changes in cell proteins could feature prominently on the gels. 2 µM haemin was

used as this had previously been demonstrated to increase dihydroartemisinin toxicity without itself being toxic to the cells (Smith *et al.*, 1997). Control cells were incubated with only dibutyryl cyclic AMP in serum-free medium.. After 24 h incubation with dihydroartemisinin, the proteins of the cells were extracted as outlined in section 2.4.3. Cell proteins were then electrophoretically resolved on 6% polyacrylamide gels as outlined in section 2.2.1. After electrophoretic resolution of cell proteins was complete the gels were placed in a fixing solution containing 25% (v/v) methanol and 10% (v/v) acetic acid (Fisons, Loughborough, UK) for 2 h and washed 3 x 15 min in distilled water. Gels were then fixed in 10% (v/v) glutaraldehyde (Fisons, Loughborough, UK) for 30 min and washed in distilled water 3 x 20 min. The gels were placed in ammoniacal silver nitrate solution (consisting of: 1.2% (v/v) conc. ammonia hydroxide (BDH, Poole, UK), 0.064% (w/v) sodium hydroxide, 0.7% (w/v) silver nitrate) for 15 min. The gel was washed three times in distilled water for 10 min before being transferred to developing solution (consisting of: 10% (v/v) absolute ethanol (Fisons, Loughborough, UK), 0.005% (w/v) citric acid, 0.018% (v/v) formaldehyde (BDH, Poole, UK)). The proteins on the gels gradually became stained with silver over a 15-30 min period, and when staining was adequate the reaction was stopped using 5% (v/v) glacial acetic acid. After each step in this process, from soaking in glutaraldehyde to developing, the gel was transferred to a clean glass dish to prevent the gel becoming damaged by sticking to the dish. The gels were stored in distilled water.

2.2.3. Coomassie Blue staining of polyacrylamide gels

An alternative to staining with an ammoniacal silver nitrate solution is to stain polyacrylamide gels with Coomassie Blue cell stain. This method was used when the effects of dihydroartemisinin and haemin on rat cortex were examined by resolving treated cortex proteins on 7.5% polyacrylamide gels. One ml aliquots of rat cortex homogenate (10 mg/ml original wet weight in 100 mM piperazine N, N'-bis (2-ethane sulphonic acid) (PIPES) buffer containing 100 units/ml penicillin and 100 μg/ml streptomycin, pH 6.8) were incubated with 7.5 μM dihydroartemisinin or 7.5 μM dihydroartemisinin and 2 μM haemin in plastic Eppendorf tubes (Fred Baker Scientific, Runcorn, UK) for 24 h at 37°C and controls were incubated with PIPES buffer alone. It was possible to use a higher concentration of dihydroartemisinin in these incubations as the cortex homogenate was a largely suspension of cells in isotonic buffer, whereas the Nb2a cells were undergoing differentiation and extending neurites in culture medium and were presumably more vulnerable to dihydroartemisinin toxicity. The higher concentration would hopefully increase binding and make drug effects on cortex proteins easier to detect. The proteins were extracted from the incubation rat brain homogenate as outlined in section 2.4.1 and separated on 7.5% polyacrylamide gels as outlined in section 2.2.1. Gels of electrophoretically separated proteins were placed in a fixing solution containing 11.4% (w/v) trichloroacetic acid, 3.4% (w/v) sulphosalicylic acid and 30% (v/v) methanol for 45 min. Fixed gels were then stained in a solution containing 0.25% (w/v) Coomassie Blue, 45.4% (v/v) methanol,

and 7.2% (v/v) glacial acetic acid for 1 h at 55°C. The stained gels were then repeatedly washed in destaining solution consisting of 30% (v/v) methanol and 10% (v/v) glacial acetic acid until the background became clear and the bands of protein visible. Gels were stored in distilled water.

2.2.4. Western blotting

Some drug-induced changes to cell proteins may be difficult to recognise on stained polyacrylamide gels because of the low concentration of particular proteins. Western blotting using monoclonal antibodies directed against cytoskeletal proteins which are common targets for neurotoxic agents may reveal modification to proteins not easily discernible on silver- or Coomassie Blue-stained gels of cell proteins. Consequently, Nb2a cells were incubated with dihydroartemisinin and haemin as outlined in section 2.2.2 and separated on 6% polyacrylamide gels as outlined in section 2.2.1. After the proteins had been separated, they were electrophoretically transferred from the gel to 0.45 μm pore size Bioblot nitrocellulose membrane (Costar, High Wycombe, UK) in a Hoefer Sci. Ins. TE series transphor unit in the presence of a transfer buffer composed of 25 mM Tris, 192 mM glycine and 20% methanol at pH 8.3. A low temperature was maintained by pumping cold water through the base of the transfer unit for the duration of the transfer process. Proteins were transferred for 1 to 1½ h at 400 mA. The nitrocellulose blots were blocked with 5% (w/v) non-fat dairy milk (Marvel, Knighton, UK) in Tris-buffered saline (TBS, 10 mM Tris and 140 mM sodium chloride, pH 7.2) for 1 h at room temperature to

block all protein binding sites on the nitrocellulose and so prevent non-specific binding of the primary and secondary antibodies. The blocked blots were washed for 5 and then 20 min in 0.05% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20) in TBS and then incubated with monoclonal antibodies directed against proteins which are common targets for neurotoxins : Rmd09 (1:5 dilution, detects non-phosphorylated NF-H neurofilaments, a gift from Prof. Brian Anderton, Institute of Psychiatry, London, UK), 611β1 (1:20 dilution, detects acetylated α-tubulin, obtained from DAKO Ltd, High Wycombe, UK) and YL 1/2 (1:50 dilution, detects tyrosinated α-tubulin, obtained from DAKO Ltd, High Wycombe, UK), and MAb101AA6 (1:10 dilution, detects spectrin, a gift from Prof. I Virtanen, University of Helsinki, Finland). All primary antibodies were diluted in 3% (w/v) BSA in TBS. In order to ensure that the bands on the blots were specific to the primary antibody, "no primary" controls were carried out in which the blots were incubated with 3 % BSA in TBS in the absence of primary antibody. The blots were incubated with the antibodies at 4°C overnight, washed 3 x 20 min in Tween 20 / TBS, and then incubated with horseradish peroxidase-conjugated rabbit anti-mouse (for MAb101AA6, 611β1 and Rmd09) or anti-rat (for YL 1/2) immunoglobulins, both a 1/1000 dilution in 5% (w/v) non-fat dairy milk in TBS, for 2 h at room temperature in darkness. The blots were then washed 3 x 15 min in Tween 20 / TBS and treated with ECL Western Blotting detection reagents (Amersham Life Science, Little Chalfont, UK). Nitrocellulose blots (both sides) were soaked with equal amounts of the two ECL reagents on a glass plate for 1-2 min and dried on 3MM chromatography paper (Whatman Lab. Division, Maidstone, UK). During treatment

with the detection reagents the horseradish peroxidase enzyme conjugated to the secondary antibodies catalysed light emission from luminol oxidation by hydrogen peroxide. The blots were placed on a glass plate and the plate covered with cling film and exposed to Kodak X-OMAT LS for 6 min. The film was then developed and blot proteins visualised in Kodak GBX developer and the process stopped in 0.1 M glacial acetic acid, fixed in Kodak GBX Fixer then washed and dried.

The density of the drug-treated and control cell protein bands on the blots were quantified using a 2222-020 LKB Ultrascan XL laser densitometer with a gel scanning program, Gel Scan XL (Pharmacia LKB, Bromma, Sweden). In addition to drug-treated and control cell proteins each gel also had an additional control sample with half the amount of control cell protein. When the blots were analysed by laser densitometry the absorbances of these half-controls were compared with those of the full controls to ensure that densitometric evaluation of drug-treated and control samples was performed within the linear range of absorbance. Any blots where the absorbance of the half-control was not approximately 50% that of the full control (*i.e.* not in the linear range) were not used.

2.2.5. Indirect immunofluorescence of fixed Nb2a cells

Indirect immunofluorescence of fixed Nb2a cells using monoclonal antibodies directed against common targets for neurotoxic agents may reveal changes in the status or distribution of proteins within the cell not apparent on Western blots, and Coomassie- or silver-stained gels. Therefore, Nb2a cells were

grown on plastic LabTek slides (Falcon/Fred Baker Scientific, Runcorn, UK), and incubated with dihydroartemisinin, as outlined in section 2.2.7 below, for this purpose. Differentiated drug-treated Nb2a cells were fixed in 4% (w/v) formaldehyde in PBS for 10 min and rinsed twice in PBS for 1 min. The cells were then permeablised by dehydrating in graded ethanols: 25%, 50%, 75%, 95% ethanols for 2 min each then left in 100% ethanol for 30 min, and rehydrated with 0.25% (v/v) Triton X-100 in PBS for 5 min and washed in PBS. After blocking with 10% (w/v) BSA in PBS for 30 min, the cells were incubated with one of the following monoclonal antibodies for 2 h at 37°C : Ta51 (1:100 dilution in 3% (w/v) BSA in PBS, detects phosphorylated NF-H neurofilaments a gift from Dr. M. Carden, University of Kent, UK), 611β1 (1:20 dilution) and YL 1/2 (1:50 dilution), and MAb101AA6 (1:10 dilution), and then rinsed 3 x 5 min in PBS. In order to ensure that any immunofluorescence was specific to the primary antibody, "no primary" controls were carried out in which the cells were incubated with 3% BSA in PBS in the absence of primary antibody. The cells were incubated with fluorescein-conjugated rabbit anti-mouse immunoglobulins (or anti-rat immunoglobulins for Ta51 and YL 1/2) for 1 h at 37°C, rinsed 3 x 5 min in PBS and for 1 min in distilled water, the plastic wells removed from the slide and the cells fixed under a coverslip with a polyvinyl alcohol-based mount. The polyvinyl alcohol base was prepared as follows: 40 ml of 0.2 M Tris-HCl, pH 8.5, was heated to 60°C. Into this was stirred 8 grams of polyvinyl alcohol, then 20 ml glycerol and 2 grams of DABCO (1,4 diazoabicyclo[2,2,2]octane) were added, the resulting mountant was centrifuged at 3000 g before use. After the cells had been fixed they were stored in

the dark at 4°C. Cells with fluorescently labelled proteins were visualised with a Zeiss Axiovert 35M microscope fitted with a Zeiss Neofluar 100 objective and photographed on Ilford HP 5 Plus film using an Olympus OM-1 camera.

2.2.6. Autoradiography

Discovery of the target protein(s) of the artemisinin derivatives by searching for drug effects on a few common targets for neurotoxic agents may not provide an answer as practical limitations mean it is only possible to examine a few selected proteins in this manner. An alternative approach is to use dihydroartemisinin itself, rather than antibodies directed against specific proteins, as a probe. The probe in this case was ^{14}C-dihydroartemisinin, which, when incubated with rat cortex homogenate, Nb2a and C6 cells may bind to target proteins allowing their detection on polyacrylamide gels exposed to X-ray film.

One ml aliquots of rat cortex homogenate (10 mg original wet weight/ml in 100 mM PIPES buffer, pH 6.8) and Nb2a and C6 cells, in 5 ml culture medium in 25 cm^3 culture flasks at densities of approximately 4x10^6 cells/flask (Nb2a cells) or 2x10^6 cells/flask (C6 cells), were incubated with 187 μM ^{14}C-dihydroartemisinin (dissolved in PIPES buffer for the homogenate assay, in serum-free medium with 0.5 mM dibutyryl cyclic AMP for the cell assays) for 24 h at 37°C. After the incubation protein was extracted from cells and homogenate as outlined in sections 2.4.3 and 2.4.1 and resolved on 15% polyacrylamide gels as outlined in section 2.2.1. The gels were then dried with a Bio-Rad Lab. Model 224 Gel Dryer, sprayed three times with

En^{3}hance (Du Pont / NEN, Stevenage, UK), and exposed to Kodak X-OMAT film at -80°C. Dried gels were firmly attached to the film by tape and marked to aid realignment when developed. After three months the film was developed as outlined in section 2.2.4.

2.2.7. Transmission electron microscopy of dihydroartemisinin-treated Nb2a cells

Transmission electron microscopy (TEM) of differentiated Nb2a cells was performed to discover what structural damage dihydroartemisinin caused to the interior of the cell body and neurite. Nb2a cells were plated onto 8-well LabTek plastic slides in 400 µl at a concentration of 50,000 cells/ml. After 24 h to allow the cells to adhere to the plate, they were induced to differentiate by removing serum from the wells and replacing it with serum-free medium containing 0.5 mM dibutyryl cyclic AMP with 2 µM dihydroartemisinin, 2 µM desoxyartemisinin or serum-free medium in the case of controls. The cells were incubated for a further 24 h with the drugs and then medium was removed and the cells fixed in 3% (v/v) glutaraldehyde (Fisons, Loughborough, UK) in 100 mM sodium cacodylate buffer, pH 7.4. (Agar Scientific, Stansted, UK) at 37°C for 2 h, washed in 100 mM sodium cacodylate buffer and postfixed in 1% (w/v) osmium tetraoxide (Agar Scientific, Stansted, UK) (also in cacodylate buffer) for 30 min at room temperature. The cells were again washed in cacodylate buffer prior to being dehydrated and embedded. They were dehydrated in a graded ethanol series: 70% ethanol (Fisons,

Loughborough, UK) for 15 min, 90% ethanol for 2 x 20 min, 100% ethanol for 3 x 30 min, and infiltrated with Epon-Araldite resin (Agar Scientific, Stansted, UK) (3 x 1h), and polymerised for 48 h at 60°C. The slide was then peeled off and the resin blocks sawn into suitable pieces for sectioning. Sections were cut parallel to the culture substrate at thickness of 120 nm using a Reichert Ultracut E Ultramicrotome and collected onto 200 mesh hexagonal thin bar grids (Agar Scientific, Stansted, UK). They were stained with 2% (w/v) uranyl acetate (Agar Scientific, Stansted, UK) and Reynold's lead citrate (Agar Scientific, Stansted, UK) (both in distilled water) and examined by transmission electron microscopy in a Philips CM10 TEM at 80 kV. Negatives were recorded on Kodak 4489 film (Agar Scientific, Stansted, UK).

2.2.8. Scanning electron microscopy of dihydroartemisinin-treated Nb2a cells

Scanning electron microscopy (SEM) of differentiated Nb2a cells was performed to discover what structural damage dihydroartemisinin caused to the exterior of the cell body and neurite. Nb2a cells grown on LabTek slides were prepared as for transmission electron microscopy through ethanol dehydration. The plastic chambers were then removed leaving the setting gasket in place, and the slide was critical point-dried in a Polaron E3000 critical point dryer. After drying, the slide was sputter-coated with gold using an Edwards E306A coating unit, and examined in a Hitachi S-520 microscope at 10 kV. Negatives were recorded on Ilford FP4 film (Agar Scientific, Stansted, UK).

2.2.9. Statistical analysis

Data form densitometric analysis of Western blots were subjected to statistical analysis. The Shapiro-Wilk test was used to assess the normality of the distribution of data. Where data were normally distributed, a two-way ANOVA with a Bonferroni modified t-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Where data were not normally distributed a two-way Mann Whitney U-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Statistical analysis was performed using Arcus Pro-Stat 3.12 computer software.

PART THREE

2.3. VALIDATION OF NB2A CELLS AS A MODEL SUITABLE FOR THE ASSESSMENT OF NEUROTOXICITY

Much of the work in this thesis uses the differentiating Nb2a neuroblastoma cell as the model with which toxicity, ultrastructural damage and binding characteristics were studied. The results of section 3.1 demonstrated that this cell line is susceptible to the toxicity of the artemisinin derivatives and that the neurite outgrowth parameter is particularly sensitive. However, the sensitivity of the Nb2a cells to the drugs and the greater toxicity toward neurite outgrowth may be a particular property of these compounds and not a true demonstration of a neurotoxic effect. Consequently, the suitability of proliferating and differentiating Nb2a cells as models for the study of dihydroartemisinin neurotoxicity was assessed.

Inhibition of Nb2a cell proliferation as a useful parameter with which to quantify dihydroartemisinin neurotoxicity was assessed by comparing the effects of dihydroartemisinin on Nb2a cell proliferation with its effects on the proliferation of two non-neuronal cell lines, the HeLa B human cervical carcinoma and the CRFK feline kidney cell lines. If Nb2a cell proliferation showed a greater sensitivity to dihydroartemisinin than the non-neuronal cell lines then this would suggest it is a useful model with which to study the postulated neurotoxicity of artemisinin.

Neurite outgrowth from differentiated Nb2a cells was more sensitive to dihydroartemisinin toxicity than cell proliferation and as differentiating Nb2a cells more closely resemble mature nerve cells, it is possible that neurite outgrowth is a more sensitive and reliable parameter with which to assess a compound's potential neurotoxicity. To test this hypothesis the effects of two neurotoxic agents and two non-specific toxic compounds on Nb2a proliferation and differentiation were compared. If Nb2a neurite outgrowth was a viable model for neurotoxicity, and if dihydroartemisinin had demonstrated neurotoxic effects then the neurotoxic agents would produce significantly greater inhibition of neurite outgrowth than of proliferation, and the non-specific toxins would not.

Inhibition by dihydroartemisinin of Nb2a neurite outgrowth was also compared with that of a variety of compounds with well-defined neurotoxic effects. This was done to quantify the potency of dihydroartemisinin as a neurotoxic agent with respect to other neurotoxic agents and in the hope of gaining an understanding of the mechanism of action of dihydroartemisinin neurotoxicity by using light and electron microscopy to identify similarities between the structural damage caused by dihydroartemisinin with that caused by the other neurotoxic agents; each neurotoxic compound used had a different mechanism of action and any similarities between dihydroartemisinin-induced damage and that of one of the neurotoxic agents would suggest a possible mechanism for dihydroartemisinin neurotoxicity. In addition, if the damage to Nb2a cells caused by the neurotoxic compounds could be correlated with the damage they caused *in vivo*, then this would provide further support for the use of Nb2a neurite outgrowth as a model for neurotoxicity.

2.3.1. Cell culture

Nb2a cells were cultured as before. CRFK feline kidney cells and HeLa B human cervix carcinoma cells were maintained in high glucose RPMI 1640 (Gibco BRL, Life Technologies Limited, Middlesex, UK), containing 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamicin, 2% (v/v) 200 mM L-glutamine and 10% (v/v) foetal calf serum. They were cultured in 25 cm^3 plastic culture flasks at a concentration of 10^6 cells/flask in a humidified Heraeus Ins. incubator with a 5% CO_2 atmosphere at 37°C. Cells took 7-10 days to reach confluency.

2.3.2. Determination of appropriate cell densities for the cell proliferation assay

Before cell proliferation assays began preliminary assays were performed to determine which HeLa B and CRFK cell densities which would allow sufficient proliferation to produce a measurable incorporation of ^{3}H-thymidine into cell DNA, but would not result in inhibition of proliferation due to cell overcrowding. HeLa B and CRFK cells were added to Microtest 96-well plastic culture plates in 200 μl of culture medium at cell densities ranging from 1000 cells/ml to 300000 cells/ml and were then incubated for 3 days. Twenty-four h before the end of the incubation 740 Bq of ^{3}H-thymidine (in medium) was added to each well. At the end of the incubation the cells were harvested by vacuum filtration onto filter paper discs and

the incorporated radioactivity was measured by liquid scintillation counting as outlined in section 2.1.3. For each experiment, a mean value was obtained from replicates of 5 wells.

2.3.3. Effects of dihydroartemisinin on the proliferation of Nb2a, CRFK and HeLa B cells

These assays were performed in order to determine if the inhibition of Nb2a cell proliferation by dihydroartemisinin is a manifestation of a neurotoxic effect or a non-specific toxicity which would equally affect non-neuronal cell lines. For measurement of cell proliferation, Nb2a cells were plated into Microtest 96-well plastic culture plates at a density of 6000 cells/ml as outlined in section 2.1.3, CRFK cells at 10000 cells/ml and HeLa B cells at 5000 cells/ml all in 200 µl/well culture medium and allowed to adhere to the plate for 24 h. Dihydroartemisinin was then added to the wells (in culture medium) to produce final concentrations ranging from 0.1 to 100 µM. The cells were then incubated with the drug for a further 2 days and 24 h before the end of the experiment, 7.4 kBq of ^{3}H-thymidine was added to each well. Each plate had five additional control wells with half the cell density of the other wells as outlined in section 2.1.4. Cells were harvested and ^{3}H-thymidine incorporation measured as outlined in section 2.1.3. For each experiment a mean value was obtained from replicates of 5 wells.

2.3.4. Comparison of the effects of known neurotoxins and non-specific toxins on the proliferation of and neurite outgrowth from Nb2a cells

The effects of two neurotoxic compounds, colchicine and 2,5-hexanedione, and two compounds with non-specific toxic effects, L-ascorbate and sodium cyanide, on Nb2a cell proliferation and neurite outgrowth were compared, to determine if neurite outgrowth is a better parameter with which to assess the neurotoxicity of a compound than cell proliferation.

Cell proliferation. The effects of the neurotoxic agents and the non-specific toxins on Nb2a cell proliferation were examined as outlined in section 2.1.4, except that in these proliferation assays the compounds were incubated with the drug for 24 h *i.e.* the compounds were added at the same time as the ^{3}H-thymidine. The neurotoxic agents were colchicine (at concentrations ranging from 0.01-5 μM) and 2,5-hexanedione (1-100 mM) (Aldrich, Poole, UK), the compounds with non-specific toxic effects used were sodium cyanide (0.1-100 mM) and L-ascorbate (100-200 μM).

Neurite outgrowth. To determine the effects of the neurotoxic agents and the compounds with non-specific toxicity on neurite outgrowth, Nb2a cells were incubated with the compounds listed below and fixed and stained as outlined in section 2.1.5. The compounds used were the same as in the cell proliferation assays, although the ranges of concentration used sometimes differed: colchicine

(1-5000 nM) and 2,5-hexanedione (0.1-10 mM), sodium cyanide (0.1-100 mM) and L-ascorbate (10-500 μM). The fixed cells were then viewed with a Zeiss Axiovert 35M light microscope linked by a video camera to a Kontron Vidas 2.0. image analyser. Subsequently, for each experiment, 5-7 different fields containing approximately 50 cells in total were chosen for each neurotoxic agent and the control. A program was written using the available functions of the image analyser in order to permit the automatic measurement of the total neuritic material produced by the cells (in pixels) in a given field and to express the results as the average length of neurites per cell.

2.3.5. Comparison of the effects of neurotoxic agents on Nb2a neurite outgrowth

In a separate study the effects of colchicine, calphostin C, 2,5-hexanedione, cytochalasin B and β,β'-iminodipropionitrile on Nb2a neurite outgrowth were compared with those of dihydroartemisinin. This was done primarily as a means of determining the sensitivity of the assay but it was also thought that comparing dihydroartemisinin toxicity with that of other neurotoxic compounds could provide clues as to the nature of dihydroartemisinin toxicity or at least help eliminate some possibilities. Consequently, Nb2a cells were incubated for 24 h in serum-free medium containing 0.5 mM dibutyryl cyclic AMP and one of the following: colchicine (1-5000 nM), cytochalasin B (1-1000 nM), calphostin C (5-500 nM), β,β'-iminodipropionitrile (Aldrich, Poole, UK) (0.5-10 μM), 2,5-hexanedione

(0.1-100 mM), dihydroartemisinin (0.1-100 μM) or serum-free medium as a control then fixed, stained as outlined in section 2.1.5 and inhibition of neurite outgrowth was assessed as outlined in section 2.3.4.

2.3.6. Electron microscopy of Nb2a cells treated with neurotoxic agents

The purpose of this approach was to see if the effects of dihydroartemisinin and the neurotoxic compounds could be correlated with ultrastructural damage to the cytoskeleton or other organelles of cells. This was done by examining cells treated with dihydroartemisinin and neurotoxic compounds by scanning and transmission electron microscopy. Colchicine and 2,5-hexanedione were selected for this study as they act on microtubules and neurofilaments respectively (Clarkson, 1986), two common targets for neurotoxic agents.

Transmission electron microscopy. Transmission electron microscopy of differentiated Nb2a cells incubated with 50 nM colchicine, 15 μM 2,5-hexanedione or serum-free medium in the case of controls was performed as outlined in section 2.2.7.

Scanning electron microscopy. Scanning electron microscopy of differentiated Nb2a cells incubated with 50 nM colchicine, 15 μM 2,5-hexanedione or serum-free medium in the case of controls was performed as outlined in section 2.2.8.

2.3.7. Calculation of data and statistical analysis

IC_{50} values were calculated as geometric means from the number of experiments as indicated, with 95% confidence limits in brackets. The Shapiro-Wilk test was used to assess the normality of the distribution of data. Where data were normally distributed, a two-way ANOVA with a Bonferroni modified t-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Where data was not normally distributed a two-way Mann Whitney U-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Statistical analysis was performed using Arcus Pro-Stat 3.12 computer software.

PART FOUR

2.4. CHARACTERISING DRUG-PROTEIN BINDING.

The artemisinin derivatives are converted to carbon-centred free radicals and other reactive products that bind extensively to proteins *in vivo* and *in vitro* (Baker *et al.*, 1993; Yang *et al.*, 1994; Smith *et al.*, 1997). It is thought that their antimalarial activity is mediated through the alkylation of specific parasite proteins by these reactive species. Autoradiographs of Nb2a cells incubated with ^{14}C-dihydroartemisinin suggest that the drugs also specifically alkylate proteins in these cells (section 3.2.4 and Kamchonwongpaisan *et al.*, 1997). Therefore, it is possible that protein binding plays an important role in mediating the toxicity of these drugs. In the following experiments, the nature of binding interactions between the artemisinin derivatives and neural cell proteins was studied. ^{14}C-dihydroartemisinin was used as the ligand with which to study the binding interactions of the artemisinin derivatives to Nb2a and C6 cells and rat brain homogenates. Initially, rat cortex, cerebellum and brain stem were incubated with ^{14}C-dihydroartemisinin to determine in which areas of the brain the greatest binding occurred; the proteins were then separated from other cell components to determine if a significant proportion of dihydroartemisinin binding was to proteins. The nature of dihydroartemisinin binding was then investigated with rat cortex, Nb2a and C6 cells under a number of different conditions.

Changes in binding with increasing ^{14}C-dihydroartemisinin concentration were examined to see if binding could be saturated and if multiple sets of binding sites existed. Haemin, which increases dihydroartemisinin toxicity to Nb2a cell neurite outgrowth (Smith *et al.*, 1997) and conversion of the artemisinin derivatives into reactive free radicals (Meshnick, 1994), was incubated with cortex homogenate and ^{14}C-dihydroartemisinin to determine if it also increased drug-protein binding. The effects of pre-incubating homogenate with agents that protect thiol and amine groups was investigated to see if these groups play an important role in dihydroartemisinin binding. Finally, arteether which is a potent antimalarial and is neurotoxic *in vivo* and *in vitro*, and desoxyartemisinin which is neither, were co-incubated with homogenate and ^{14}C-dihydroartemisinin to examine a possible link between therapeutic activity, toxicity and protein binding.

2.4.1. Comparison of dihydroartemisinin binding in different areas of rat brain.

Homogenised rat cerebellum, cortex and brain stem were incubated with ^{14}C-dihydroartemisinin for 2 h and 24 h to determine if binding of ^{14}C-dihydroartemisinin differed in several areas of the rat brain and if any changes significant in binding occurred over a 24 h period. The brain was removed from adult male Wistar rats (weighing approximately 250 grams), the brainstem, cerebellum and cortex were dissected out and processed immediately at 0°C. One ml aliquots of brainstem, cerebellum and cortex homogenates (10 mg original wet weight/ml in 100 mM PIPES buffer, containing 100 units/ml penicillin and 100

μg/ml streptomycin, pH 6.8) were incubated for 2 h or 24 h at 37°C with approximately 3.3 kBq of ^{14}C-dihydroartemisinin in plastic Eppendorf tubes. After 2 h or 24 h the incubation was halted and the homogenised tissue was centrifuged at 12000 g in a Jouan MR14.11 centrifuge and the supernatant removed. The pellet of brain cells was then twice resuspended in PIPES buffer and centrifuged at 12000 g. This repeated washing and resuspension was performed to remove unbound ^{14}C-dihydroartemisinin. The rat brain homogenate was then homogenised (by hand) in 50 μl PIPES buffer. After homogenisation 950 μl acetone (Fisons, Loughborough, UK) at -20°C was added and the protein precipitated from the homogenised brain material, which was then centrifuged at 12000 g. The acetone supernatant was removed from the pellet and the protein was then resuspended in PIPES buffer. ^{14}C-dihydroartemisinin binding was determined by taking aliquots of resuspended protein, and of the acetone supernatant, and measuring the radioactivity present in each by liquid scintillation counting with a Packard Tricarb 4640 scintillation counter.

Homogenisation of rat brainstem, cerebellum and cortex was performed very gently. The purpose was to break up the tissue, not individual brain cells and the incubations were carried out in an isotonic buffer to prevent the cells rupturing. Nevertheless, some brain cells must have been damaged during processing and their proteins released into the incubation buffer. Consequently, a preliminary assay was performed to determine if a significant degree of drug binding to free protein took place in the PIPES buffer and which may not be centrifuged into the pellet of cells when the incubation was terminated. Rat brain homogenate was incubated with

^{14}C-dihydroartemisinin and drug-protein binding assessed as above. The protein in the supernatant collected from the centrifugation of the pellet of cells was also precipitated in acetone at -20°C and binding determined by liquid scintillation counting. Only ~5% of total ^{14}C-dihydroartemisinin binding was to proteins in the supernatant, therefore, in subsequent assays (section 2.4.1 to section 2.4.5), drug binding to supernatant protein was not regarded as significant and was not assessed.

Another preliminary assay was performed to determine if any unbound ^{14}C-dihydroartemisinin was being precipitated in the acetone along with the protein-bound drug. Incubations were performed as above but without rat brain homogenate. At the end of the incubation the mixture was centrifuged at 12000 g. Following centrifugation 950 µl of the incubation mixture was removed, 950 µl PIPES buffer mixed with the remaining 50 µl and centrifuged at 12000 g. This procedure was repeated once and then the remaining 50 µl mixed with 950µl acetone at -20°C and centrifuged at 12000 g. All the acetone / PIPES was then gently removed and the tube washed with PIPES buffer. Liquid scintillation counting of this PIPES buffer revealed only background levels of radioactivity, indicating that there was no significant precipitation of unbound ^{14}C-dihydroartemisinin by acetone and therefore only protein-bound drug would be counted.

2.4.2. Characterising ^{14}C-dihydroartemisinin-protein binding

Rat cortex homogenate, Nb2a and C6 cells were incubated with increasing concentrations of ^{14}C-dihydroartemisinin (0.94 μM to 187 μM) to determine if drug-protein binding were saturable and, by Rosenthal plots of the data (Rosenthal, 1967), to ascertain if multiple sets of binding sites for the drugs existed. This was investigated using data from the control incubations of the haemin assays (*i.e.* incubations containing only ^{14}C-dihydroartemisinin and cells / homogenate), outlined below in section 2.4.3.

2.4.3. Effects of haemin on protein binding of ^{14}C-dihydroartemisinin

Haemin catalyses the conversion of artemisinin derivatives into reactive products and increases their toxicity to differentiating Nb2a cells (Cumming *et al.*, 1997, Smith *et al.*, 1997). The effects of haemin on ^{14}C-dihydroartemisinin binding to rat cortex, Nb2a and C6 cells were investigated to discover if it also increased drug-protein binding.

Nb2a cells. Nb2a neuroblastoma cells, cultured as outlined in 2.1.2 and proliferating in 5 ml medium in 25 cm^3 culture flasks at a density of approximately 4x10^6 cells/flask, were induced to differentiate by withdrawing serum and adding 0.5 mM dibutryl cyclic AMP in serum-free medium. ^{14}C-dihydroartemisinin (0.94 μM to 187 μM) with or without haemin (2 μM) in serum-free medium was

also added to the flasks. The haemin concentration was 2 μM as a previous study (Smith *et al.*, 1997) demonstrated that at this concentration, haemin potentiates the toxicity of dihydroartemisinin in Nb2a cells without itself having any adverse effects on the cells. After 24 h the cells were removed from the flask, centrifuged at 12000 g and the supernatant removed. The pellet of cells was resuspended and centrifuged at 12000 g twice in PIPES buffer (100 mM, containing 100 units/ml penicillin and 100 μg/ml streptomycin, pH 6.8). Repeated washing and resuspension were performed to remove free haemin and ^{14}C-dihydroartemisinin. The cells were then homogenised (by hand) in 50 μl PIPES buffer. After homogenisation 950 μl acetone (Fisons, Loughborough, UK) at -20°C was added and the protein precipitated from the homogenised cells, which was then centrifuged at 12000 g. The protein pellet was resuspended in PIPES buffer and aliquots used to determine the ^{14}C-dihydroartemisinin-protein binding by liquid scintillation counting with a Packard Tricarb 4640 scintillation counter.

C6 cells. Cells were treated as above, except that the density of proliferating C6 cells, cultured as outlined in 2.1.2, was approximately 2x10^6 cell/flask.

Rat cortex homogenate. Cortex was prepared from adult male Wistar rats (approximately 250 grams) and processed immediately as outlined in section 2.4.1. One ml aliquots of rat cortex homogenate (10mg original wet weight/ml in PIPES buffer were incubated for 24 h at 37°C with ^{14}C-dihydroartemisinin (0.94 μM to 187 μM) in PIPES buffer with or without 2 μM haemin in plastic Eppendorf tubes.

Protein was washed, extracted and ^{14}C-dihydroartemisinin binding to protein was assessed as outlined in 2.4.1.

A preliminary assay to determine if any ^{14}C-dihydroartemisinin-haemin adducts formed during the incubations were precipitated by the acetone was performed. This was identical to the preliminary assay performed in section 2.4.1 except that 2 μM haemin was also added to the incubation. Again, liquid scintillation counting revealed that there was no significant ^{14}C-dihydroartemisinin-haemin adduct precipitation by the acetone and therefore only protein-bound drug would be counted.

2.4.4. Effects of blocking reagents on protein binding of ^{14}C-dihydroartemisinin

In this study cortex homogenate was pre-incubated with the thiol and amine group blocking agents iodoacetamide and sodium cyanate in order to determine if these common protein moieties play an important role in ^{14}C-dihydroartemisinin binding.

Preliminary incubations of rat cortex homogenate with the blocking agents were performed to determine the concentrations that gave maximum inhibition of ^{14}C-dihydroartemisinin binding. Rat cortex homogenate was prepared as outlined in section 2.4.1. One ml aliquots were pre-incubated with iodoacetamide (0.1 M to 2 M) for 20 min at room temperature, then centrifuged at 12000 g and resuspended in PIPES buffer (100 mM, containing 100 units/ml penicillin and 100 μg/ml

streptomycin, pH 6.8) twice and incubated with 667 Bq of ^{14}C-dihydroartemisinin for 24 h at 37°C. Protein was extracted and binding assessed as outlined in section 2.4.1. The optimum concentration of iodoacetamide was calculated to be 1 M. Subsequently, cortex homogenate was pre-incubated with 1 M iodoacetamide then centrifuged at 12000 g and resuspended in PIPES buffer twice, pre-incubated with sodium cyanate (0.1 to 2 M) for 1 h at 37°C, then centrifuged at 12000 g and resuspended in PIPES buffer twice, then incubated with 667 Bq of ^{14}C-dihydroartemisinin for 24 h at 37°C. Binding to cortex protein was determined as above and the optimum concentration of sodium cyanate determined.

Rat cortex homogenate was prepared as outlined in section 2.4.1. One ml aliquots were then incubated with iodoacetamide (1 M in PIPES buffer) for 20 min at room temperature, centrifuged at 12000 g and resuspended in PIPES buffer twice. After incubation with iodoacetamide the resuspended homogenate was then incubated with sodium cyanate (Aldrich, Poole, UK) (1 M in PIPES buffer) for 1 h at 37°C, and then once more centrifuged at 12000 g and resuspended in PIPES buffer twice. Aliquots of cortex treated with iodoacetamide alone were instead incubated with PIPES buffer for 1 h at 37°C. Controls were subjected to both incubation steps using PIPES buffer instead of iodoacetamide and sodium cyanate. The cortex homogenate was then incubated with ^{14}C-dihydroartemisinin (0.94 μM to 187 μM; 24 h at 37°C) and ^{14}C-dihydroartemisinin binding to protein assessed as outlined in section 2.4.1.

2.4.5. Effects of arteether and desoxyartemisinin on ^{14}C-dihydroartemisinin protein binding

The relationship between the pharmacological activity and toxicity of the artemisinin derivatives and protein binding was examined by investigating the effects of arteether, an artemisinin derivative that is both a potent antimalarial and toxic to rats and cultured neural cells, and desoxyartemisinin, an inactive, non-toxic artemisinin metabolite, on ^{14}C-dihydroartemisinin binding to cortex proteins. Rat cortex homogenate was prepared as outlined in section 2.4.1. One ml aliquots were incubated for 24 h at 37 °C with either 37.5 μM ^{14}C-dihydroartemisinin and 37.5 μM arteether or 37.5 μM ^{14}C-dihydroartemisinin and 37.5 μM desoxyartemisinin and the effects of arteether and desoxyartemisinin on ^{14}C-dihydroartemisinin-protein binding were assessed. Protein extraction from cortex homogenate and ^{14}C-dihydroartemisinin-protein binding was determined as outlined in section 2.4.1.

2.4.6. Statistical analysis and calculation of binding parameters.

The Shapiro-Wilk test was used to assess the normality of the distribution of data. Where data were normally distributed, a two-way ANOVA with a Bonferroni modified t-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Where data were not normally distributed a Mann Whitney U-test was used to establish statistical significance, rejecting the null hypothesis when $p \leq 0.05$. Statistical analysis was performed using Arcus Pro-Stat

3.12 computer software. Binding parameters K_D (dissociation constant) and B_{max} (maximum binding capacity of cells / homogenate) were calculated using GraFit 3.01 computer software (Erithacus Software Ltd., London, UK).

CHAPTER THREE

RESULTS

PART ONE

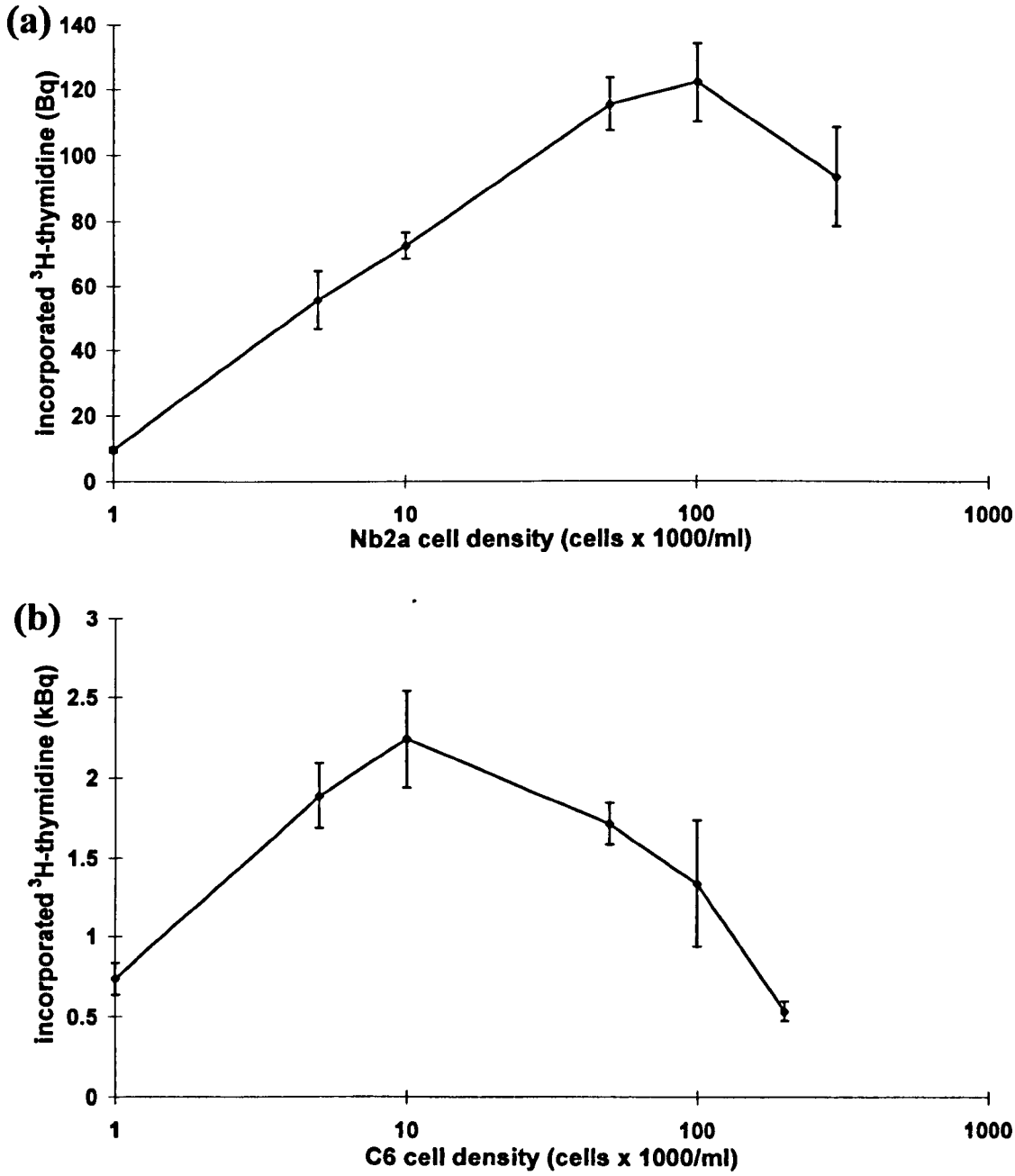
3.1. THE EFFECTS OF ARTEMISININ DERIVATIVES ON CULTURED NEURAL CELLS

Little is known about the process by which the artemisinin derivatives induce damage to the central nervous system of dogs and rats (Brewer *et al.*, 1994a, 1994b). The neuropathological lesions are characterised by swelling of axonal processes and spheroid formation with axonal degeneration and necrosis in myelinated axons. These neuropathies suggest that the targets for this toxicity are predominantly axons and their associated glial cells. The *in vitro* use of cultured cells as a tool to screen compounds for toxicity and pharmacological activity relatively quickly and cheaply, and as models to study their mechanism of action of various compounds is widespread. In these cell culture studies the effects of the artemisinin derivatives and their metabolites on cell growth and axonal maintenance were assessed using two neurally derived cell lines, the Nb2a mouse neuroblastoma and the C6 rat glioma. The toxicity of the artemisinin derivatives to cell growth was assessed by measuring their effect on the incorporation of ^{3}H-thymidine into the DNA of proliferating cells. The effects of the artemisinin derivatives on axonal maintenance were examined by measuring their effects on neurite outgrowth from differentiating Nb2a cells. The purpose of this work was to determine which, if any, artemisinin derivatives are toxic to the cells, to compare artemisinin derivative toxicity between neuronal cells and glial cells and to compare toxicity between cell growth and axonal maintenance.

3.1.1. Determination of the appropriate cell densities for the proliferation assays

Nb2a and C6 cell proliferation assays took place over two and five days. During the course of these incubations the cells grew and divided many times. If the plate-well in which they were growing became overcrowded there would not have been sufficient nutrients in the medium to feed all the cells and proliferation would cease and the cells would die. Such an occurrence during a proliferation assay, in which the cells were being incubated with potentially toxic compounds, would cause serious errors in the results. In order to ensure that any inhibition of cell proliferation that occurred in these assays was solely the result of incubating the cells with artemisinin derivatives and not partially due to an adverse increase in cell density preliminary assays were performed to determine Nb2a and C6 cell densities which would allow sufficient proliferation over the course of the assay but would not result in overcrowding and inhibition of proliferation. Nb2a cells were plated onto 96-well plates at densities ranging from 1000 to 300000 cells/ml and C6 cells were plated at densities ranging from 1000 to 200000 cells/ml. They were treated in the same manner as control cells in the proliferation assays reported in sections 3.1.2 & 3.1.3. The results of these assays (figure 3.1a & 3.1b) revealed that the appropriate cell density for Nb2a cell proliferation assays was 6000 cells/ml and the appropriate cell density for C6 cell proliferation assays was 4000 cells/ml. An appropriate cell

Figure 3.1. Assays to determine appropriate cell densities for subsequent cell proliferation assays. **(a)** Assay to determine an appropriate Nb2a cell density for proliferation assays. Nb2a cells at were plated onto 96-well plates at densities ranging from 1000 to 300000 cells/ml and incubated for 3 days. Twenty-four hours before the end of the incubation 740 Bq of ^{3}H-thymidine was added to each well. Cells were then harvested by vacuum filtration and radioactivity incorporated into cell DNA measured by liquid scintillation counting. **(b)** Assay to determine an appropriate C6 cell density for proliferation assays. As above except cells were plated at densities of 1000 to 200000 cells/ml, incubated for 6 days and 7.4 kBq of ^{3}H-thymidine was added 24 h before the end of the experiment. An appropriate cell density for proliferation assays is one which is near the centre of the positive gradient of the curve. Values represent means ± standard deviations.



density is one which falls in the middle of the positive gradient slope of the curve, where cell density and incorporation of ^{3}H-thymidine have a linear relationship.

3.1.2. Effects of the artemisinin derivatives on Nb2a cell proliferation

The effects of artemisinin, dihydroartemisinin, arteether, artemether, sodium artelinate, desoxyarteether and desoxyartemisinin on neuronal cell growth were assessed by measuring their inhibition of the incorporation of ^{3}H-thymidine into proliferating Nb2a cells at concentrations ranging from 0.1 μM to 100 μM. All artemisinin derivatives, with the exception of the desoxy-compounds, significantly ($p < 0.05$) inhibited cell proliferation in a dose-dependent manner (figure 3.2a & 3.2b). The lowest statistically significant ($p < 0.05$) inhibition of proliferation occurred with artemisinin at 0.1 μM, the lowest significant inhibition ($p < 0.05$) with arteether, artemether and dihydroartemisinin was at a concentration of 1 μM. Desoxyartemisinin did not significantly ($p < 0.05$) inhibit cell growth at any concentration used, while desoxyarteether and sodium artelinate had no effects at concentrations below 10 μM. The rank order of toxicity of the compounds, based on the IC_{50} values (concentration that inhibits cell proliferation by 50%) was: artemisinin > dihydroartemisinin > arteether > artemether > sodium artelinate > desoxyarteether ≈ desoxyartemisinin (table 3.1). Number of experiments performed (N) = 4 (four separate experiments) for dihydroartemisinin, artemether, arteether and artemisinin, N = 5 for sodium artelinate and desoxyarteether and N = 6 for desoxyartemisinin.

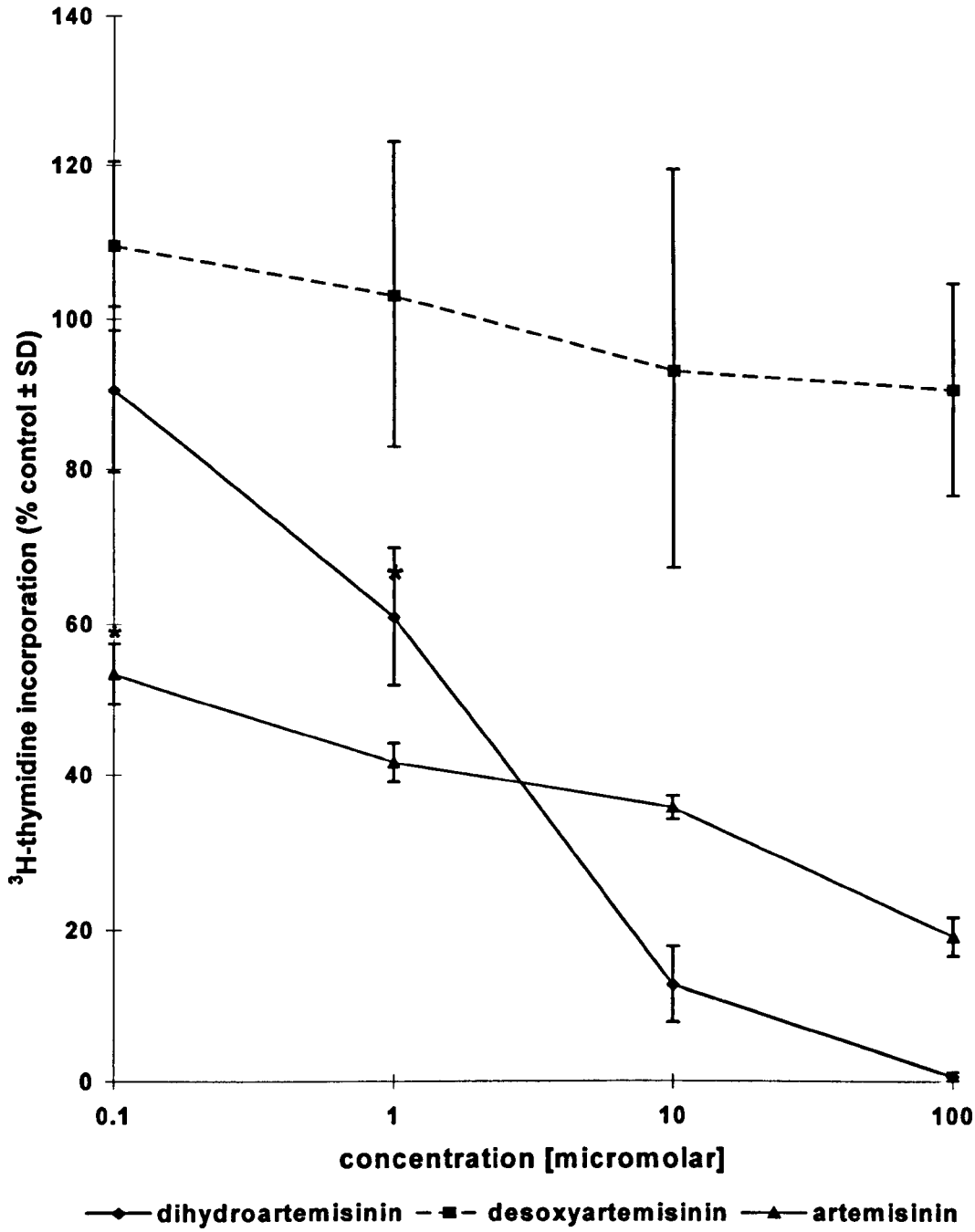


Figure 3.2a. The effects of artemisinin and derivatives on proliferation of Nb2a neuroblastoma cells in culture. Cells were incubated for 2 days with compounds at the concentrations shown. Proliferation was measured by including ^{3}H-thymidine in the medium during the final 24 h; the incorporation of radioactivity by the cells was measured by liquid scintillation counting and expressed as a percentage of that in cells incubated in the absence of drug. Values represent mean ± standard deviation. N = 4 for dihydroartemisinin and artemisinin, N = 6 for desoxyartemisinin. The lowest concentration of drug that produced a statistically significant ($p < 0.05$) inhibition of proliferation is indicated (*).

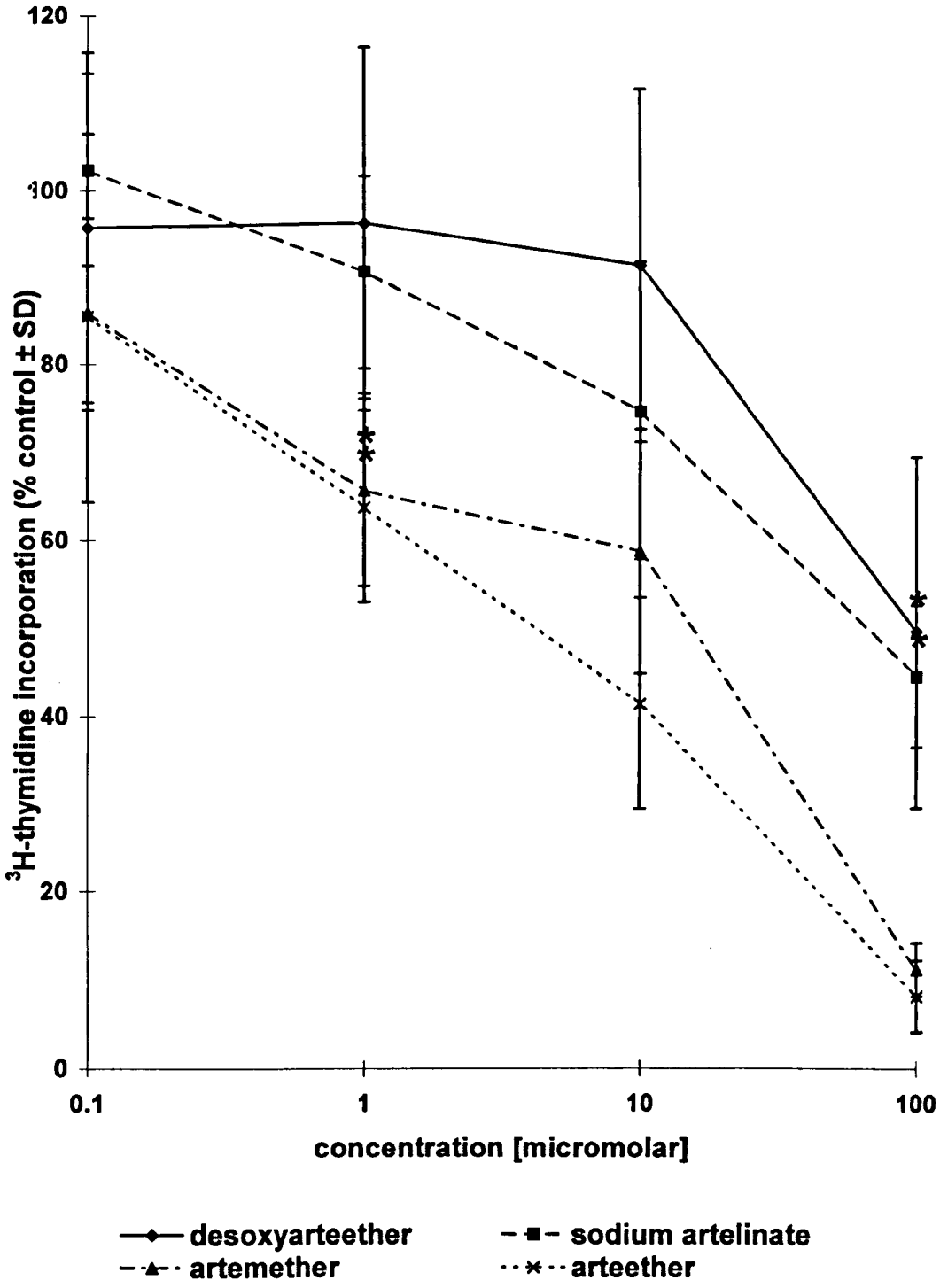


Figure 3.2b. The effects of artemisinin and derivatives on proliferation of Nb2a neuroblastoma cells in culture. Incubations were performed as outlined in figure 3.2a. Values represent mean ± standard deviation. N = 4 for artemether and arteether, N = 5 for sodium artelinate and desoxyarteether. The lowest concentration of drug that produced a statistically significant ($p < 0.05$) inhibition of proliferation is indicated (*).

3.1.3. Effects of the artemisinin derivatives on C6 cell proliferation

The effects of dihydroartemisinin, arteether, artemether and desoxyartemisinin on glial cell growth were assessed by measuring their inhibition of the incorporation of ^{3}H-thymidine into proliferating C6 cells at concentrations ranging from 0.1 μM to 100 μM. All the artemisinin derivatives tested, with the exception of desoxyartemisinin, significantly ($p< 0.05$) inhibited cell proliferation in a dose-related manner (figure 3.3). Artemether and arteether were significantly ($p< 0.05$) toxic at concentrations as low as 10 μM. Dihydroartemisinin, surprisingly, was less toxic to C6 cell proliferation than either artemether or arteether causing significant ($p< 0.05$) inhibition only at a concentration of 100 μM. IC_{50} values calculated for inhibition of C6 proliferation suggested that C6 cells possess a greater sensitivity to artemether and arteether than Nb2a neuroblastoma cells and, conversely, that dihydroartemisinin was less toxic to C6 cells than Nb2a cells (table 3.1). There was, however, a large variation in the C6 cell proliferation data which meant that the artemisinin derivatives only caused *significant* inhibition of proliferation at higher concentrations than in Nb2a cell assays. The rank order of toxicity, based on the IC_{50} values, was : arteether > artemether > dihydroartemisinin > desoxyartemisinin. N = 4 for dihydroartemisinin, artemether and arteether, N = 6 for desoxyartemisinin.

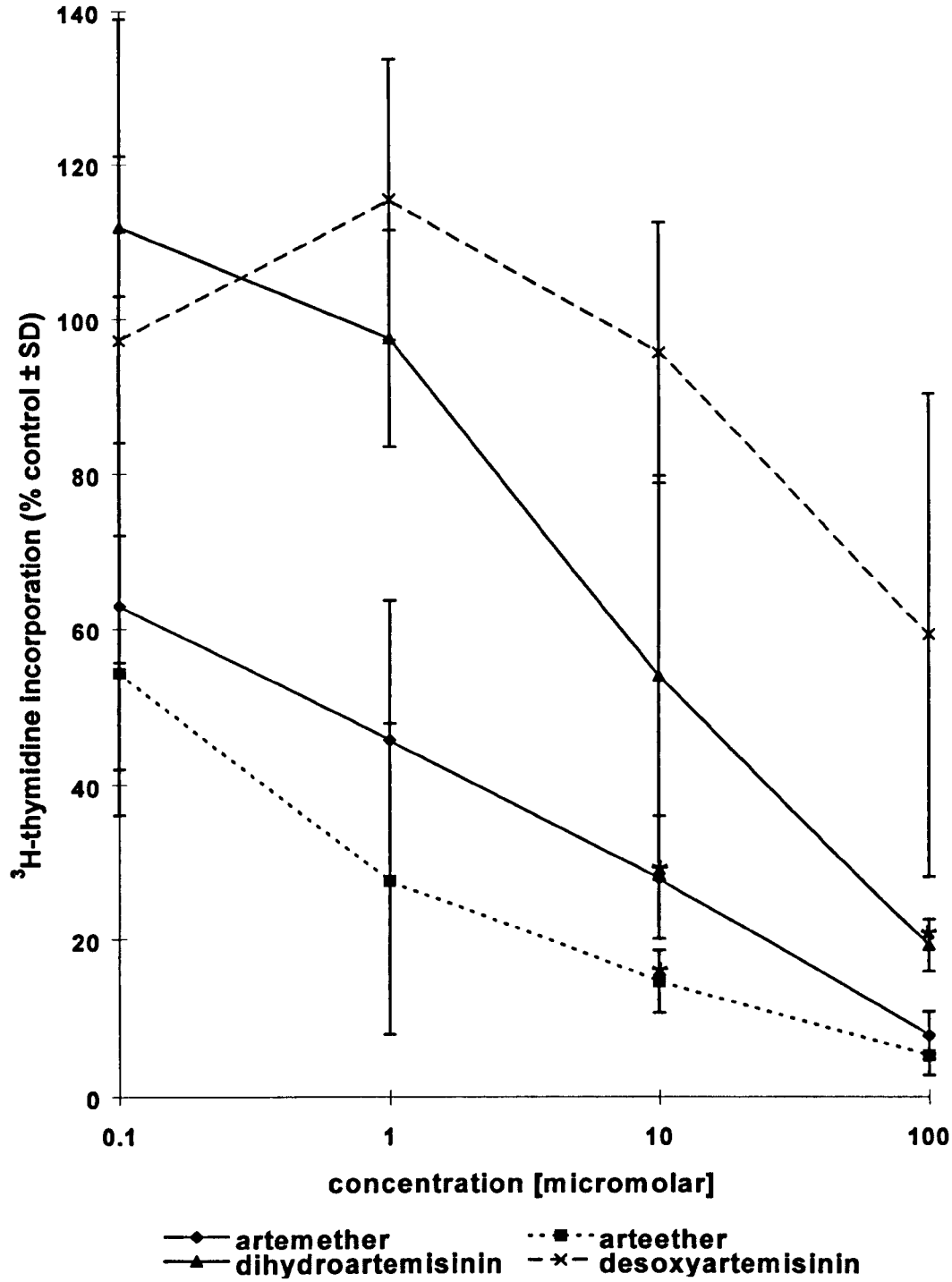


Figure 3.3. The effects of artemisinin derivatives on proliferation of C6 glioma cells in culture. Cells were incubated for 5 days with compounds at the concentrations shown. Proliferation was measured as in Fig. 3.2a. Values represent mean ± standard deviation. N = 4 for dihydroartemisinin, artemether and arteether, N = 6 for desoxyartemisinin. The lowest concentration of drug that produced a statistically significant ($p < 0.05$) inhibition of proliferation is indicated (*).

3.1.4. Effects of the artemisinin derivatives on Nb2a cell neurite outgrowth

The effects of artemisinin, dihydroartemisinin, artemether, arteether and desoxyartemisinin on axonal maintenance were assessed by measuring their inhibition of neurite outgrowth from differentiating Nb2a cells at concentrations ranging from 1 nM to 100 μM. Treatment of the differentiating Nb2a cells with arteether, artemether, artemisinin and dihydroartemisinin at concentrations ranging from 1 nM to 100 μM led to a significant ($p< 0.05$) dose-related decrease in the number of neurites / extensions formed by the cells. Dihydroartemisinin was the most toxic of the artemisinin derivatives, causing significant ($p< 0.05$) inhibition of neurite outgrowth at concentrations as low as 1 nM (figure 3.4), whilst artemisinin caused significant ($p< 0.05$) inhibition at a concentration of 10 nM, and artemether and arteether at a concentration of 100 nM ($p< 0.05$). Desoxyartemisinin had no significant effect on extension / neurite formation at any concentration used. IC_{50} values (concentration that inhibited cell neurite outgrowth by 50%) are given on table 3.2. All the artemisinin derivatives assessed (except desoxyartemisinin) significantly ($p< 0.05$) inhibited neurite outgrowth at concentrations lower than the lowest concentrations at which they significantly ($p< 0.05$) inhibited cell proliferation, and the neurite outgrowth IC_{50} values of dihydroartemisinin and artemether were significantly ($p< 0.01$) lower than their proliferation IC_{50} values. As neurite outgrowth occurs in differentiating neuroblastoma cells which more closely resemble cells of the mature nervous system in terms of cell shape and neurite maintenance than do proliferating cells, the compound's greater inhibition of neurite

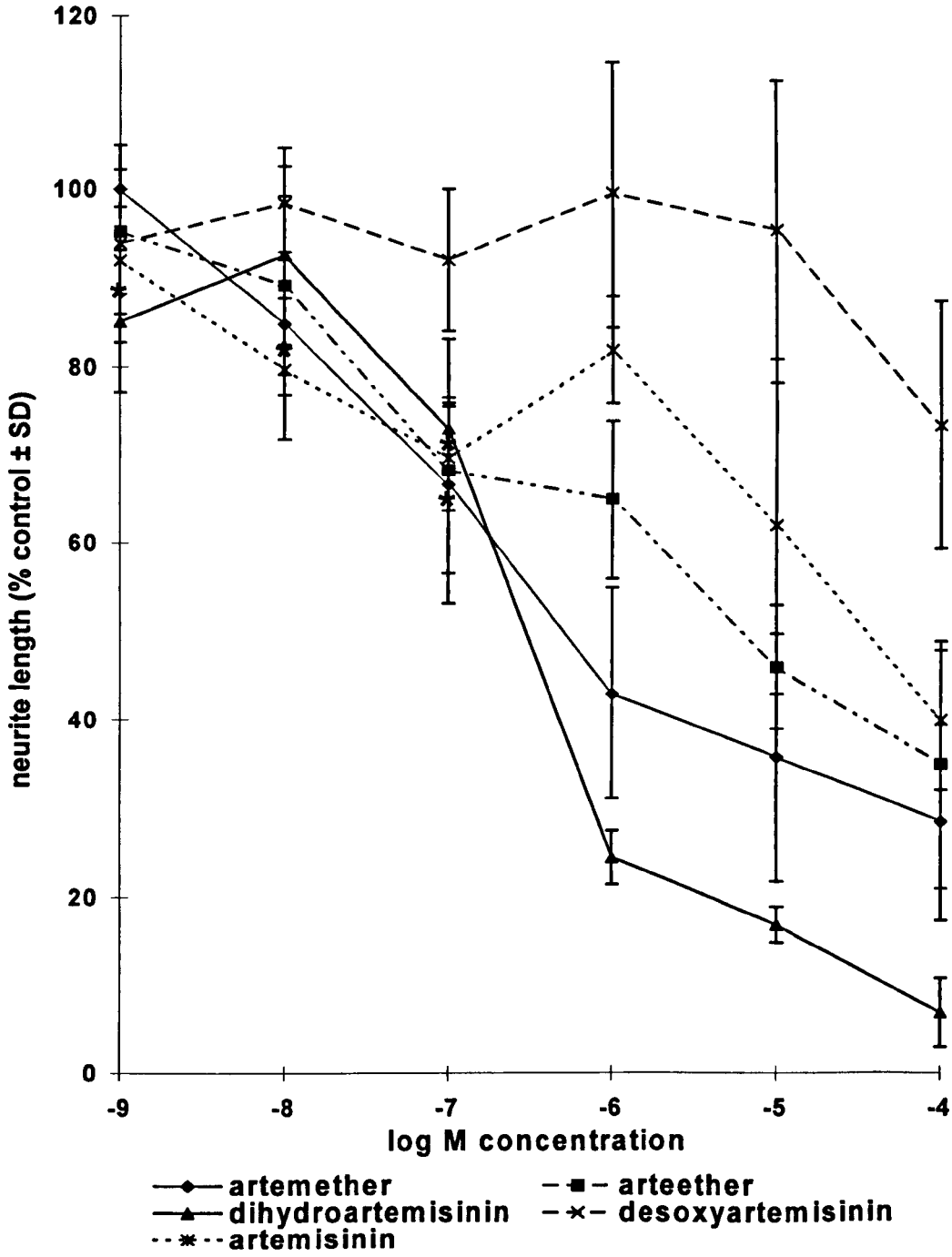


Figure. 3.4. The effects of artemisinin derivatives on neurite outgrowth. Nb2a cells were induced to differentiate and extend neurites in the presence of dibutyryl cyclic AMP (dbcAMP) and drugs at concentrations as indicated. In each experiment, the number of neurites / extensions was counted for each cell and expressed as a percentage of that in cells incubated in the absence of drug. Values represent mean ± standard deviation. N = 8 for all compounds from 1-100 nM and N = 4 for all compounds from 1-100 μM. The lowest concentration of drug that produced a statistically significant ($p < 0.05$) inhibition of neurite outgrowth is indicated (*).

outgrowth suggests a neurotoxic effect, rather than a general toxicity toward the Nb2a cell. N = 8 for all compounds from 1-100 nM and N = 4 for all compounds from 1-100μM.

Table 3.1. IC_{50} values of artemisinin and derivatives on Nb2a and C6 cell proliferation.

COMPOUND	IC_{50} VALUE [μM] (95% CONFIDENCE LIMITS)	IC_{50} VALUE [μM] (95% CONFIDENCE LIMITS)
	NB2A CELL PROLIFERATION	**C6 CELL PROLIFERATION**
ARTEMISININ	0.34 (0.06 - 0.78)	~
DIHYDROARTEMISININ	1.58 (-1.70 - 7.10)	6.44 (-28.8 - 85.1)
ARTEETHER	4.26 (-2.95 - 15.7)	0.32 (0.28 - 0.37)
ARTEMETHER	13.6 (0.73 - 29.9)	0.74 (-0.27 - 2.24)
SODIUM ARTELINATE	44.2 (17.9 - 76.1)	~
DESOXYARTEETHER	> 100	~
DESOXYARTEMISININ	> 100	> 100

Proliferation of Nb2a and C6 cells was determined as outlined in figures 3.2 & 3.3 and an IC_{50} value calculated for each drug. Geometric mean values are expressed from the number of experiments indicated in figures 3.2 and 3.3, with 95% confidence limits in brackets.

Table 3.2. IC_{50} values of artemisinin and derivatives on Nb2a neurite outgrowth.

COMPOUND	IC_{50} VALUE [μM] (95% CONFIDENCE LIMITS)
	NB2A CELL NEURITE OUTGROWTH
ARTEMISININ	20.3 (10.6 - 35.8)
DIHYDROARTEMISININ	0.29 (0.26 - 0.32)
ARTEETHER	4.39 (1.02 - 11.3)
ARTEMETHER	0.88 (-1.11 - 4.41)
DESOXYARTEMISININ	> 100

Nb2a cell neurite outgrowth was determined as outlined in figure 3.4 and an IC_{50} value calculated for each drug. N is indicated in figure 3.4, IC_{50} values given as geometric mean with 95% confidence limits.

PART TWO

3.2. MORPHOLOGICAL AND IMMUNOCYTOCHEMICAL EFFECTS OF DIHYDROARTEMISININ ON DIFFERENTIATING NB2A NEUROBLASTOMA CELLS

Nb2a cell proliferation and neurite outgrowth are useful parameters that provide a means of measuring and comparing the magnitude of the effects of the different artemisinin derivatives. They did not, however, provide any indication of the cellular targets of the compounds. Consequently, in order to determine if the artemisinin derivatives caused ultrastructural damage to the cells and their neurites and to identify potential target proteins for the drugs, the effects of dihydroartemisinin in differentiated Nb2a neuroblastoma were studied by transmission and scanning electron microscopy, Western blotting, immunocytochemistry and autoradiography.

Microtubules, neurofilaments and spectrin are all important components of the neural cell cytoskeleton and neurite axon. Western blots of proteins from differentiated Nb2a cells incubated with dihydroartemisinin and fixed dihydroartemisinin-treated differentiated Nb2a cells were probed with antibodies directed against these important cytoskeletal proteins. Two of these proteins, neurofilaments and microtubules, are common targets for neurotoxic agents. Polyacrylamide gels of the proteins of differentiated cells and rat cortex incubated with dihydroartemisinin were stained with silver and Coomassie Blue cell stain, to

determine if aggregation or degradation of proteins had taken place and autoradiography of polyacrylamide gels of cells and rat brain proteins incubated with ^{14}C-dihydroartemisinin was also performed to identify the possible target proteins of the artemisinin derivatives.

Electron microscopy allows close examination of drug effects on the surface and the interior of cells, to visualise structural damage to cell membrane, cytoskeleton and organelles. Consequently, the effects of dihydroartemisinin on these cell structures was examined by scanning and electron microscopy. Dihydroartemisinin is the most toxic of the artemisinin metabolites, and most artemisinin derivatives undergo conversion to dihydroartemisinin at some point during metabolism. This makes it the best artemisinin derivative with which to study the mechanism of action of the artemisinin derivatives

3.2.1. Ammoniacal silver nitrate and Coomassie Blue staining of polyacrylamide gels

Neurotoxic compounds often cause aggregation or some other modification to cytoskeletal proteins which may affect their mass. Such changes can be observed on SDS / polyacrylamide gels which separate proteins according to their molecular weights. Dihydroartemisinin, as a compound with neurotoxic effects, may cause such changes. Consequently, differentiating Nb2a cells were incubated with either 2 μM dihydroartemisinin or 2 μM dihydroartemisinin and 2 μM haemin and rat cortex homogenate (N = 4) was incubated with either 7.5 μM dihydroartemisinin or

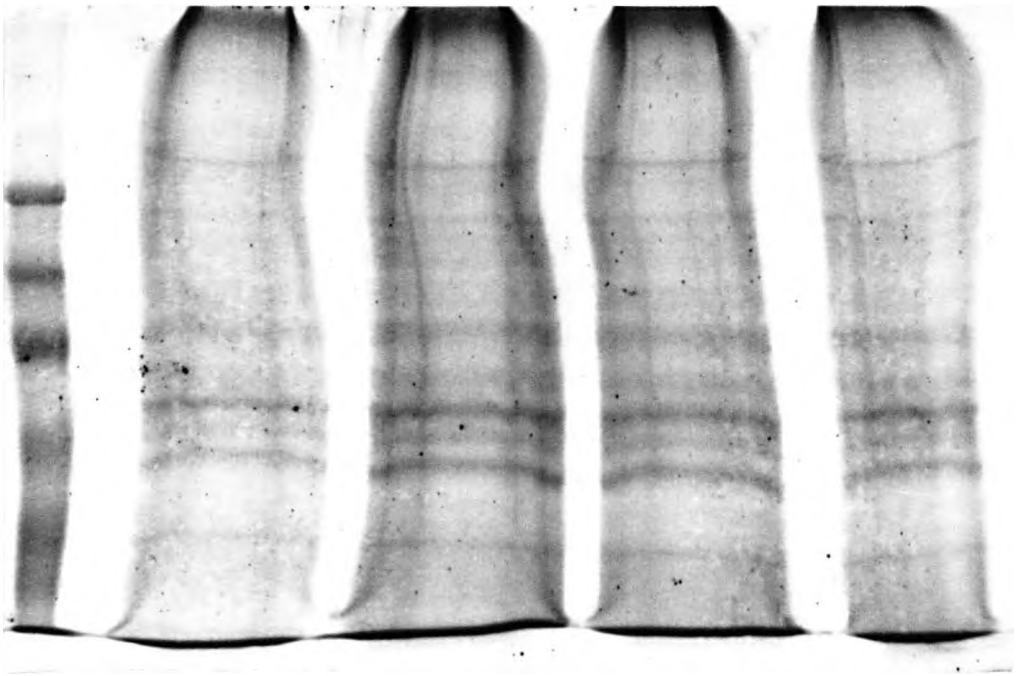
7.5 μM dihydroartemisinin and 2 μM haemin. 2 μM haemin was used as haemin significantly increased dihydroartemisinin toxicity to Nb2a cells and had been shown to significantly increase drug-protein binding (Smith *et al.*, 1997 and section 3.4.3). The cells / cortex proteins were then harvested, separated on polyacrylamide gels, and the proteins stained with ammoniacal silver nitrate or Coomassie Blue cell stain. Neither incubation produced any noticeable changes in the proteins of the cell / cortex (figures 3.5 & 3.6). There was no significant alteration in the amount of any of the protein, no extra proteins appeared and no proteins disappeared, which would indicate protein aggregation or degradation. This supports the findings of the light and electron microscopy studies (see below and section 3.3) which suggested that the artemisinin derivatives did not act in the same way as a variety of well known neurotoxicants that induce or the aggregation depolymerisation of cytoskeletal protein filaments.

3.2.2. Western blotting

Not all drug-induced changes in proteins are readily observable on polyacrylamide gels, as there are hundreds of different proteins in a cell. Western blotting using monoclonal antibodies directed against cytoskeletal proteins that are common targets for neurotoxic agents may reveal modification to cell cytoskeletal proteins not discernible on silver- or Coomassie Blue-stained gels.

Figure 3.5. Silver-stained SDS-polyacrylamide gel of Nb2a cells incubated with dihydroartemisinin and haemin. A typical gel of one of four similar experiments. Nb2a cells were incubated for 24 h with either 2 μM dihydroartemisinin or 2 μM dihydroartemisinin and 2 μM haemin in serum-free medium containing 0.5 mM dbcAMP. The cells were then harvested, cell proteins denatured in SDS, urea and mercaptoethanol, separated on polyacrylamide gels and stained with ammoniacal silver nitrate. Sample lanes on blot from left to right: **(1)** dihydroartemisinin and haemin, **(2)** dihydroartemisinin, **(3)** control, **(4)** molecular weight standards. The mass of molecular weight standards are: 116 kilodaltons (kDa), 84 kDa, 58 kDa and 45 kDa. There was no apparent alteration in the amount of any of the proteins, nor were any existing proteins deleted and no additional proteins appeared.

Figure 3.6. Coomassie Blue-stained SDS-polyacrylamide gel of rat cortex protein incubated with dihydroartemisinin and haemin. A typical gel of one of four similar experiments. Rat cortex homogenate was incubated for 24 h with either 7.5 μM dihydroartemisinin or 7.5 μM dihydroartemisinin and 2 μM haemin in PIPES buffer. The cortex protein was then separated on gels as outlined in figure 3.5 and stained with Coomassie Blue cell stain. Sample lanes on blot from left to right: **(1)** molecular weight standards, **(2)** dihydroartemisinin and haemin, **(3)** dihydroartemisinin, **(4)** control, **(5)** control. The mass of molecular weight standards are: 116 kilodaltons (kDa), 84 kDa, 58 kDa, 45 kDa, 36.5 kDa and 26.6 kDa. There was no apparent alteration in the amount of any of the proteins, nor were any existing proteins deleted and no additional proteins appeared.



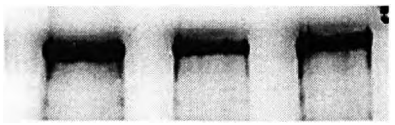
Consequently, blots of dihydroartemisinin-treated or dihydroartemisinin and haemin-treated Nb2a cells (N = 6) were probed with monoclonal antibodies directed against unphosphorylated neurofilament protein NF-H (antibody Rmd09), acetylated and tyrosinated α-tubulin (611β1 and YL 1\2 respectively) and spectrin (MAb101AA6), and compared to control cells (figures 3.7a-d). Densitometric analysis of the blots (Fig. 3.7e) revealed that neither dihydroartemisinin nor dihydroartemisinin and haemin caused significant alterations in the amount or distribution of these proteins ($p > 0.05$). Control blots that were not incubated with primary antibody did not possess any labelled proteins which demonstrated that the primary antibodies, rather than the secondary antibodies, were responsible for the labelling of cell proteins separated on polyacrylamide gels.

Some Western blotting was repeated using 4% polyacrylamide gels, rather than the 6% gels used above in order to analyse higher molecular weight materials which, if dihydroartemisinin had caused aggregation of proteins, may not be able to enter higher density gels and would as a consequence go undetected. Use of lower density gels however did not reveal any previously unseen high molecular weight protein aggregates (results not shown).

Having failed to detect any dihydroartemisinin-induced differences in these common targets for neurotoxins, Western blotting was repeated using monoclonal antibodies developed by Dr Jorge Ferreira (Ferreira *et al.*, 1995) and Dr Mondher Jaziri (Jaziri *et al.*, 1993). These antibodies were developed to measure the amounts of the artemisinin-related compounds synthesised by the various parts of the *A.*

Figure 3.7. Western blots of differentiated Nb2a cells incubated with dihydroartemisinin or dihydroartemisinin and haemin. Nb2a cell incubations and protein separation were performed as outlined in figure 3.5. Cell proteins were transferred from gels to nitrocellulose paper which were probed with monoclonal antibodies directed against **(a)** spectrin (MAb101AA6), **(b)** non-phosphorylated neurofilament NF-H (Rmd09), **(c)** tyrosinated α-tubulin (YL 1 / 2) and **(d)** acetylated α-tubulin (611β1). Bands on blots, left to right: **(1)** dihydroartemisinin and haemin, **(2)** dihydroartemisinin, **(3)** control. **(e)** Densitometric analysis of these blots revealed that neither dihydroartemisinin nor dihydroartemisinin and haemin significantly ($p > 0.05$) affected any of these proteins. N = 6, two-way ANOVA and Bonferroni modified t-test statistical analysis used. Abbreviations: dihydroartemisinin and haemin = DHA + H; dihydroartemisinin = DHA; control = CON.

(a) spectrin.

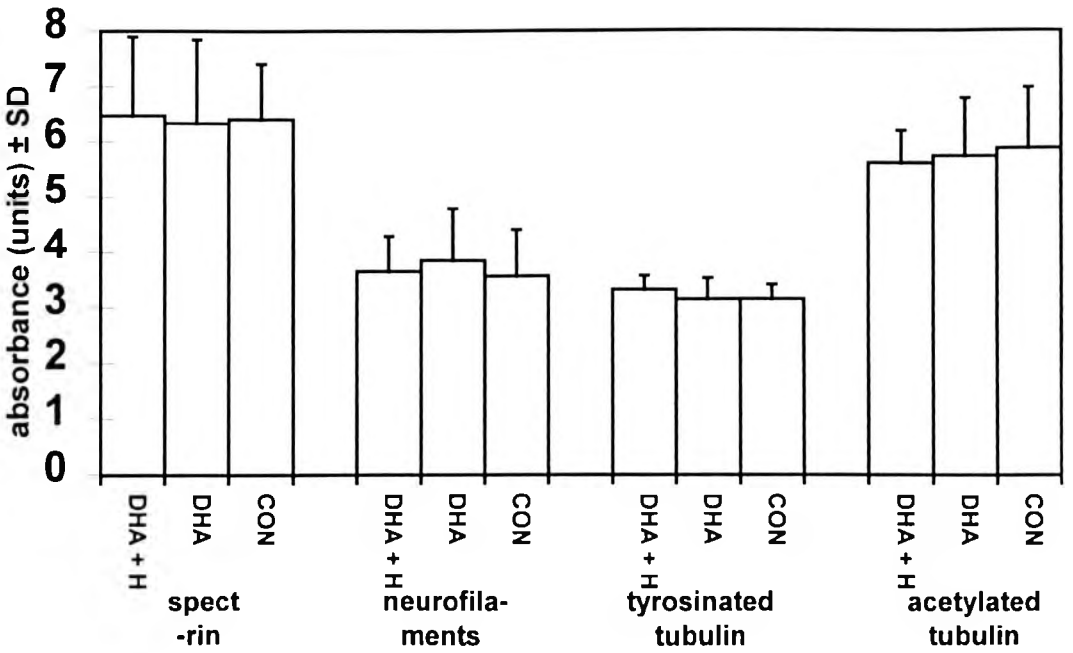


(b) non-phosphorylated NF-H.

(c) tyrosinated α-tubulin.

(d) acetylated α-tubulin.

(e) densitometric analysis of Western blots.

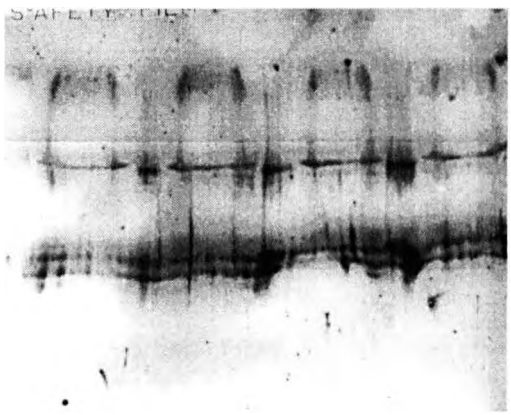


annua plant. However, they failed to detect any artemisinin derivatives on Western blots and also failed to detect dihydroartemisinin on dot blots of protein incubated with the drug, but they did recognise globulins in controls and drug-treated cells (figure 3.8). The antibody probably recognises the tricyclic ring structure of the artemisinin molecule, which here opens up to produce a reactive free radical molecule which can bind to proteins. Consequently, the antibodies may not be able to detect the dihydroartemisinin molecule when it is bound to neural proteins and this may explain the lack of reactivity of this antibody.

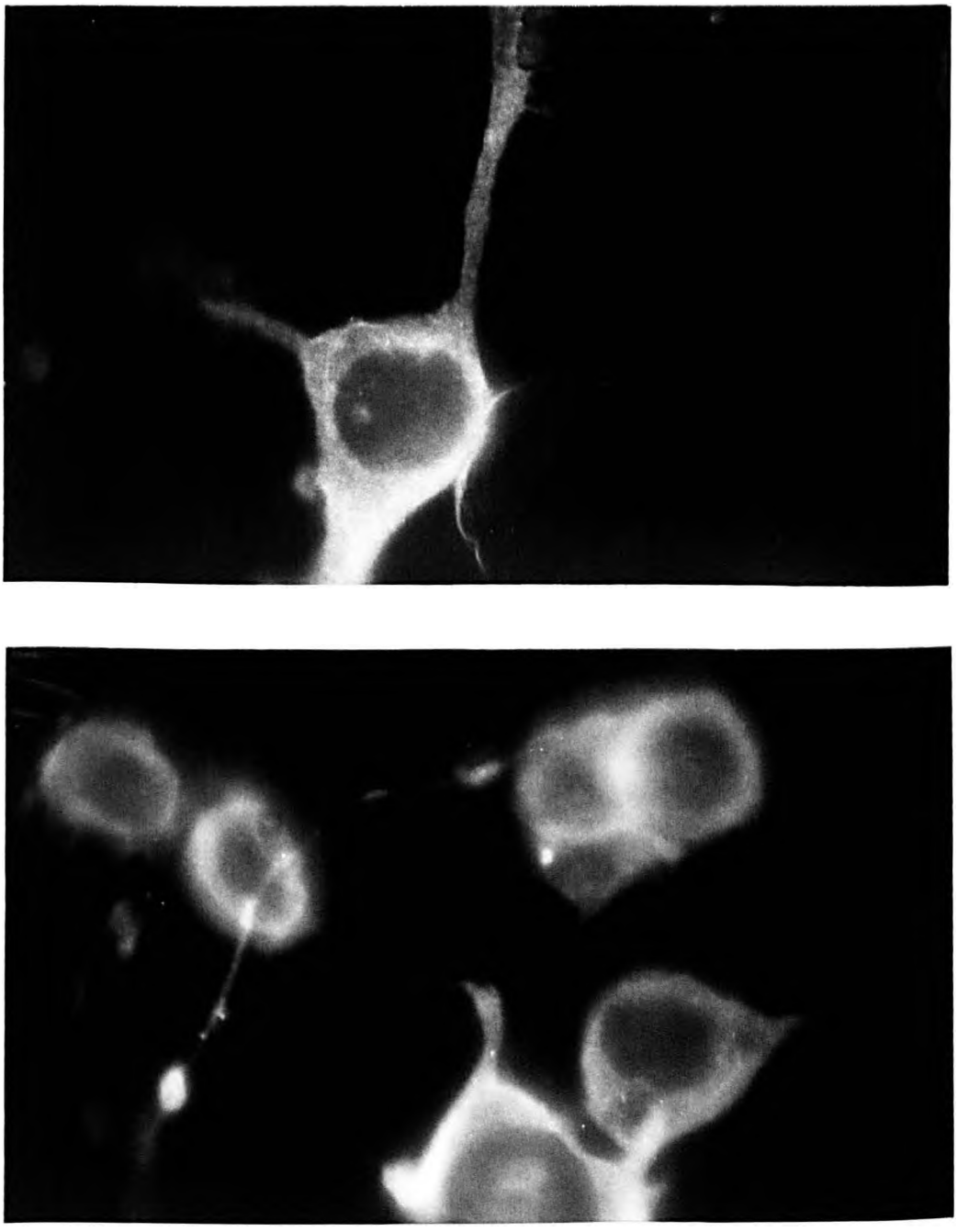
3.2.3. Indirect immunofluorescence of fixed Nb2a cells

Some drug effects on cell proteins are not detectable on gels. Indirect immunofluorescence of fixed Nb2a cells using monoclonal antibodies directed against common targets for neurotoxic agents may reveal changes in the status or distribution of proteins within the cell not apparent on Western blots or silver-stained gels. Consequently, fixed differentiated Nb2a cells were incubated with monoclonal antibodies directed against the cytoskeletal proteins: spectrin (MAb101AA6), phosphorylated neurofilament NF-H (Ta51), acetylated α-tubulin (611β1), and tyrosinated α-tubulin (YL 1/2) (N = 4). Fluorescent microscopic examination of these cells revealed no drug effects on the distribution of spectrin, neurofilament proteins, and tubulin in the neurites or soma of Nb2a cells. In both drug-treated and control cells spectrin was distributed throughout the cell and neurites but its main location was beneath the membrane of the cell body

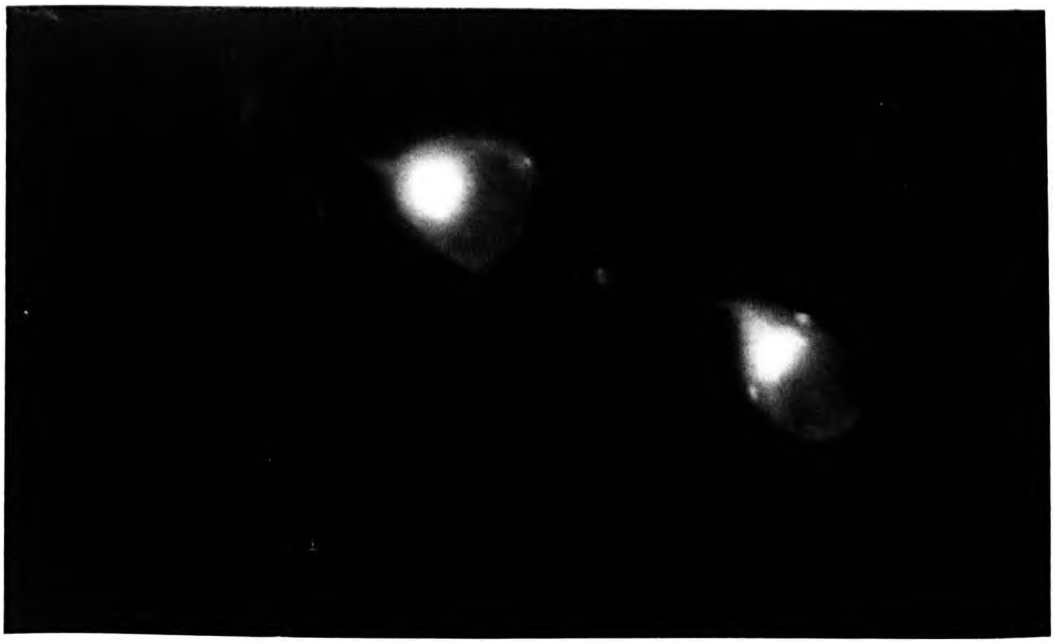
Figure 3.8. Western Blot of dihydroartemisinin-treated rat cortex and brain stem proteins probed with V41 artemisinin antibodies. Homogenate incubations and Western blotting were performed as outlined in figures 3.6 and 3.7. Cortex and brainstem proteins were probed with V41 antibodies which detect artemisinin derivatives. The proteins detected by the antibodies were present in both dihydroartemisinin-treated homogenate and in control homogenate where no dihydroartemisinin was present. Western blots of Nb2a cells produced a similar result. Bands on blots, left to right: **(1)** dihydroartemisinin-treated brainstem **(2)** control brainstem **(3)** dihydroartemisinin-treated cortex **(4)** control cortex.

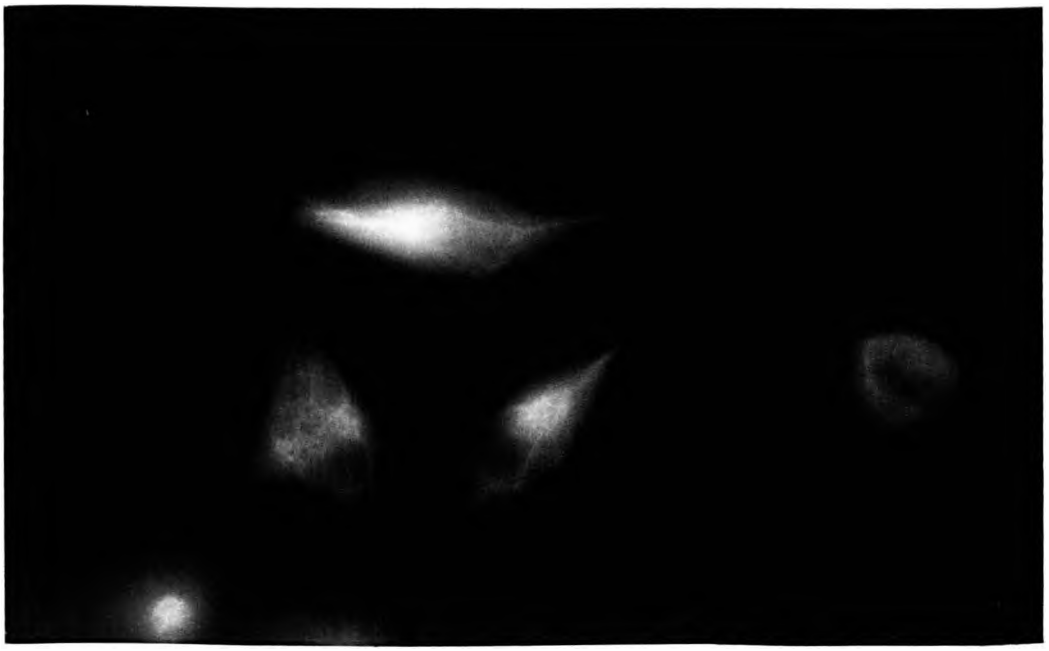


Figures 3.9a & b. Indirect immunofluorescence of fixed differentiated Nb2a cells. A typical photograph of one of four similar experiments. Nb2a cells grown on LabTek slides were incubated with 2 μM dihydroartemisinin and 0.5 mM dbcAMP in serum-free medium for 24 h, fixed with formaldehyde and incubated with monoclonal antibodies directed against spectrin (antibody: MAb101AA6). This was followed by incubation with fluorescein-conjugated rabbit anti-mouse immunoglobulins and fixed under a coverslip with a polyvinyl alcohol-based mount. Dihydroartemisinin did not appear to affect the amount or distribution of these proteins within the cells. **Top:** dihydroartemisinin-treated cells, and **bottom:** control.

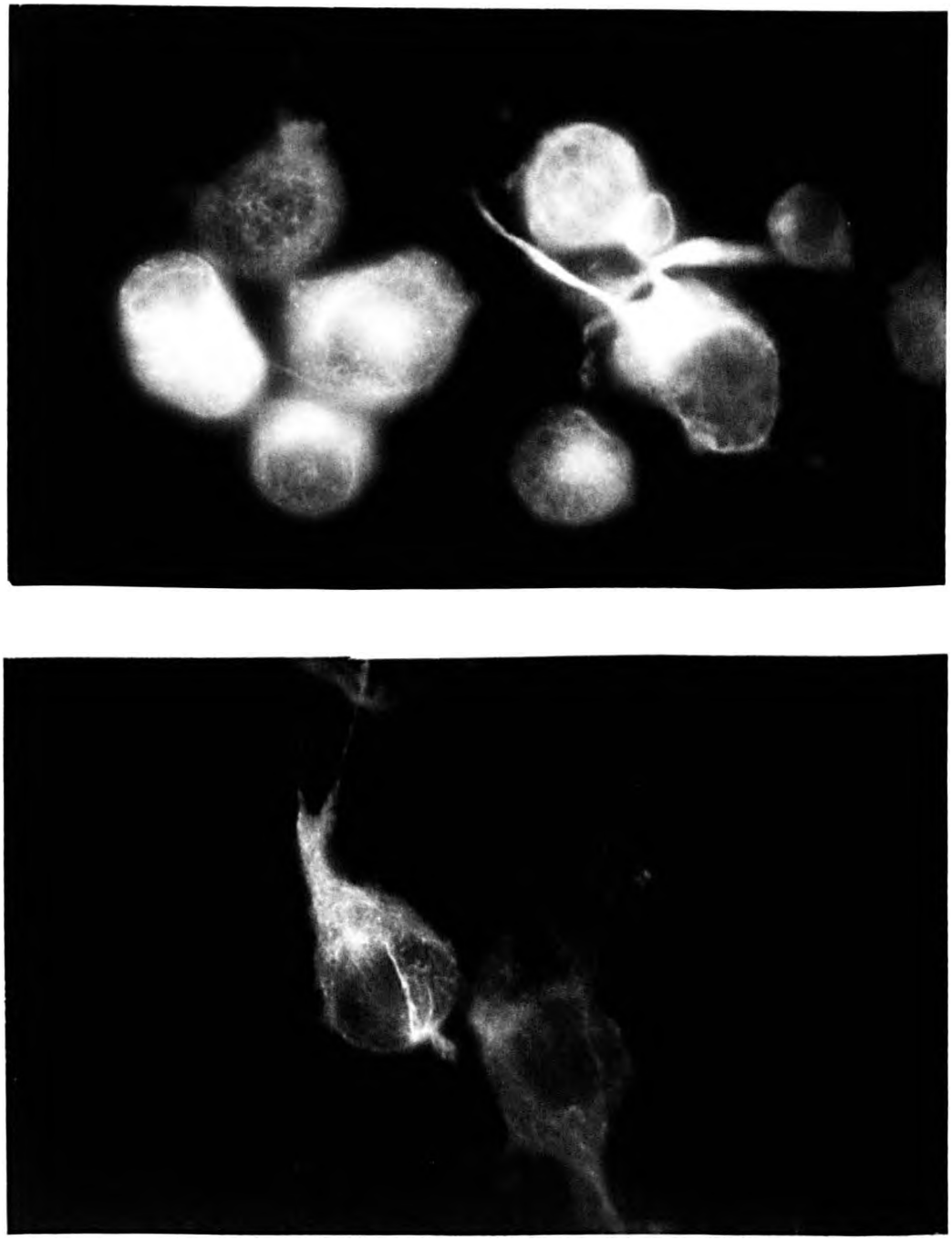


Figures 3.9c & d. Indirect immunofluorescence of fixed differentiated Nb2a cells. A typical photograph of one of four similar experiments. Nb2a cells grown on LabTek slides were incubated with 2 μM dihydroartemisinin and 0.5 mM dbcAMP in serum-free medium for 24 h, fixed with formaldehyde and incubated with monoclonal antibodies directed against phosphorylated neurofilament NF-H (Ta51). This was followed by incubation with fluorescein-conjugated rabbit anti-rat immunoglobulins and fixed under a coverslip with a polyvinyl alcohol-based mount. Dihydroartemisinin did not appear to affect the amount or distribution of these proteins within the cells. **Top:** dihydroartemisinin-treated cells, and **bottom:** control.

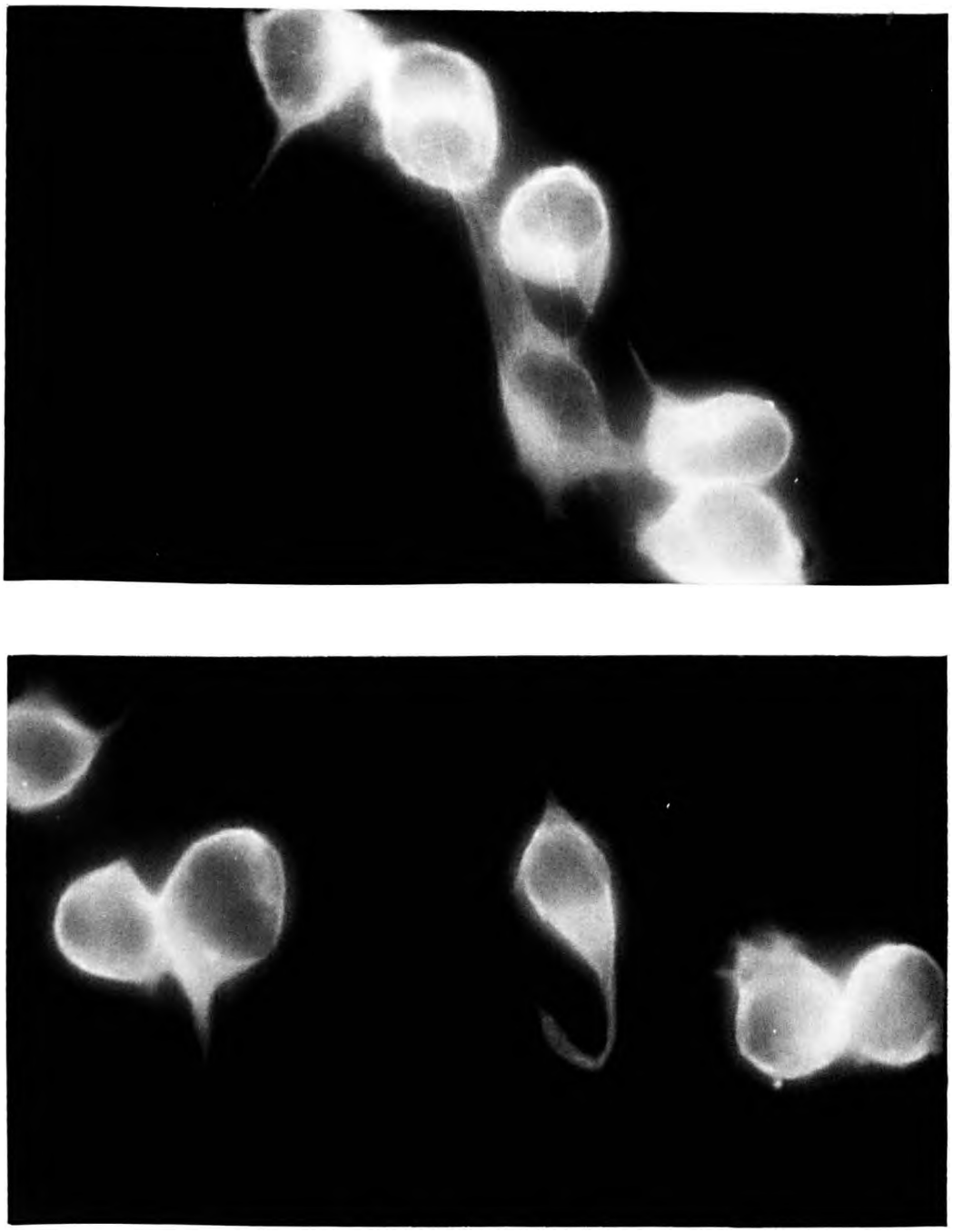




Figures 3.9e & f. Indirect immunofluorescence of fixed differentiated Nb2a cells. A typical photograph of one of four similar experiments. Nb2a cells grown on LabTek slides were incubated with 2 μM dihydroartemisinin and 0.5 mM dbcAMP in serum-free medium for 24 h, fixed with formaldehyde and incubated with monoclonal antibodies directed against acetylated α-tubulin (611β1). This was followed by incubation with fluorescein-conjugated rabbit anti-mouse immunoglobulins and fixed under a coverslip with a polyvinyl alcohol-based mount. Dihydroartemisinin did not appear to affect the amount or distribution of these proteins within the cells. **Top:** dihydroartemisinin-treated cells, and **bottom**: control.



Figures 3.9g & h. Indirect immunofluorescence of fixed differentiated Nb2a cells. A typical photograph of one of four similar experiments. Nb2a cells grown on LabTek slides were incubated with 2 μM dihydroartemisinin and 0.5 mM dbcAMP in serum-free medium for 24 h, fixed with formaldehyde and incubated with monoclonal antibodies directed against tyrosinated α-tubulin (YL 1/2). This was followed by incubation with fluorescein-conjugated rabbit anti-rat immunoglobulins and fixed under a coverslip with a polyvinyl alcohol-based mount. Dihydroartemisinin did not appear to affect the amount or distribution of these proteins within the cells. **Top:** dihydroartemisinin-treated cells, and **bottom:** control.



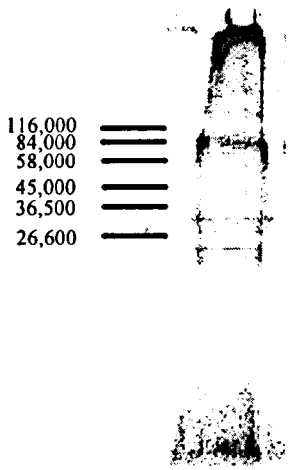
(figures 3.9a & b); phosphorylated neurofilaments were mainly located in the cell bodies at the base of the neurites, although there was also Ta51 staining in the neurites (figures 3.9c & d); acetylated tubulin was also present in both the neurites and cell body but was more evenly distributed throughout the cell than either spectrin or neurofilaments and individual microtubules, or bundles of microtubules, are visible in the cell body (figures 3.9e & f); finally, tyrosinated tubulin staining, like that of spectrin, was distributed throughout the cell body and neurites but was particularly prominent under the cell membrane, individual bundles of filaments were not visible (figures 3.9g & h). "No primary antibody" control assays did not stain the cells, confirming that staining was specific and that labelling by the primary antibodies was responsible for the immunofluorescent staining of cell proteins.

3.2.4. Autoradiography

Identification of the target proteins of the artemisinin derivatives by searching for drug effects on the common targets for neurotoxic agents has proved inconclusive. An alternative approach is to use dihydroartemisinin itself, rather than antibodies directed against specific proteins, as the probe. This was attempted using the Jaziri / Ferreira artemisinin antibodies on Western blots and here using ^{14}C-labelled dihydroartemisinin to radiolabel the target proteins of the drugs.

Nb2a cells, C6 cells and rat cortex homogenate proteins that had been incubated with ^{14}C-labelled dihydroartemisinin were separated on 15% polyacrylamide gels (N = 4) and exposed to film at -80°C for 1 to 3 months. Gels of

Figure 3.10. SDS-polyacrylamide gel autoradiograph of rat brain proteins labelled with ^{14}C-dihydroartemisinin. A typical gel from one of four similar experiments. Rat cortex homogenate was incubated with 187 μM of ^{14}C-dihydroartemisinin as outlined in figure 3.28, separated on polyacrylamide gels as outlined in figure 3.5 and exposed to photographic film for three months. Several proteins were radiolabelled with ^{14}C-dihydroartemisinin. These proteins had masses of approximately 24 kilodaltons (kDa), 32 kDa and 84 kDa. Nb2a and C6 cell gels did not possess strongly labelled protein bands.

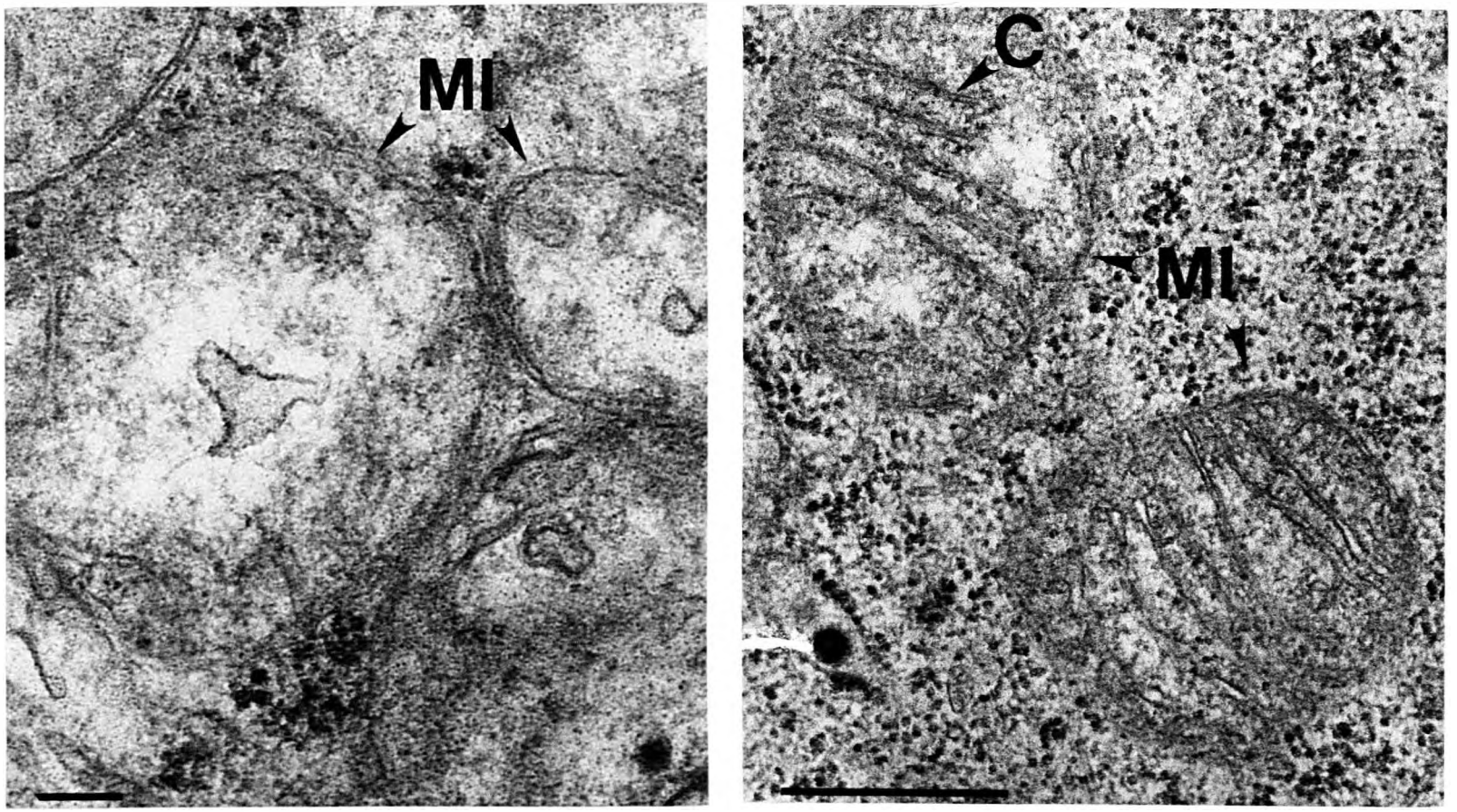


rat cortex homogenate had several radiolabelled protein bands which were of apparent molecular weights of approximately 84 kilodaltons (kDa), 32 kDa and 24 kDa (figure 3.10). Although C6 and Nb2a cells bound ^{14}C-dihydroartemisinin, the proteins did not resolve into bands on the gels which would allow estimation of either the number or mass of the proteins.

3.2.5. Transmission electron microscopy of dihydroartemisinin-treated Nb2a cells

Transmission electron microscopy of differentiated Nb2a cells treated with 2 μM dihydroartemisinin (N = 4) was performed to examine the interior of the cells for structural damage. The transmission electron micrographs revealed that after 24 h exposure the mitochondria of the cells in both the cell body and in the neurites were damaged by dihydroartemisinin. The cristae of the mitochondria were absent or deformed in comparison to control cells and the outer membranes of these organelles were often ruptured (figures 3.11a & b). Dihydroartemisinin also affected the rough endoplasmic reticulum (RER) of the cells; in cells treated with dihydroartemisinin there was little visible RER in comparison with control cells (figures 3.12c & d), possibly due to ribosome dissociation, and that which was visible was small and often slightly swollen (figures 3.12a & b). Surprisingly, given the toxicity of the artemisinin derivatives towards neurite outgrowth, no dihydroartemisinin effects were seen on the microtubules or neurofilaments in cell neurites (figure 3.13a & b). Dihydroartemisinin did not appear to affect the nuclei of the cells (not pictured).

Figures 3.11a & b. Transmission electron micrographs of the effect of dihydroartemisinin on Nb2a cell mitochondria (MI). Typical micrographs of four similar experiments. Nb2a cells were plated onto 8-well LabTek slides and cells were induced to differentiate in serum-free medium containing dibutyryl cyclic AMP and 2 μM dihydroartemisinin. Cells were fixed in glutaraldehyde, postfixed in osmium tetraoxide, infiltrated with Epon araldite resin, and sections cut parallel to the culture substrate then stained uranyl acetate and Reynolds lead citrate and examined by transmission electron microscopy in a Philips CM10 TEM at 80 kV. **Left: (a)** Mitochondria of Nb2a cell incubated with 2 μM dihydroartemisinin for 24 h. The cristae (C) of the mitochondria are absent or deformed. The membranes of some the mitochondria also appeared to be damaged. Bar = 100 nm. **Right: (b)** Mitochondria of control cells. The membranes of the cells are intact and the cristae are regular in appearance. Bar = 500 nm.



Figures 3.12a & b. Transmission electron micrographs of the effect of dihydroartemisinin on Nb2a rough endoplasmic reticulum (RER)(labelled ER). Typical micrographs of four similar experiments. Incubations and preparation of cells for transmission electron microscopy were as outlined in figure 3.11. **Left: (a).** Nb2a cell incubated with 2 μM dihydroartemisinin for 24 h. Bar = 500 nm. Note the swelling and shortening of RER. **Right: (b).** Cytoplasm of control Nb2a cell. Normal RER with its associated ribosomes. Bar = 500 nm.

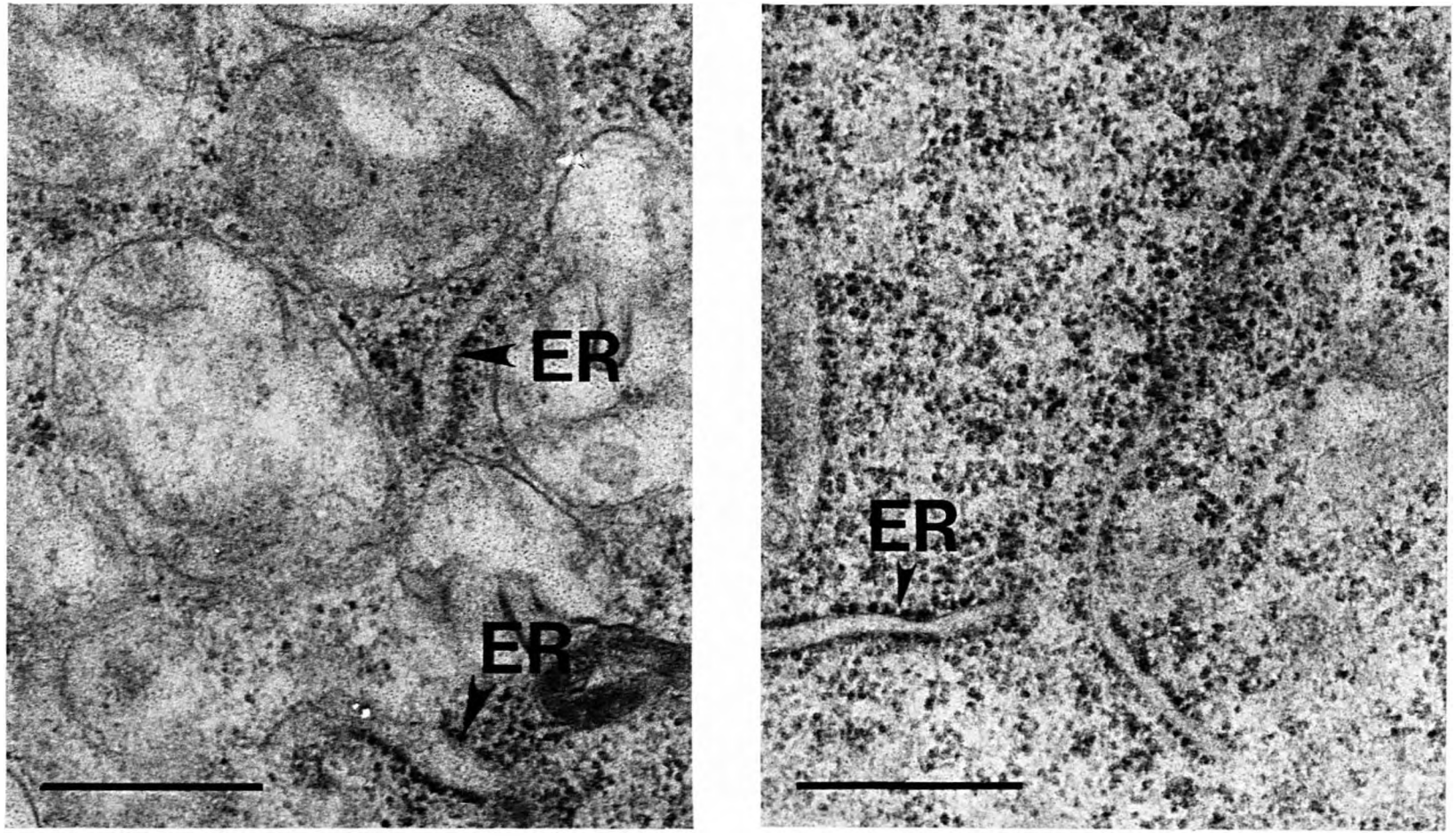


Figure 3.12c. Transmission electron micrograph of the effect of dihydroartemisinin on Nb2a rough endoplasmic reticulum (RER). A typical micrograph from one of four similar experiments. Incubations and preparation of cells for transmission electron microscopy were as outlined in figure 3.11. In cells treated with dihydroartemisinin there seemed to be less RER (arrows) in the cytoplasm compared to control cells (figure 3.12d, following page), and that which remained was often swollen. Bar = 1 μm.

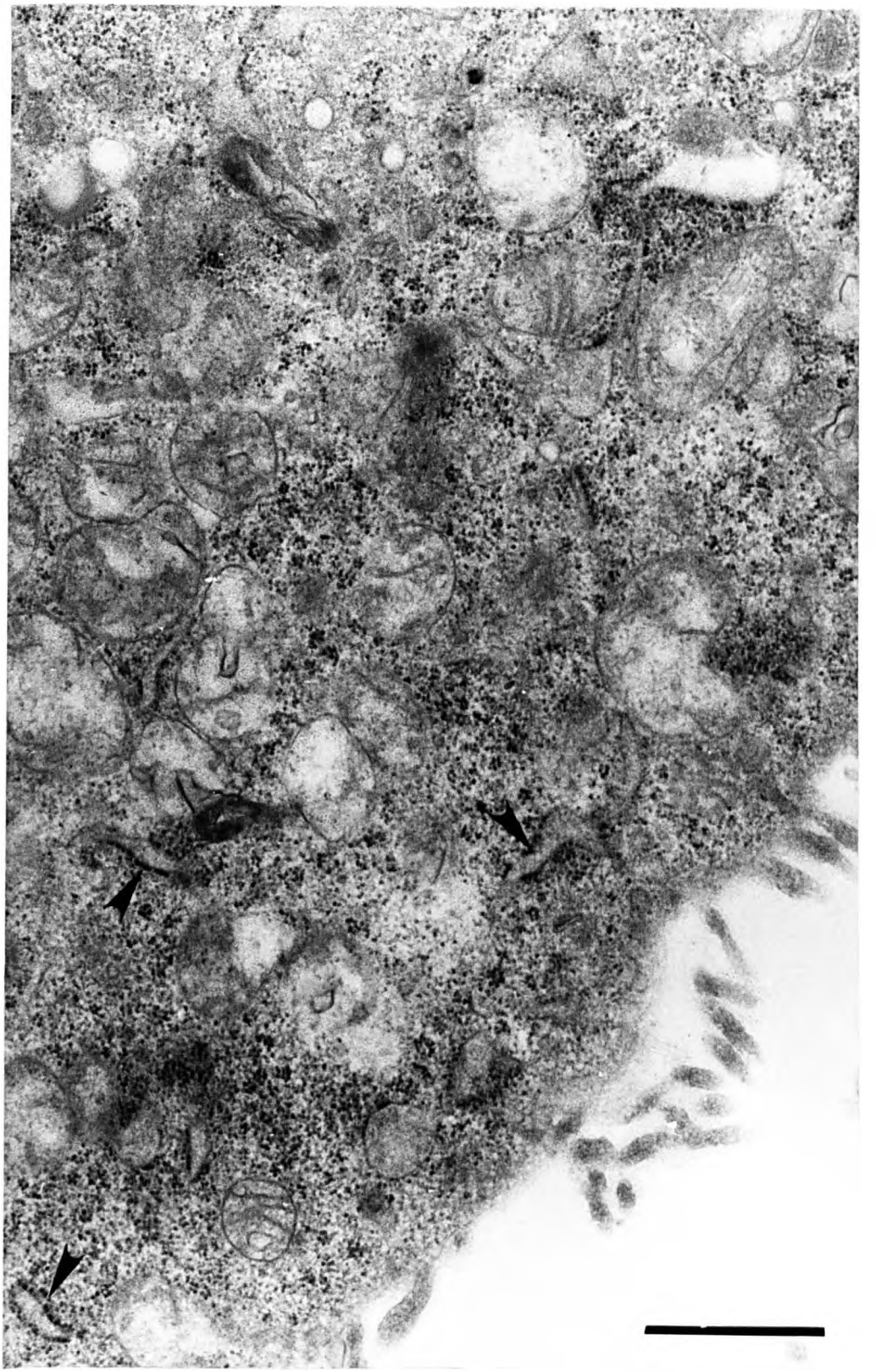
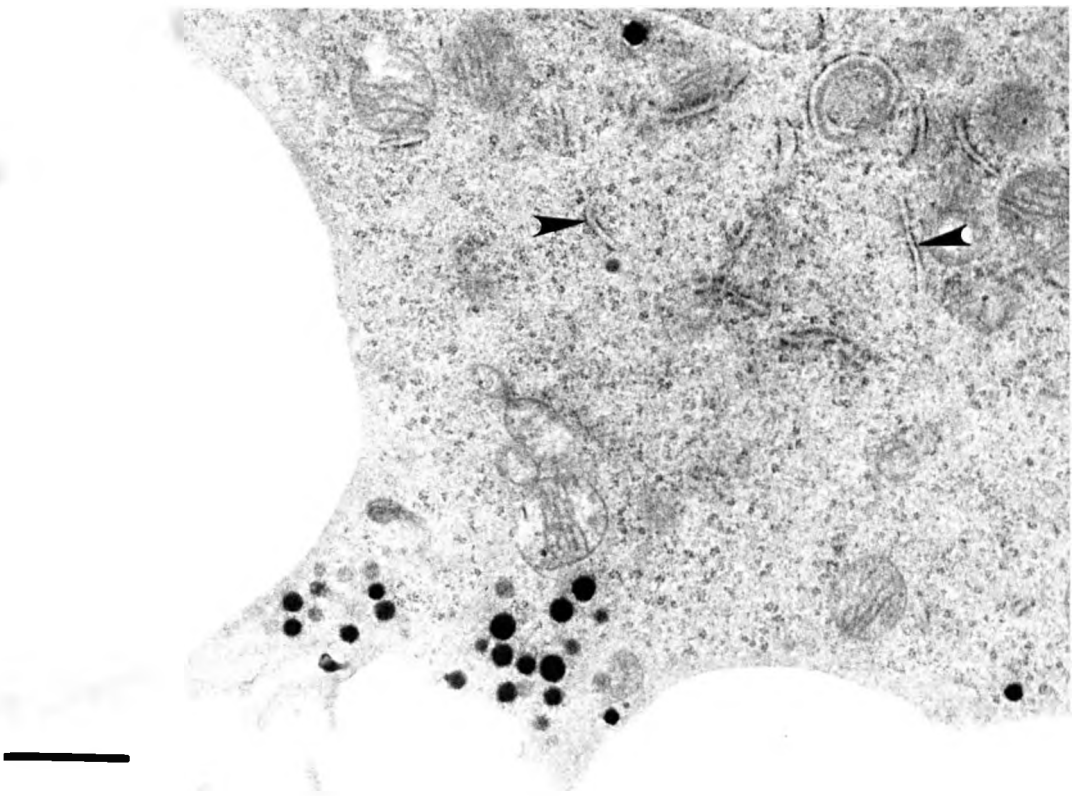
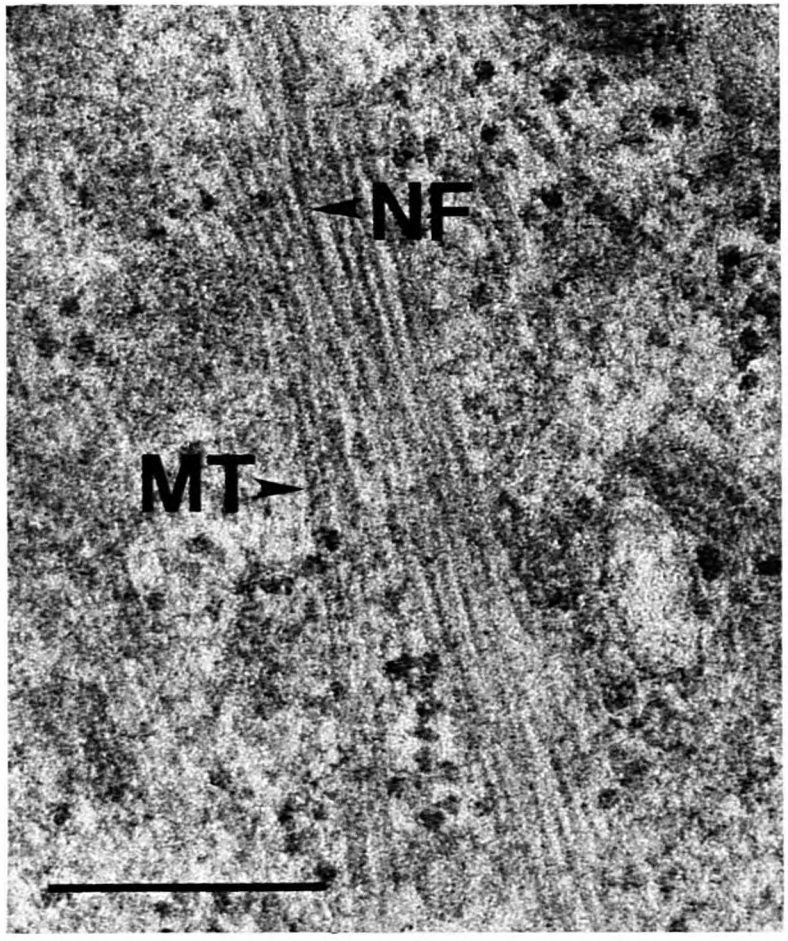


Figure 3.12d. Transmission electron micrograph of the rough endoplasmic reticulum (RER) (arrows) of a control cell. A typical micrograph from one of four similar experiments. Incubations and preparation of cells for transmission electron microscopy were as outlined in figure 3.11. Bar = 1 μm.



Figures 3.13a & b. Transmission electron micrographs of the effect of dihydroartemisinin on the neurite cytoskeleton of differentiated Nb2a cells. Typical micrographs of four similar experiments. Incubations and preparation of cells for transmission electron microscopy were as outlined in figure 3.11. **Left: (a).** Neurite of differentiated Nb2a cell incubated with 2 μM dihydroartemisinin for 24 h. The cytoskeletal proteins of the neurite, the microtubules (MT) and neurofilaments (NF), are undamaged by the drug. Bar = 250 nm. **Right: (b)** Neurite cytoskeleton of control cell. Bar = 250 nm.



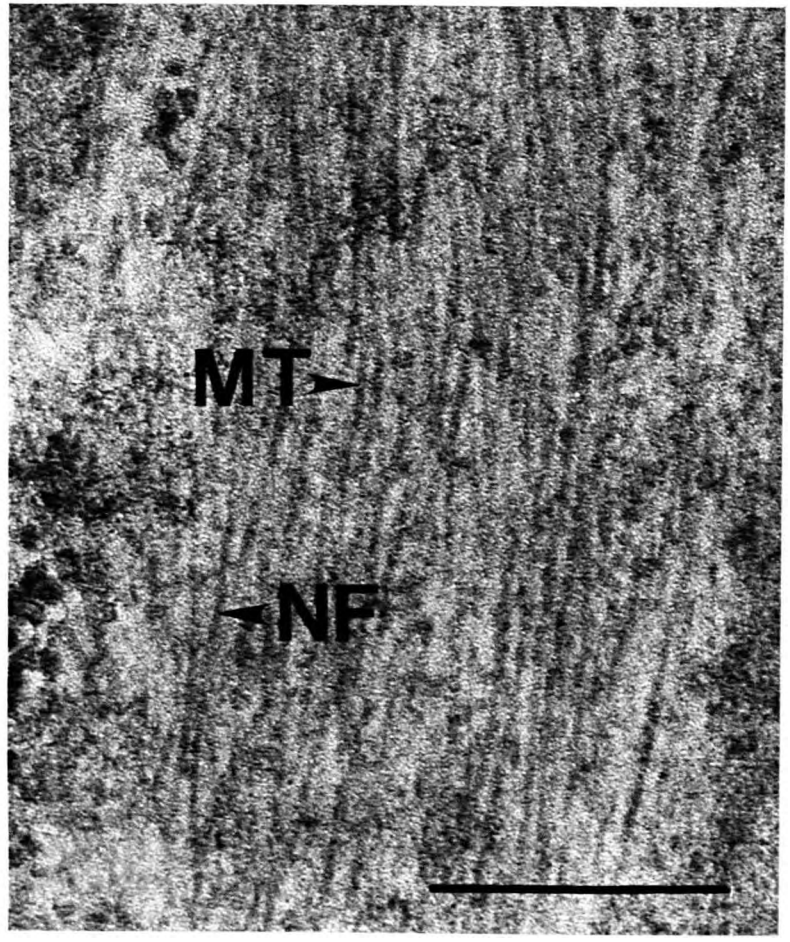
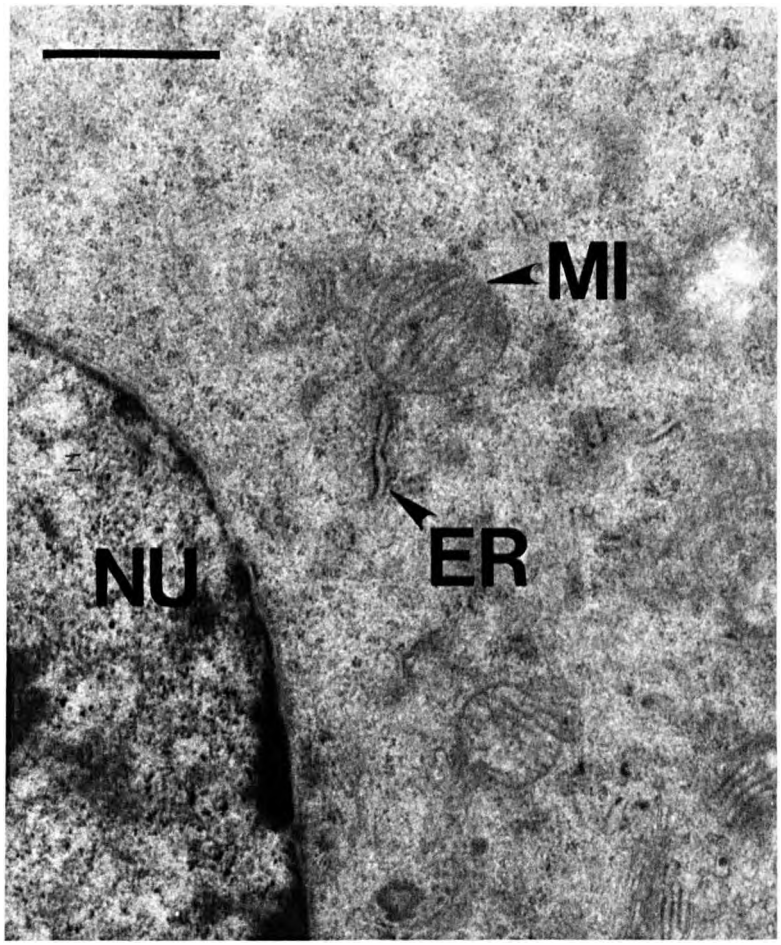


Figure 3.14. Transmission electron micrograph of Nb2a cell treated with 2 μM desoxyartemisinin. A typical micrograph from one of four similar experiments. Incubation and preparation of cells for transmission electron microscopy were as outlined in figure 3.11. No damage to any of the cell organelles or membranes is apparent. NU = nucleus. Bar = 1 μm.

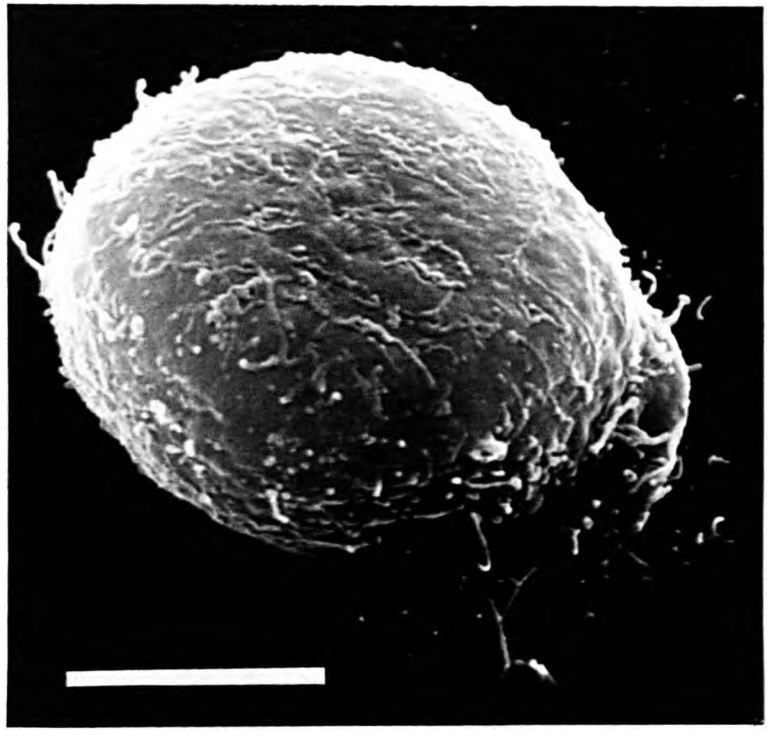


Nb2a cells treated with 2 μM desoxyartemisinin (N = 4), the artemisinin metabolite that lacks the endoperoxide bridge pharmacophore, showed no signs of drug-induced damage and the targets for dihydroartemisinin toxicity, the RER and mitochondria, appeared unaffected by this compound (figure 3.14).

3.2.6. Scanning electron microscopy of dihydroartemisinin-treated Nb2a cells

Scanning electron microscopy of differentiated Nb2a cells treated with 2 μM dihydroartemisinin (N = 4) was performed to examine the exterior of the cells for structural damage. The scanning electron micrographs revealed that dihydroartemisinin had a very noticeable effect on the cell membranes (figure 3.15a). The surface of control cell bodies and neurites were covered with processes commonly known as microspikes (Brinton *et al.*, 1994; Smith, 1994a; Schwartz-Albiez *et al.*, 1995; Jackson *et al.*, 1996), that projected away from the cell surface (figure 3.15b). These processes were far less numerous on the cell bodies, and to a lesser extent, the neurites of the drug-treated cells. Some dihydroartemisinin-treated cells had these processes but they lay flat against the cell body and appeared damaged (figure 3.15c). The growth cones of cell neurites were not obviously affected by dihydroartemisinin and no damage other than that to microspikes / filopodia was apparent on the surface of cell neurites (figures 3.16a & b). Again cells treated with desoxyartemisinin (N = 4) showed no abnormalities (figure 3.17).

Figures 3.15a & b. Scanning electron micrographs of Nb2a cells treated with 2 μM dihydroartemisinin. Typical micrographs of four similar experiments. For scanning electron microscopy, cultured cells grown on LabTek slides were prepared as for transmission electron microscopy through fixing with glutaraldehyde. The plastic chambers were then removed leaving the setting gasket in place, and the slide was critical point-dried in a Polaron critical point dryer. After drying, the slide was sputter-coated with gold, and examined in a Hitachi S-520 microscope at 10 kV. **Left: (a)** Dihydroartemisinin-treated Nb2a cell. There are few apical microspikes projecting from the cell membrane or filopodial microspikes projecting along the extracellular matrix. Bar = 6 μm. **Right: (b)** Control cells with many filopodia-like processes projecting from the cell surface. Bar = 12 μm.



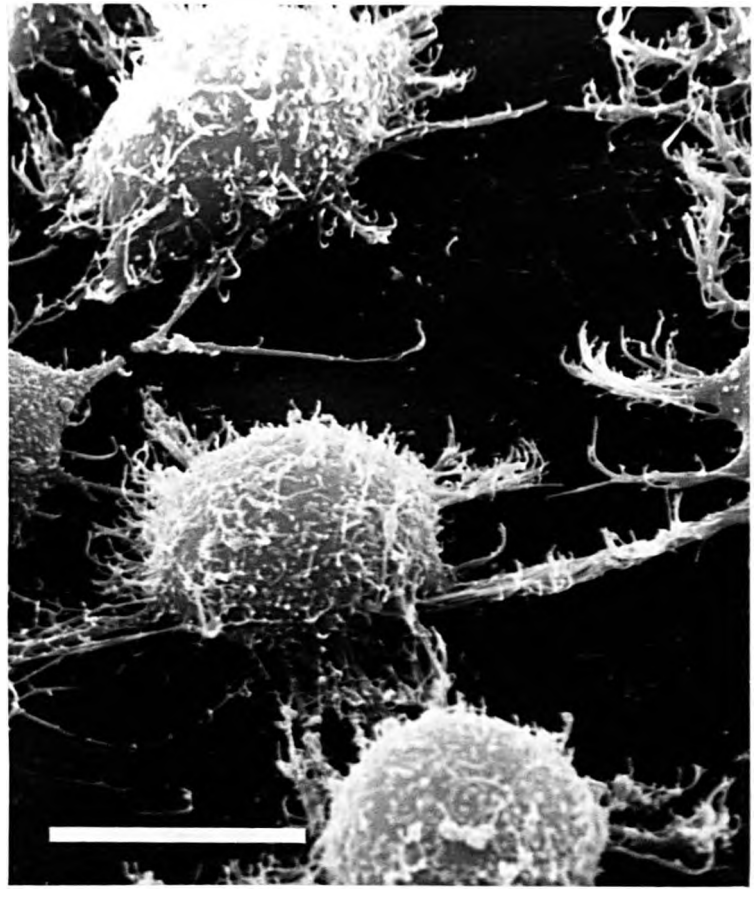
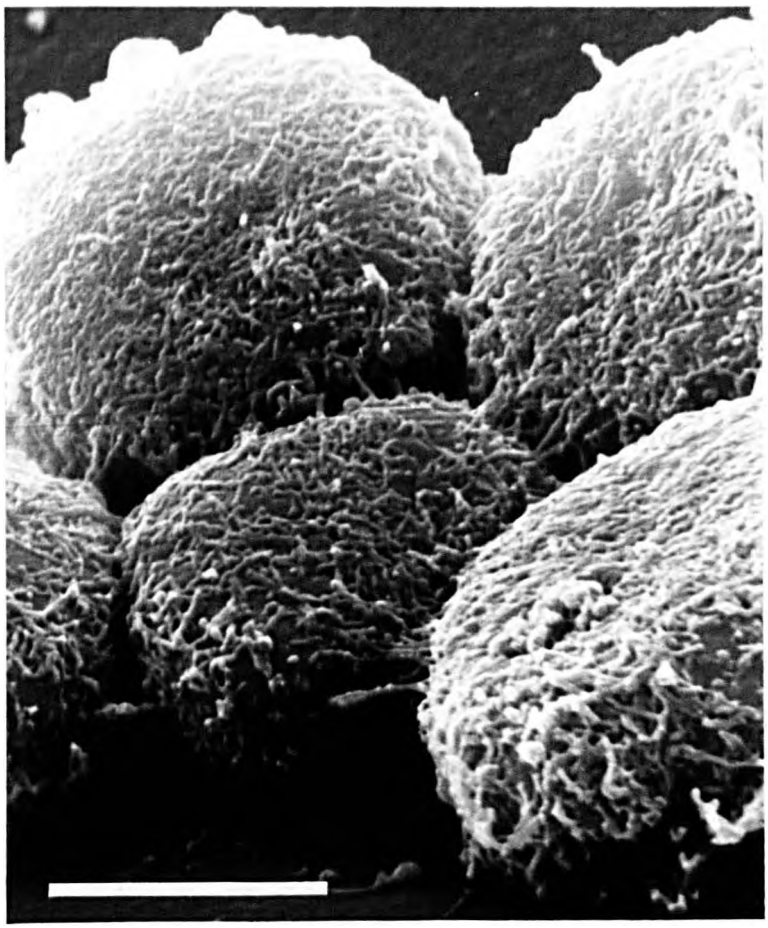
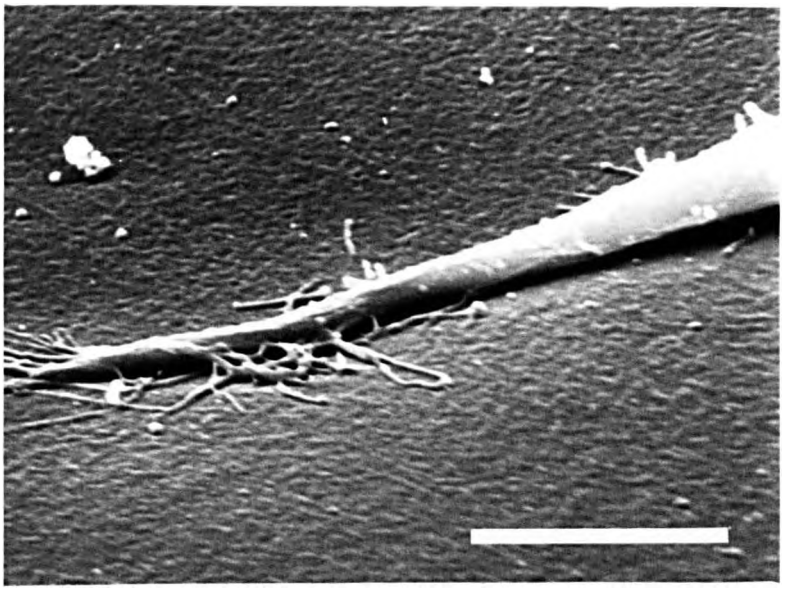


Figure 3.15c. Scanning electron micrographs of Nb2a cells treated with 2 μM dihydroartemisinin. A typical micrograph from one of four similar experiments. Incubation and preparation of cells for scanning electron microscopy were as outlined in figure 3.15. Some drug-treated cells retain their microspike processes, but they appear damaged and lie flat against the membrane surface. Bar = 6 μm.



Figures 3.16a & b. Scanning electron micrographs of the neurites of Nb2a cells treated with 2 μM dihydroartemisinin. Typical micrographs of four similar experiments. Incubation and preparation of cells for scanning electron microscopy were as outlined in figure 3.15. **Top: (a)** Growth cone of dihydroartemisinin-treated Nb2a cell. No damage or deformation of the growth cone of the neurite is apparent. Bar = 6 μm. **Bottom: (b)** Growth cone of control cell. Bar = 6 μm.



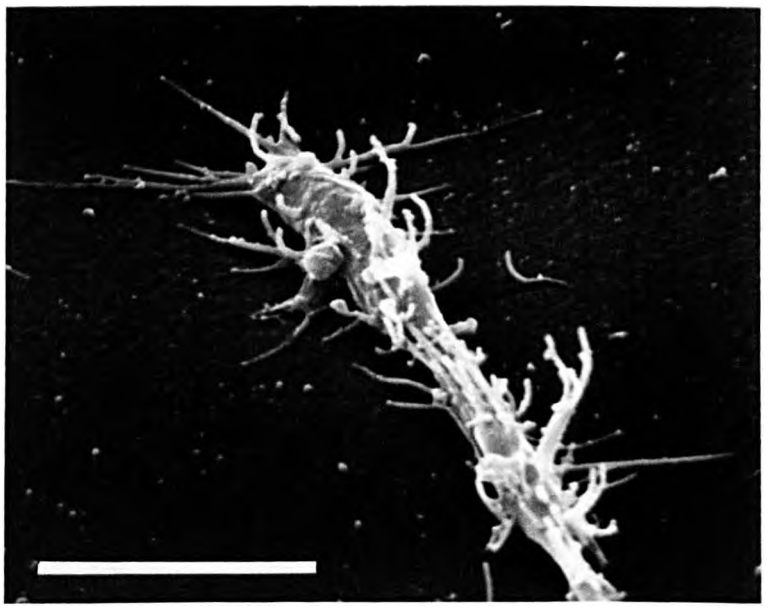
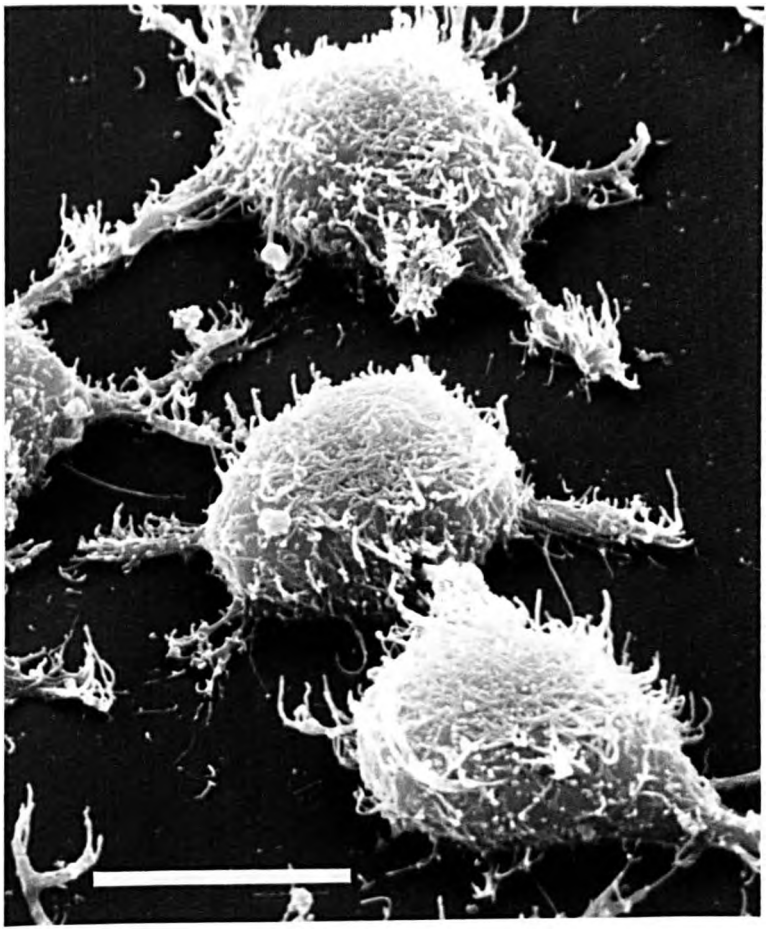


Figure 3.17. Scanning electron micrograph of Nb2a cells treated with 2 μM desoxyartemisinin. A typical micrograph from one of four similar experiments. Incubation and preparation of cells for scanning electron microscopy were as outlined in figure 3.15. Desoxyartemisinin-treated Nb2a cells appear unharmed by the drug. Bar = 12 μm.



PART THREE

3.3. VALIDATION OF NB2A CELLS AS A MODEL SUITABLE FOR THE ASSESSMENT OF NEUROTOXICITY

The intention of this study was to use the Nb2a neuroblastoma cell as the primary model for studying the neurotoxicity of the artemisinin derivatives *in vitro*. The results of section 3.1 suggested that this cell line was very susceptible to the toxicity of the artemisinin derivatives. Was this neurotoxicity or a general deleterious effect on the cells ?

Nb2a cell proliferation as a parameter for the measurement of artemisinin derivative neurotoxicity was assessed by comparing the effects of dihydroartemisinin on Nb2a cell proliferation with its effects on the proliferation of two non-neuronal cell lines. If Nb2a proliferation was a good parameter for assessing the neurotoxicity of artemisinin derivatives then one would expect proliferating Nb2a cells to show a greater sensitivity to their effects than non-neuronal cell lines.

The sensitivity of the neurite outgrowth parameter to the toxicity of the artemisinin derivatives in comparison with cell proliferation suggests that it may be the better model for neurotoxicity. To test this hypothesis the effects of two neurotoxic agents and two non-specific toxic agents on Nb2a cell proliferation and

neurite outgrowth were examined. If Nb2a neurite outgrowth was a good parameter for neurite outgrowth then the neurotoxins would inhibit neurite outgrowth to a greater degree than cell proliferation and the non-specific toxins would not.

The effects of dihydroartemisinin on Nb2a cell neurite outgrowth were compared with those of a range of known neurotoxic agents. This was carried out partly to determine the potency of dihydroartemisinin as a neurotoxic agent in comparison to other neurotoxins and partly to gain an insight into the mechanism of dihydroartemisinin toxicity; each of the neurotoxic agents has a different putative mechanism of action and by comparing their effects on the differentiated Nb2a cells with those of dihydroartemisinin by light and (and in some cases) electron microscopy it was hoped that some similarities between the effects of dihydroartemisinin and those of other known neurotoxins would be observed. Additionally, if the effects of the neurotoxic agents on differentiating Nb2a cells resembled their effects *in vivo* then this would provide further support for the use of Nb2a neurite outgrowth as a useful parameter with which to measure neurotoxicity.

3.3.1. Comparison of the Nb2a mouse neuroblastoma with two non-neuronal cell lines

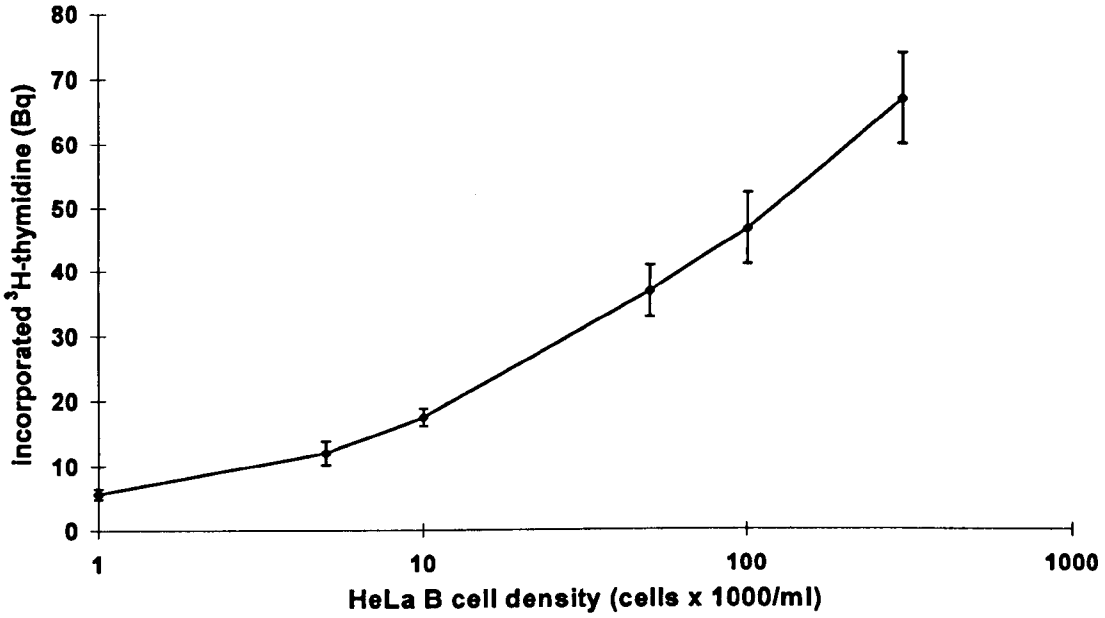
These assays (N = 6) were performed as it was necessary to determine if the proliferating Nb2a neuroblastoma cell was a good model in which to study the neurotoxic effects of dihydroartemisinin, to see if the toxicity already observed in Nb2a cells was a manifestation of neurotoxicity of dihydroartemisinin or a non-

specific toxicity which would equally affect non-neuronal cell lines. The effects of the artemisinin derivative dihydroartemisinin on Nb2a cell proliferation was compared with its effect on the proliferation of two other non-neuronal immortalised cell lines, CRFK feline kidney carcinoma and HeLa B, a human cervical carcinoma; this was done by measuring the inhibitory effects of dihydroartemisinin on ^{3}H-thymidine incorporation by the three cell lines. HeLa B and CRFK cells were plated onto 96-well plates at densities ranging from 1000 to 300000 cells/ml and appropriate cell densities for these assays were determined as outlined in section 2.3.2. In subsequent proliferation assays HeLa B cells were used at a cell density of 5000 cells/ml and CRFK cells were used at a density of 10000 cells/ml (figures 3.18a & b). Dihydroartemisinin toxicity towards the proliferation of these cell lines gave IC_{50} values of 0.98 μM for Nb2a cells, 1.37 μM for HeLa B cells and 4.09 μM for CRFK cells. There was no significant difference in the IC_{50} values for Nb2a and HeLa B proliferation inhibition ($p > 0.05$) but the IC_{50} value for CRFK proliferation inhibition was significantly greater than the IC_{50} values for Nb2a and HeLa B proliferation ($p = 0.001$ in both cases).

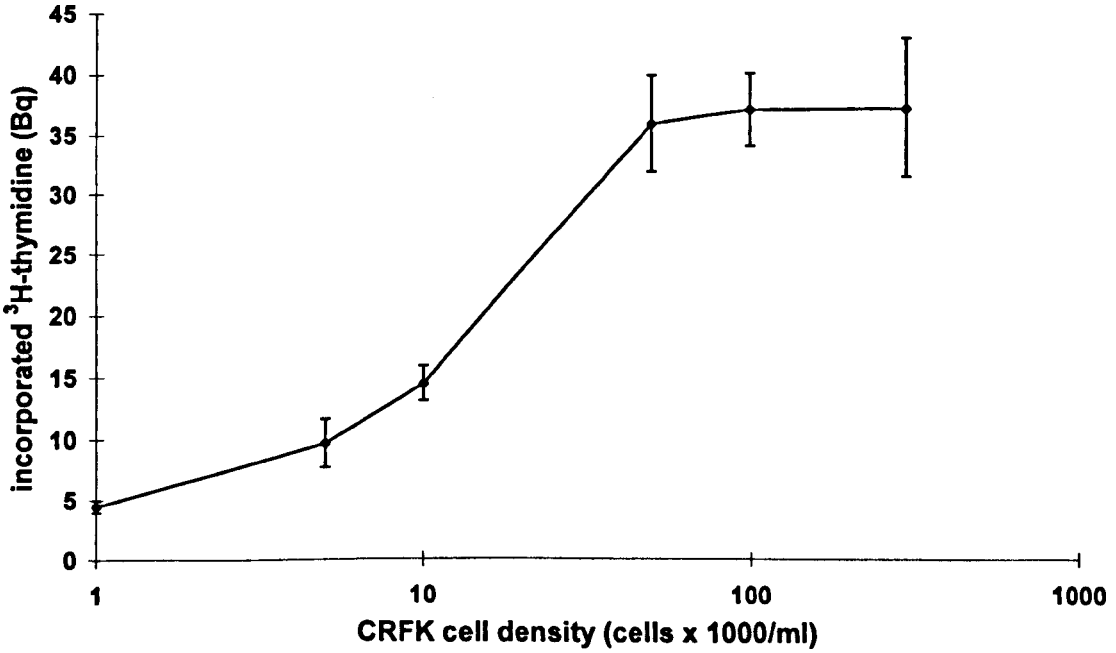
These results demonstrated that although Nb2a cells seemed marginally more sensitive to the toxic effects of dihydroartemisinin (figure 3.19 and table 3.3) there was no real difference between proliferating neuronal and non-neuronal cell sensitivity to the compound. These cells are immortalised tumour cell lines whose shared characteristics as cancer cells may outweigh any differences of phenotype. Consequently, Nb2a cell proliferation may not be the best model for assessing neurotoxicity *in vitro* and a preferable approach could be measurement of drug

Figure 3.18. Assays to determine appropriate cell densities for subsequent cell proliferation assays. **(a)** Assay to determine an appropriate HeLa B cell density for proliferation assays. **(b)** Assay to determine an appropriate CRFK cell density for proliferation assays. HeLa B and CRFK cells were plated onto 96-well plates at densities ranging from 1000 to 300000 cells/ml and incubated for 3 days. Twenty-four hours before the end of the incubation 740 Bq of ^{3}H-thymidine was added to each well. Cells were then harvested by vacuum filtration and radioactivity incorporated into cell DNA measured by liquid scintillation counting. An appropriate cell density for proliferation assays is one which is near the centre of the positive gradient of the curve. Values represent means ± standard deviations.

(a)



(b)



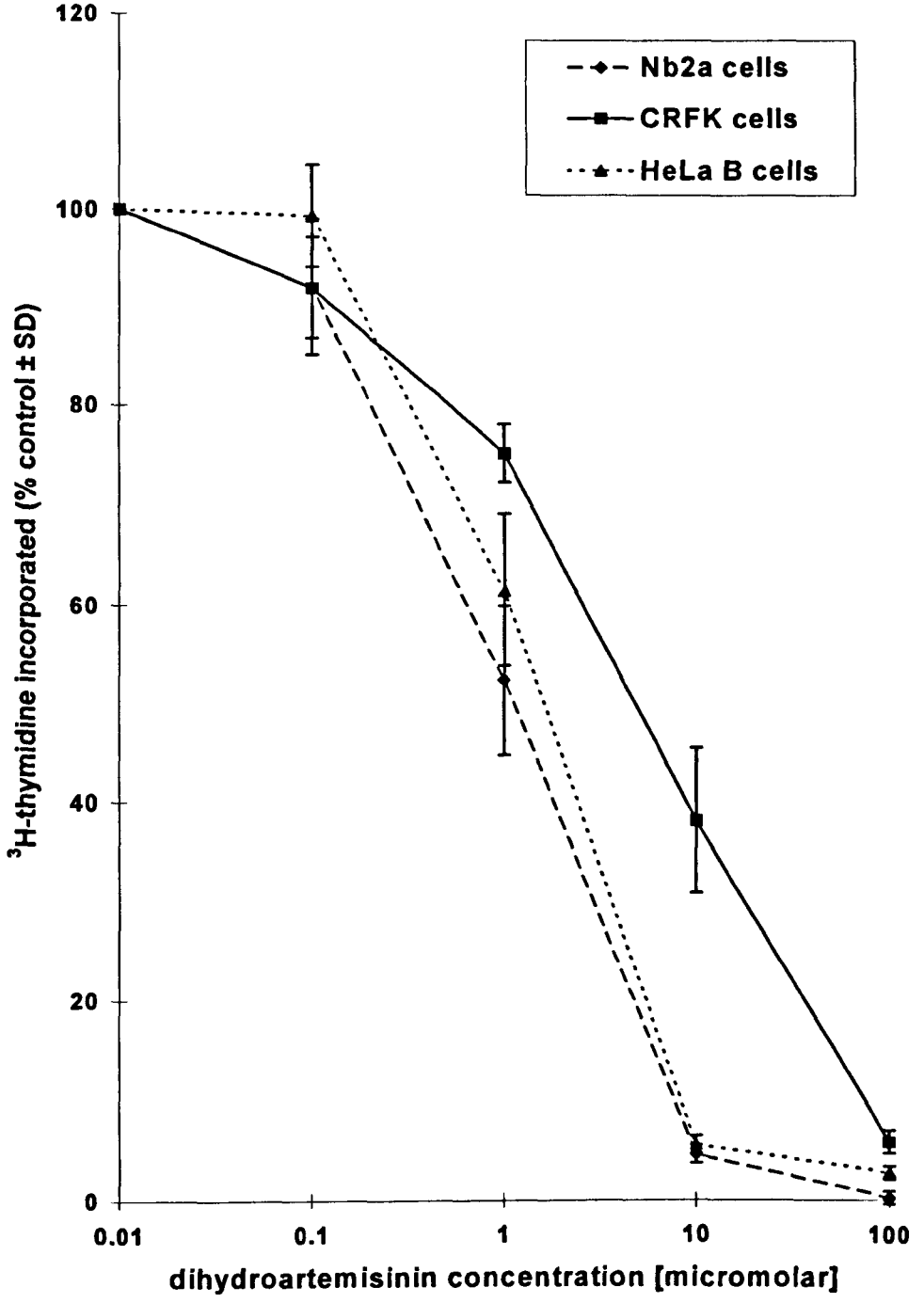


Figure 3.19 The effects of dihydroartemisinin on the proliferation of Nb2a neuroblastoma cells, CRFK feline kidney cells and HeLa B cervical carcinoma cells in culture. Cells were incubated for 2 days with dihydroartemisinin at the concentrations shown. Proliferation was measured by including ^{3}H-thymidine; the incorporation of radioactivity by the cells was measured by liquid scintillation counting and expressed as a percentage of that in cells incubated in the absence of drug. N = 6 for each cell line. Values represent mean ± standard deviation.

Table 3.3. IC_{50} values of inhibitory effects of dihydroartemisinin on Nb2a, CRFK and HeLa B cell proliferation.

CELL LINE	IC_{50} VALUE [μM] (95% CONFIDENCE LIMITS)
NB2A	0.98 (0.37 - 1.77)
HELA B	1.37 (0.64 - 2.58)
CRFK	4.09 (0.69 - 12.1)

N = 6, IC_{50} values given as a geometric mean with 95% confidence limits. Proliferation of Nb2a, CRFK and HeLa B cells was determined as outlined in figure 3.19 and an IC_{50} value calculated for each cell line.

effects on some characteristic of neuronal cells such as the growth and maintenance of neurites from differentiating cells, as the results of section 3.1 seem to suggest. However, a direct comparison between cell lines based on neurite outgrowth was not possible of course, as proliferating CRFK and HeLa B cells cannot be made to express neurites and differentiated Nb2a cells cannot be made to proliferate. In other words we cannot make the same measurements on the same cells in a differentiated and undifferentiated state.

3.3.2. Comparison of the effects of known neurotoxins and non-specific toxins on the proliferation of and neurite outgrowth from Nb2a cells

The results of the assays in section 3.1 in which the artemisinin derivatives displayed significantly greater toxicity towards Nb2a neurite outgrowth than Nb2a proliferation suggest that inhibition of neurite outgrowth could be a measure of the neurotoxic effects of a compound. To test this hypothesis the effects of two neurotoxins and two non-specific toxins on cell proliferation and neurite outgrowth were compared, to see if the neurotoxins, like the artemisinin derivatives, also had markedly greater effects on inhibition of neurite outgrowth than on proliferation and that the non-specific toxic agents did not. If this was so then it would support the notion that the artemisinin derivatives have neurotoxic effects and that Nb2a neurite outgrowth was a useful parameter with which to assess neurotoxicity.

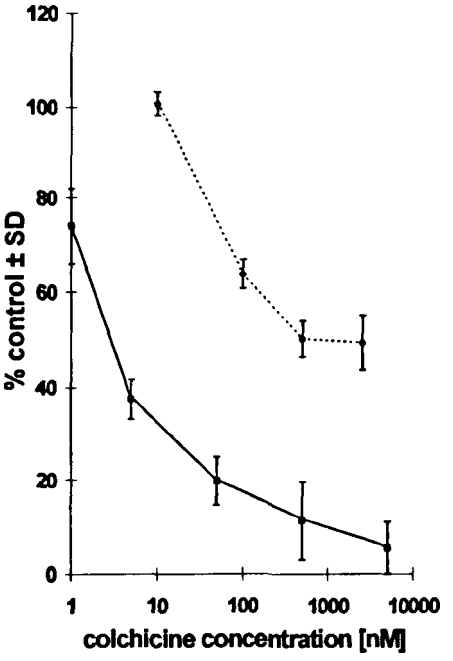
Two compounds that are known to show neurotoxicity are colchicine, the inhibitor of microtubule assembly, and 2,5-hexanedione, the n-hexane metabolite

that forms covalent pyrrole cross-links between neurofilaments (Margolis & Wilson, 1977; Genter St. Clair *et al.*, 1988; Lanning *et al.*, 1994). With both compounds, their inhibitory effects were significantly greater ($p< 0.05$) on cell neurite outgrowth than on cell proliferation (N = 6), roughly x 175 greater in the case of colchicine, [IC_{50} values 2.71 nM for neurite outgrowth and 506 nM for cell proliferation] and x 10 greater in the case of 2,5-hexanedione, [IC_{50} values 1.08 mM for neurite outgrowth and 10.9 mM for cell proliferation] (figures 3.20a & b and table 3.4). The effects of these neurotoxic agents correspond with those of the artemisinin derivatives which were also significantly more toxic to neurite outgrowth than to proliferation, suggesting that the artemisinin derivatives have neurotoxic effects.

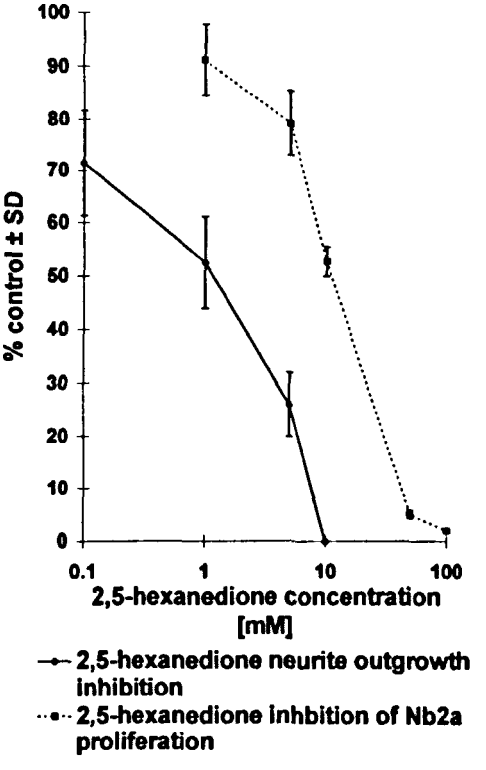
The assays were repeated using two non-specific toxic agents. Sodium cyanide, which disrupts oxygen cycling in the cell, and the pro-oxidant L-ascorbic acid (Johnson *et al.*, 1994; De Laurenzi *et al.*, 1995; Despande *et al.*, 1997). In both cases, there was no significant ($p> 0.05$) difference in the toxicity of the compounds to cell proliferation and neurite outgrowth of Nb2a cells (N = 6) (figures 3.20c & d). The IC_{50} values (table 3.4) for each of the measures of toxicity (proliferation and neurite outgrowth) were very similar, [1.66 mM for proliferation and 1.63 mM for neurite outgrowth of Nb2a cells incubated with sodium cyanide, and 175 μM for proliferation and 164 μM for neurite outgrowth of cells incubated with L-ascorbic acid].

The increased sensitivity of the neurite outgrowth parameter to neurotoxins and dihydroartemisinin (and relative lack of sensitivity to non-specific toxins) suggests that inhibition of neurite outgrowth is a genuine measure of neurotoxicity

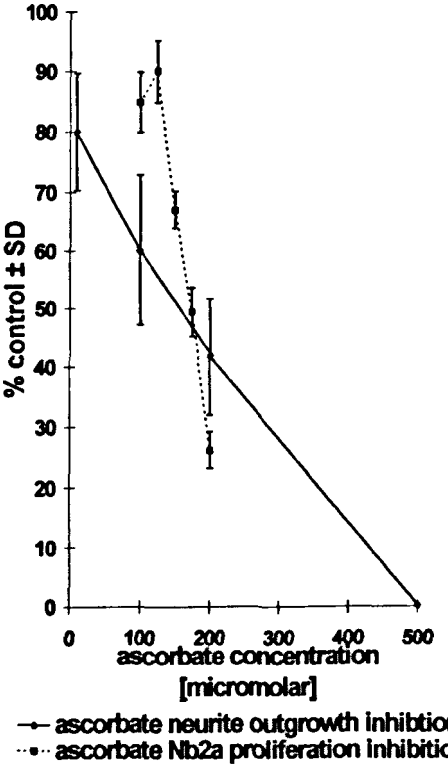
Figure 3.20. (overleaf) The effects of **(a)** colchicine, **(b)** 2,5-hexanedione, **(c)** L-ascorbate, and **(d)** sodium cyanide on Nb2a cell proliferation and neurite outgrowth. Cells were incubated for 1 day with these compounds at the concentrations shown. Proliferation was measured by including ^{3}H-thymidine the in culture medium; the incorporation of ^{3}H-thymidine into cell DNA was measured by liquid scintillation counting and expressed as a percentage of incorporation into control cells incubated in the absence of drug. Nb2a cells were induced to differentiate in the presence of dibutyryl cyclic AMP and drugs at concentrations as indicated. In each experiment, neurite outgrowth from approximately 50 cells was counted using a Kontron image analyser and expressed as a percentage of neurite outgrowth by control cells in the absence of drug. N = 6 for each compound. Values represent mean ± standard deviation.


% control ± SD
colchicine concentration [nM]
colchicine Nb2a proliferation inhibition
colchicine neurite outgrowth inhibition

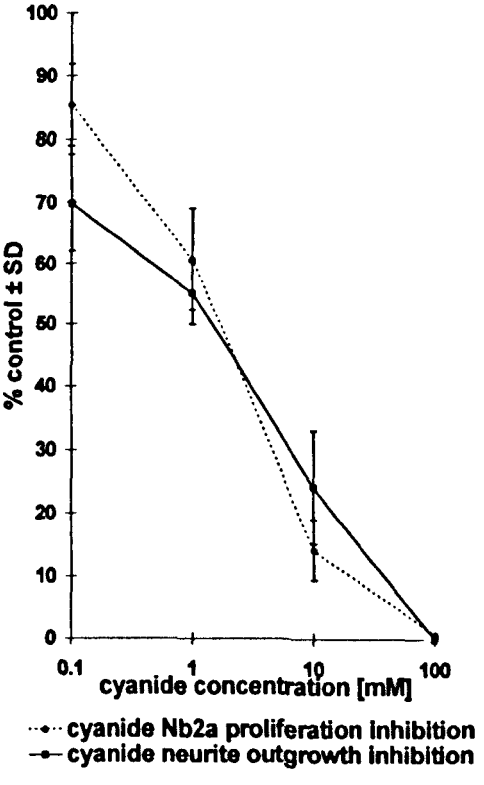
a)


% control ± SD
2,5-hexanedione concentration [mM]
2,5-hexanedione neurite outgrowth inhibition
2,5-hexanedione inhbition of Nb2a proliferation

(b)


% control ± SD
ascorbate concentration [micromolar]
ascorbate neurite outgrowth inhibtion
ascorbate Nb2a proliferation inhibition

(c)


% control ± SD
cyanide concentration [mM]
cyanide Nb2a proliferation inhibition
cyanide neurite outgrowth inhibition

(d)

Table 3.4. IC_{50} values of neurotoxic agents and non-specific toxins on Nb2a cell proliferation and neurite outgrowth

COMPOUND	IC_{50} VALUE (95% CONFIDENCE LIMITS)	IC_{50} VALUE (95% CONFIDENCE LIMITS)
	PROLIFERATION	**NEURITE OUTGROWTH**
COLCHICINE	506 (337- 683) nM	2.71 (1.11 - 4.87) nM
2,5-HEXANEDIONE	10.9 (10.1 - 11.9) mM	1.08 (0.66 - 1.55) mM
L-ASCORBATE	175 (171 - 180) μM	164 (113 - 229) μM
SODIUM CYANIDE	1.66 (1.09 - 2.40) mM	1.63 (1.16 - 2.23) mM

Proliferation of and neurite outgrowth from Nb2a cells were determined as outlined in figure 3.20 and an IC_{50} value calculated for each toxic agent. N = 6, values given as a geometric mean with 95% confidence limits.

rather than a reflection of the general well-being of the cell. This has also been confirmed by studies in this laboratory (Smith, unpublished) where the effects of dihydroartemisinin on the general metabolism of the differentiated cell (as measured by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a coloured product) were compared to the effects of dihydroartemisinin on neurite outgrowth: the IC_{50} value was found to be significantly higher for inhibition of general metabolism. Consequently, compounds that inhibit neurite outgrowth to a significantly greater degree than they inhibit proliferation probably exert neurotoxic effects on the cells.

In summary, these results demonstrate that dihydroartemisinin shares the characteristics of neurotoxins rather than non-specific toxins, which concurs with the findings of *in vivo* toxicity studies (Brewer *et al.*, 1994a, 1994b). They also support the idea that differentiated Nb2a cells more closely resemble neuronal cells than undifferentiated Nb2a cells (Prasad, 1991) and that neurite outgrowth from differentiated Nb2a cells is a better parameter for the study of neurotoxicity than proliferation of undifferentiated Nb2a cells.

3.3.3. Comparison of dihydroartemisinin toxicity with that of neurotoxic agents

In a separate study colchicine, calphostin C, 2,5-hexanedione, cytochalasin B and β,β`-iminodipropionitrile effects on Nb2a neurite outgrowth were examined and compared with those of dihydroartemisinin (N = 6). This was done primarily as a means of determining the sensitivity of the assay. The above compounds were

selected as they mediate their toxicity by a variety of different mechanisms of action, and it was hoped that comparing dihydroartemisinin toxicity with those of the neurotoxins might provide clues to the nature of dihydroartemisinin toxicity or at least help eliminate some possibilities. The IC_{50} values for the effects of neurotoxins on neurite length are given in table 3.5. The IC_{50} for dihydroartemisinin was within the range of those of the known neurotoxins, not as toxic as colchicine, calphostin C or cytochalasin B, but more toxic than 2,5-hexanedione and β,β'-iminodipropionitrile.

The appearance of the cells was examined by light microscopy, but with one exception no obvious morphological differences were visible to the eye. There was no deformation of neurites or cell bodies; drug effects always seemed to take the form of shortening the length and reducing the number of neurites projecting from the cell body (figure 3.21a-g). However, 1 μM cytochalasin B (figure 3.21c), which completely inhibited neurite outgrowth caused the cells to form peculiar "chains", but this did not also occur in dihydroartemisinin-treated cells. Consequently, examination of the cells by light microscopy provided no clues to the mechanism of action of the artemisinin derivatives.

Another approach to validating the assay was to see if the effects of dihydroartemisinin and the neurotoxins could be correlated with ultrastructural damage within the cells. Differentiated Nb2a cells incubated with dihydroartemisinin, colchicine or 2,5-hexanedione (N = 4) were prepared for scanning and transmission electron microscopy. Colchicine and 2,5-hexanedione were selected as they act on microtubules and neurofilaments respectively

Table 3.5. IC_{50} values of artemisinin and a group of neurotoxic agents on Nb2a neurite outgrowth

COMPOUND	**NEURITE OUTGROWTH INHIBITION**
	IC_{50} VALUE (95% CONFIDENCE LIMITS)
DIHYDROARTEMISININ	0.25 (0.18 - 0.34) μM
COLCHICINE	2.71 (1.11 - 4.87) nM
CYTOCHALASIN B	3.36 (-3.46 - 25.3) nM
CALPHOSTIN C	23.8 (21.6 - 26.1) nM
β,β`-IMINODIPROPONITRILE	8.48 (3.93 - 15.3) μM
2,5-HEXANEDIONE	1.08 (0.66 - 1.55) mM

Nb2a cell neurite outgrowth was determined as outlined in figure 3.4 and an IC_{50} value calculated for each toxic agent. N = 6, values given as geometric mean with 95% confidence limits.

Figure 3.21. Effects of neurotoxic agents on differentiating Nb2a cells. Light microscopy images of the effects of **(a)** calphostin C (50 nM), **(b)** colchicine (0.5 μM), **(c)** cytochalasin B (1 μM), **(d)** 2,5-hexanedione (10 mM) and **(e)** iminodipropionitrile (10 μM), **(f)** dihydroartemisinin (1 μM) on neurite outgrowth from differentiating Nb2a cells. Typical fields of cells from one of six similar experiments. Cells were incubated with a neurotoxic agent and dibutyryl cyclic AMP and neurite outgrowth was determined as outlined in figure 3.20. Bar = 10 μm.

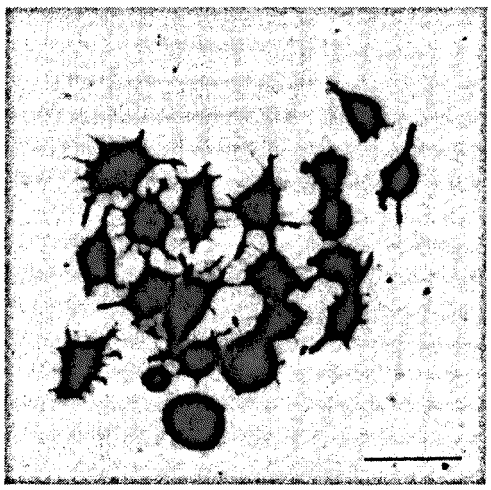
(a) Calphostin 50 nM

(b) Colchicine 0.5 μM

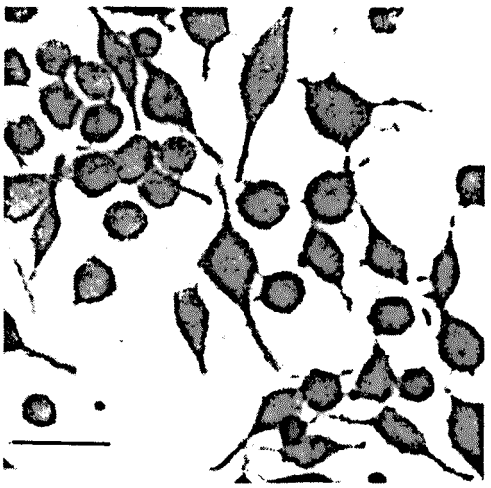
(c) Cytochalasin B 1 μM

(d) 2,5-hexanedione 10 mM

(e) β,β`-iminodipropionitrile 10 μM



(f) dihydroartemisinin 1 μM



(g) control cells

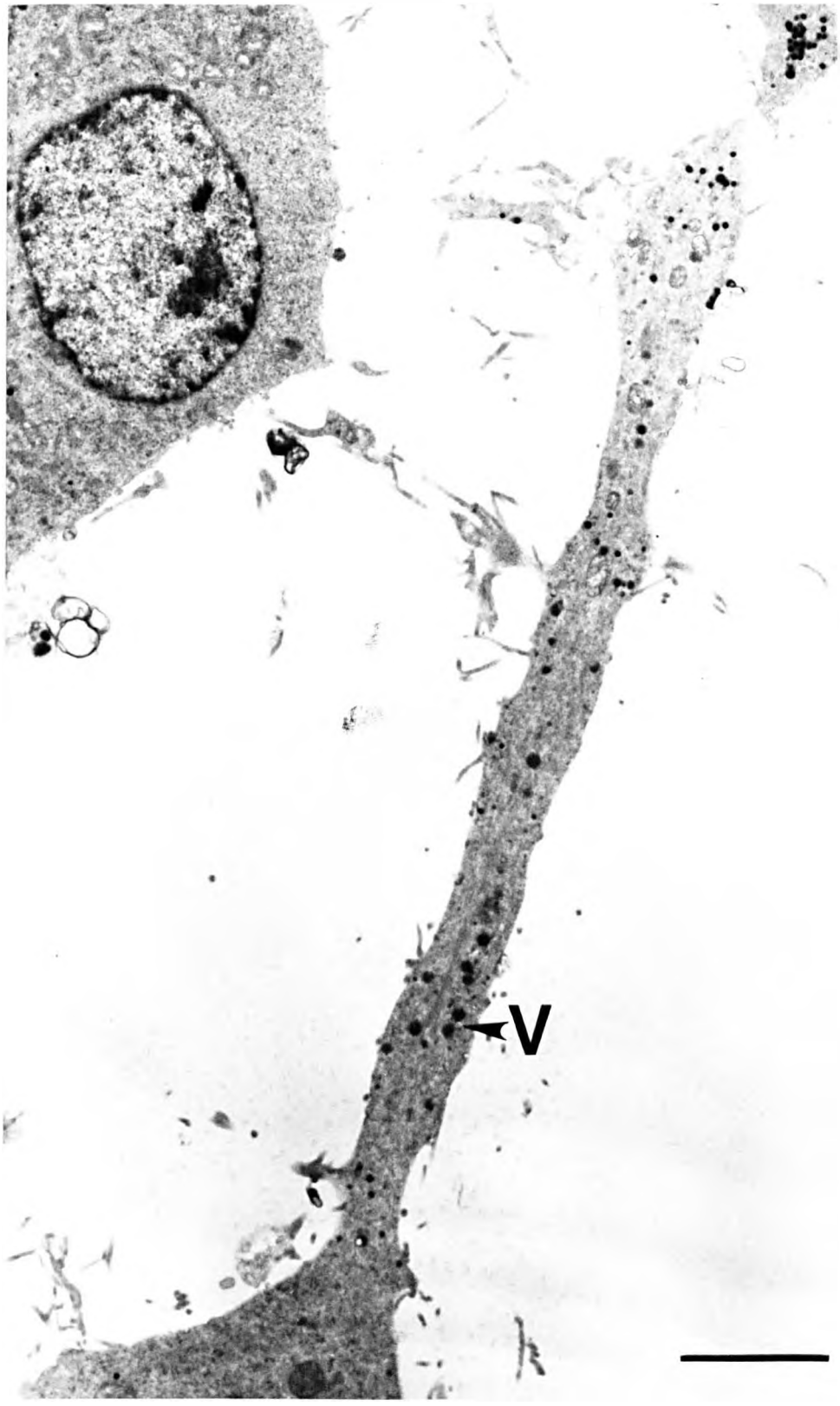
(Clarkson, 1986), two common targets for neurotoxic agents. Transmission electron microscopy of dihydroartemisinin-treated cells revealed an apparent loss of endoplasmic reticulum, along with dilation and dissociation of ribosomes from that endoplasmic reticulum which remained; and the cristae of mitochondria in both cell bodies and neurites were either deformed or absent. Scanning electron microscopy of dihydroartemisinin-treated cells showed that the microspikes / filopodia projecting from the cell body and neurites were far fewer in number and / or damaged. Electron microscopy of dihydroartemisinin-treated cells is discussed in greater detail in section 3.2.

Transmission electron micrographs of cells treated with 50 nM colchicine revealed that there was an accumulation of vesicles at the base of some neurites compared to control cells where vesicles were distributed throughout the neurite, which may be due to a disruption of their axonal transport (figures 3.22a & b). This might be expected of a compound that inhibits the assembly of microtubules, a vital component of the axonal cytoskeleton. Scanning electron micrographs of colchicine-treated cells showed they had far fewer neurites than control cells (which had already been observed by light microscopy) and that filopodia around the base of the cell body took the form of very short or stunted neurites. This ostensible stunting of neurite outgrowth could also be caused by microtubule disruption (figure 3.23a).

Scanning electron microscopy of cells treated with 15 mM 2,5-hexanedione did not reveal any obvious alterations in the surface structures of the cell bodies or deformation of the neurites (figure 3.24a). They resembled control cells except for the absence or shortening of neurites which was already observed and quantified by

Figure 3.22a. Transmission electron micrograph of a neurite of a differentiated Nb2a cell incubated with 50 nM colchicine for 24 h. Incubation and preparation of cells for transmission electron microscopy were performed as outlined in figure 3.11. Vesicles (V) accumulated at the base of some neurites which may be due to disruption of axonal transport. A typical micrograph from one of four similar experiments. Bar = 1 μm.

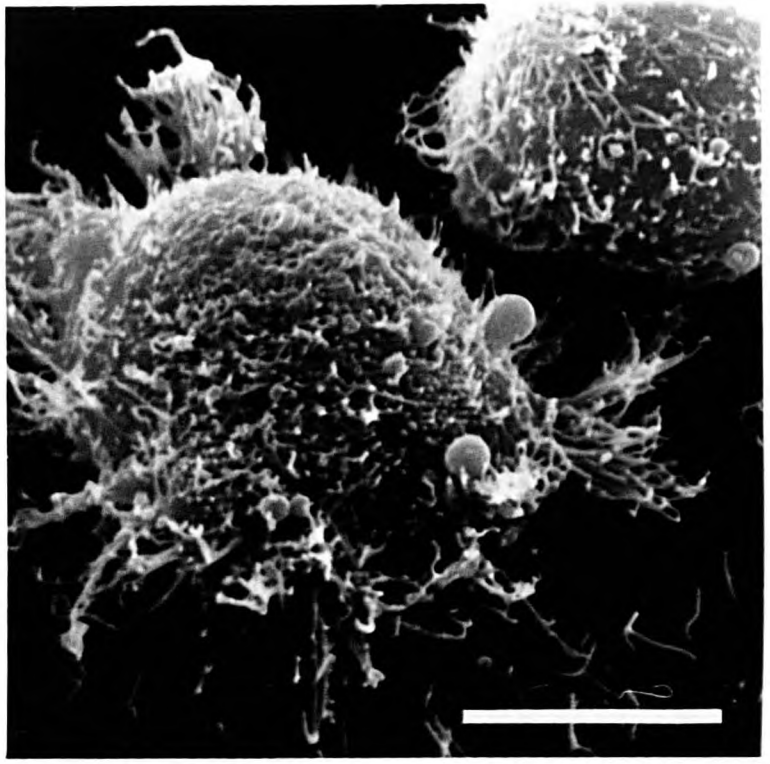
Figure 3.22b. Transmission electron micrograph of a neurite of a control differentiated Nb2a cell. Incubation and preparation of cells for transmission electron microscopy were performed as outlined in figure 3.11. Vesicles are present throughout the neurite. A typical micrograph from one of four similar experiments. Bar = 5 μm.

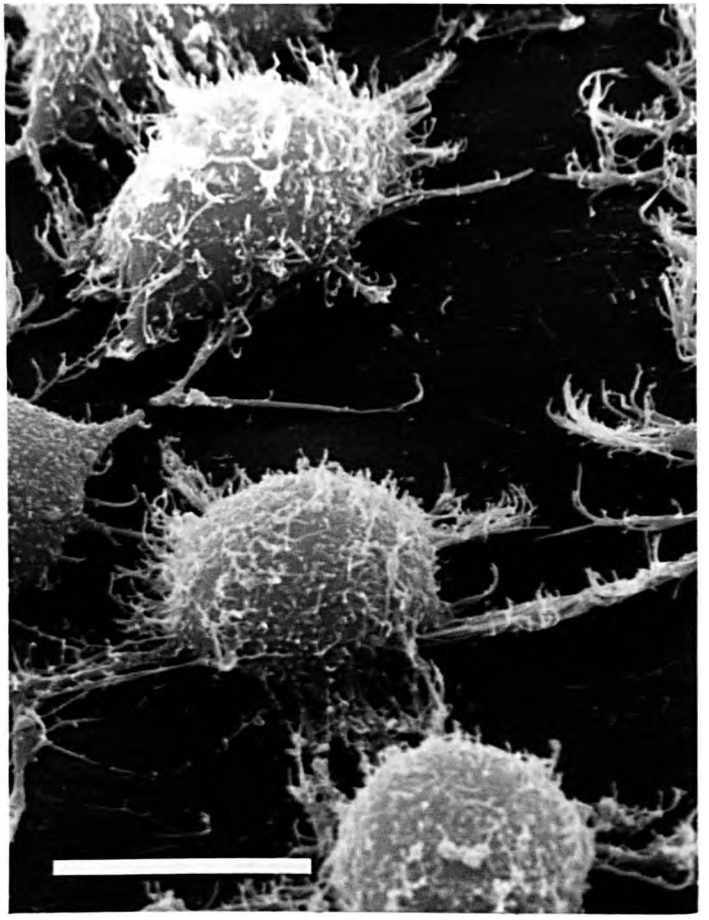


light microscopy. Transmission electron micrographs of cells treated with 2,5-hexanedione showed that their neurites often appeared to contain more microtubules and neurofilaments, packed together, than control cells (figure 3.25a). This is consistent with 2,5-hexanedione actions *in vivo* where it causes the aggregation of axonal neurofilaments. No such effects were observed in cells incubated with dihydroartemisinin.

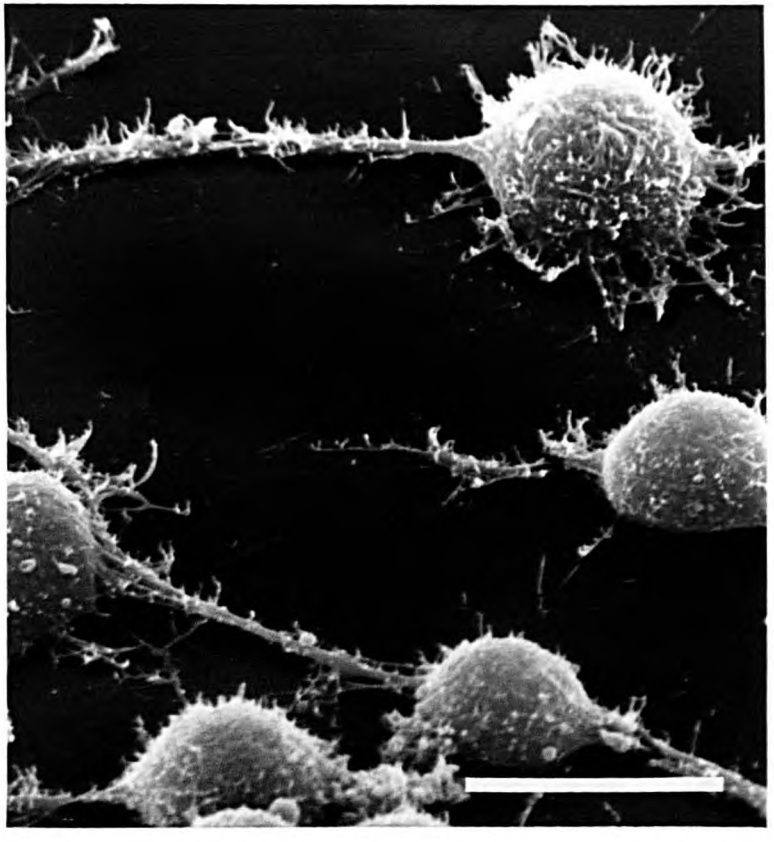
Electron microscopy demonstrated that the effects of the neurotoxic agents colchicine and 2,5-hexanedione did not resemble the effects of dihydroartemisinin on differentiated Nb2a cells. Whilst this does not provide any hints as to the mechanism of action of the artemisinin derivatives, it does at least suggest that their toxicity is not mediated through colchicine- or 2,5-hexanedione-like effects on microtubules or neurofilaments. The measurement of neurite outgrowth and the electron micrographs did, however, go some way toward validating the use of Nb2a cells for testing potential neurotoxins. The effects of the colchicine and 2,5-hexanedione on differentiating Nb2a cells appeared to be related to their mechanisms of action *in vivo* (Clarkson, 1986) and this provides support for the use of the neurite outgrowth parameter and differentiated Nb2a cells as *in vitro* models for the study of neurotoxic agents.

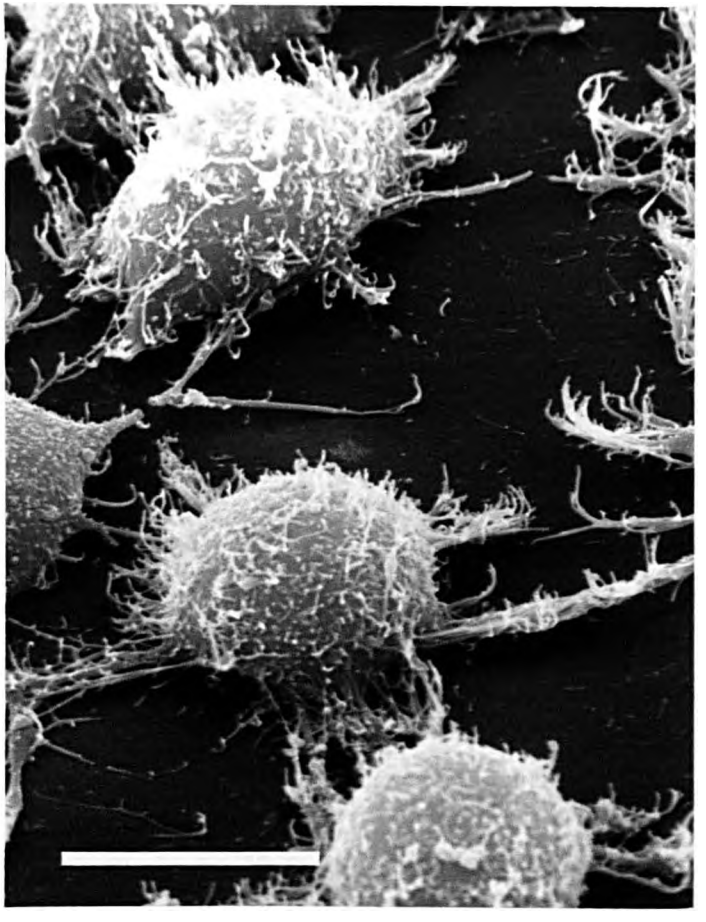
Figures 3.23a & b. Scanning electron micrographs of, **left: (a)** differentiated Nb2a cells incubated with 50 nM colchicine for 24h. Bar = 8.6 μm. **Right: (b)** control cells. Bar = 12 μm. Incubation and preparation of cells for scanning electron microscopy were performed as outlined in figure 3.15. Cells treated with colchicine **(left)** have few mature neurites but many short, stunted, neurite-like filopodia around the base of the cell, possibly caused by disruption of neuritogenesis and damage to the neurite cytoskeleton. Typical micrographs of four similar experiments.



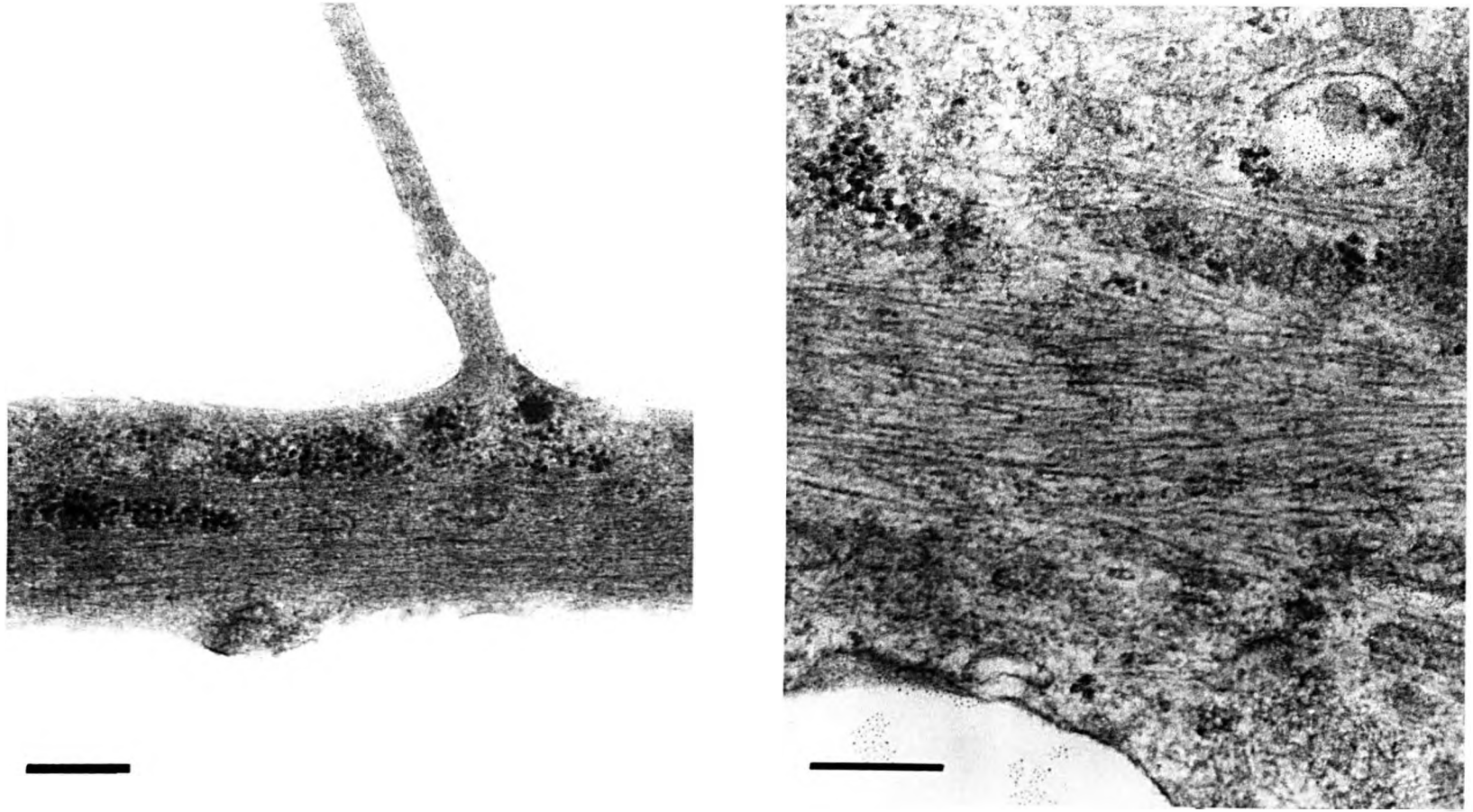


Figures 3.24a & b. Scanning electron micrographs of, **left: (a)** differentiated Nb2a cells incubated with 15 mM 2,5-hexanedione for 24 h. Bar = 17.6 μm. **Right: (b)** control cells. Bar = 12 μm. Incubation and preparation of cells for scanning electron microscopy were as outlined in figure 3.15. There are no obvious deformations or abnormalities of the neurites or cell bodies of cells treated with 2,5-hexanedione **(left)**. Typical micrographs of four similar experiments.





Figures 3.25a & b. Transmission electron micrograph of, **left: (a)** differentiated Nb2a cell incubated with 15 mM 2,5-hexanedione 24 h. Bar = 250 nm. **Right: (b)** control cells. Bar = 250 nm. Incubation and preparation of cells for transmission electron microscopy were as outlined in figure 3.11. The neurites of cells incubated with 2,5-hexanedione **(left)** often appeared to contain more microtubules and neurofilaments, packed together, than control cells **(right)**. This may be analogous to *in vivo* toxicity where 2,5-hexanedione exerts its toxic effects by covalently crosslinking neurofilaments. Typical micrographs of four similar experiments.



PART FOUR

3.4. CHARACTERISING DIHYDROARTEMISININ - PROTEIN BINDING

The preceding sections of this thesis demonstrated that the artemisinin derivatives are toxic to Nb2a and C6 cells, that they damage the membrane and organelles of the cells, and that cultured neural cells are a suitable model for the study of neurotoxic agents. In this section the nature of artemisinin derivative binding within the cell was investigated. ^{14}C-labelled dihydroartemisinin was used to study the mode of action of the artemisinin derivatives in binding assays with several different neural preparations : Nb2a and C6 cells, as these had been useful models for cell growth and axonal maintenance, and rat brain homogenates, as *in vivo* toxicity studies demonstrated that rats are susceptible to the toxicity of the artemisinin derivatives.

Initially, the binding of dihydroartemisinin to several areas of rat brain over time was assessed as was the relative contributions to total ^{14}C-dihydroartemisinin-binding by proteins and other cell material, to determine where the greatest binding was taking place. This established that the majority of binding involved proteins. The nature of ^{14}C-dihydroartemisinin-protein binding was then investigated. This was done by examining the effects of increasing the concentration of ^{14}C-dihydroartemisinin in incubations with cells and homogenate to determine if there were multiple sets of binding sites for dihydroartemisinin. Haemin, which increases the toxicity of the artemisinin derivatives *in vitro*, was added to the cell

and homogenate incubations to see if it similarly increased the drug-protein binding. The effects on binding of protecting protein thiol and amine groups by pre-incubating homogenate with blocking agents was examined to ascertain if these groups played an important role in dihydroartemisinin binding. Finally, unlabelled arteether, which is both a potent antimalarial and is toxic *in vivo* and *in vitro*, or unlabelled desoxyartemisinin, which is neither toxic nor an antimalarial, were co-incubated with homogenate and ^{14}C-dihydroartemisinin to further examine the relationship between binding and toxicity.

3.4.1. Comparison of dihydroartemisinin binding in different areas of rat brain

Homogenised rat cerebellum, cortex and brain stem were incubated with ^{14}C-dihydroartemisinin for 2 h and 24 h (N = 6) to ascertain if there was a difference in the degree of ^{14}C-dihydroartemisinin-binding in different areas of the rat brain and to discover if there were any changes in binding over 24 h. The results revealed that drug-protein binding increased significantly ($p< 0.001$) eight- to ten-fold during the intervening 22 h (figure 3.26), suggesting that in each of the three brain areas binding increased steadily throughout the incubation. There was no significant difference ($p> 0.05$) between the amount of ^{14}C-dihydroartemisinin-protein binding that took place in the brainstem, cerebellum and cortex homogenates (figure 3.26). After unbound ^{14}C-dihydroartemisinin was removed, the proteins of the homogenate were precipitated in acetone and the protein pellet separated from the acetone supernatant. Liquid scintillation counting of both the resuspended proteins and the

acetone revealed that there was a considerable amount of ^{14}C-dihydroartemisinin present in the acetone, which contained the homogenate lipids, DNA, other non-protein components of cells and presumably a quantity of unbound drug that was present within the cells before the acetone precipitation of proteins. After 2 h there was no significant ($p > 0.05$) difference between the amount of ^{14}C-dihydroartemisinin bound to proteins and the other cellular material in the acetone - approximately 0.7% of the total ^{14}C-dihydroartemisinin used in the incubations in each case. However, after 24 h there was significantly ($p < 0.01$) greater ^{14}C-dihydroartemisinin-protein binding than ^{14}C-dihydroartemisinin binding to the other cell material in the acetone : approximately 5.5% of total drug bound to proteins and 2% in the acetone (table 3.6, figure 3.26). These results demonstrate that although some binding to other cell components does take place, *e.g.* by membrane lipid peroxidation (although not by binding to DNA (Yang *et al.*, 1994)), the majority of ^{14}C-dihydroartemisinin binding is to cell proteins. Protein modification is often the mechanism by which neurotoxic agents act and it was therefore dihydroartemisinin binding to cell proteins which was then examined in detail.

3.4.2. Characterising ^{14}C-dihydroartemisinin-protein binding

Changes in the nature of ^{14}C-dihydroartemisinin binding to rat cortex homogenate and cells with increasing ^{14}C-dihydroartemisinin concentration (0.94

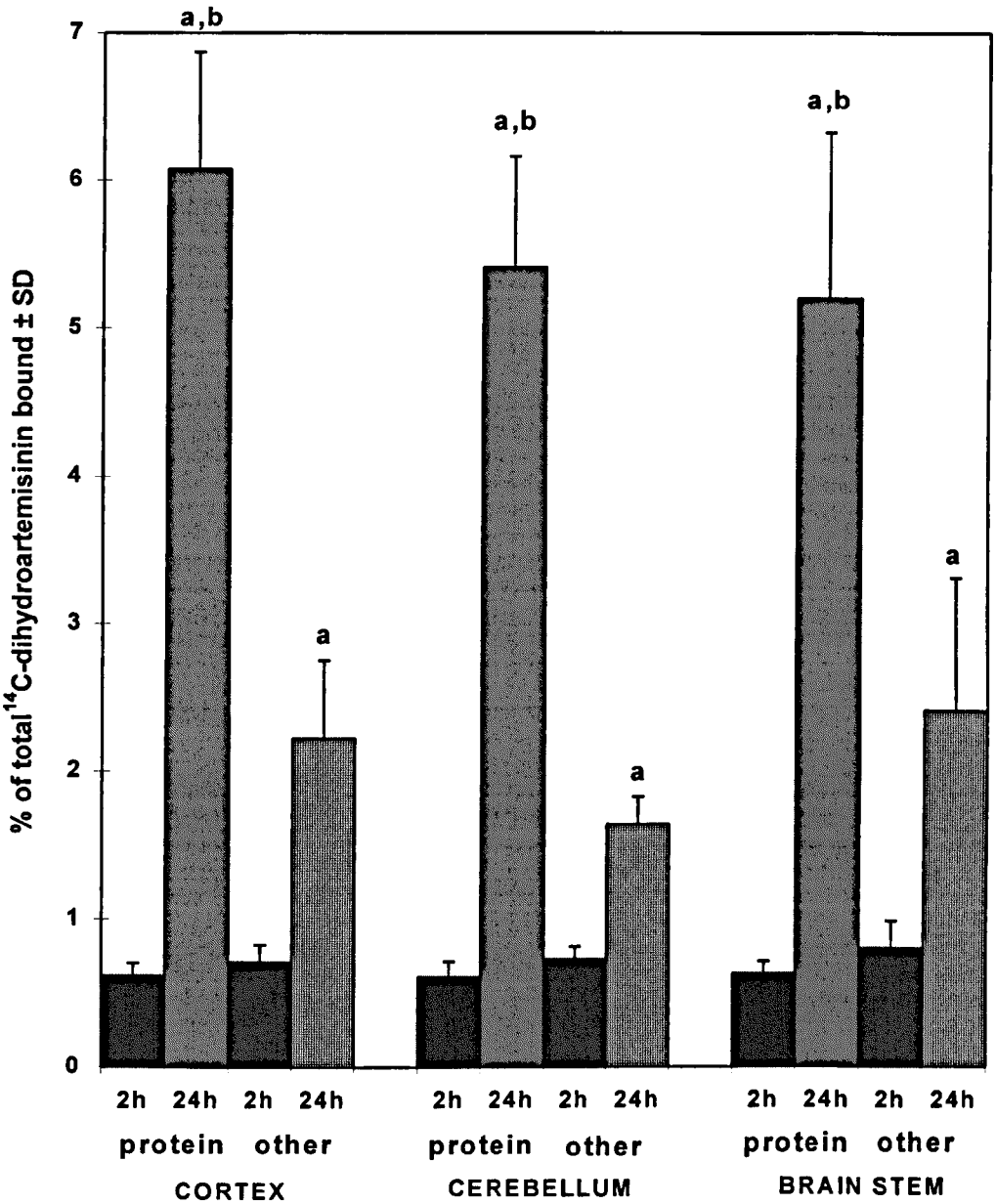


Figure 3.26. Binding of ^{14}C-dihydroartemisinin to rat cortex, cerebellum and brain stem homogenates. Rat brain homogenate (10 mg wet weight in PIPES buffer, 100 mM, pH 6.8) was incubated with 3.3 kBq of ^{14}C-dihydroartemisinin for 2 or 24 h. The homogenate was then centrifuged, washed to remove unbound ^{14}C-dihydroartemisinin and the proteins precipitated in acetone. The protein was then resuspended in PIPES buffer and aliquots were taken from this, along with the acetone supernatant, to determine the ^{14}C-dihydroartemisinin binding by liquid scintillation counting. "**a**" indicates a statistically significant ($p < 0.05$) increase in drug binding to either protein or other cell material over the 22 h period between 2 h and 24 h. "**b**" indicates a significantly greater degree of drug binding to proteins than to other cell material in each area of rat brain after 24 h. N = 6. Binding given as mean ± standard deviation.

Table 3.6. ^{14}C-dihydroartemisinin binding to rat cortex, cerebellum, and brain stem homogenates.

	% TOTAL ^{14}C-DIHYDROARTEMISININ BOUND ± SD	
CORTEX HOMOGENATE	2 h	24 h
PROTEIN BINDING	0.62 ± 0.08	6.08 ± 0.79
OTHER BINDING	0.71 ± 0.12	2.22 ± 0.53
CEREBELLUM HOMOGENATE		
PROTEIN BINDING	0.61 ± 0.10	5.41 ± 0.75
OTHER BINDING	0.73 ± 0.08	1.64 ± 0.19
BRAIN STEM HOMOGENATE		
PROTEIN BINDING	0.64 ±0.08	5.20 ± 1.12
OTHER BINDING	0.90 ± 0.18	2.41 ± 0.89

^{14}C-dihydroartemisinin binding to rat cortex, cerebellum and brainstem homogenates was determined as outlined in figure 3.26 and expressed as a percentage of the total ^{14}C-dihydroartemisinin used in the incubation. N = 6, values given as mean ± standard deviation.

μM to 187 μM), and the possible existence of multiple sets of binding sites were examined using data from the control incubations of the haemin assays (N = 4) (below). Rosenthal plots (Rosenthal, 1967) revealed that in rat cortex homogenate, Nb2a cells and C6 cells there were two discrete sets of binding sites for dihydroartemisinin: high affinity / low capacity binding sites and low affinity / high capacity binding sites (figures 3.27a, b & c). Curvature of the plot suggested that they had very different affinities and capacities for dihydroartemisinin. As there was no significant difference in protein binding to rat cerebellum, cortex and brain stem, any could have been used and cortex was chosen as it was the most plentiful.

3.4.3. Effects of haemin on ^{14}C-dihydroartemisinin-protein binding

Haemin catalyses the conversion of artemisinin derivatives into reactive products and increases their toxicity to differentiating Nb2a cells (Smith *et al.*, 1997). In this study the effects of haemin on ^{14}C-dihydroartemisinin binding to rat cortex homogenate, Nb2a and C6 cells were examined to determine if it also increased drug-protein binding. The binding parameters k_D and B_{max} were derived from these data (table 3.7).

Rat cortex homogenate. Haemin significantly ($p< 0.001$) increased the total binding of ^{14}C-dihydroartemisinin to cortex proteins at all concentrations of ^{14}C-dihydroartemisinin used (N = 4) (figure 3.28). Binding parameters calculated from these data demonstrated that in the high affinity / low capacity phase of binding

haemin significantly ($p< 0.01$) increased the B_{max} of the homogenate protein but did not significantly alter the k_D, whereas in the low affinity / high capacity phase of binding, it significantly reduced the k_D (*i.e.* increased the affinity) ($p< 0.05$) but did not significantly affect the B_{max} (table 3.7).

Nb2a cells. Haemin significantly ($p< 0.01$ & $p< 0.001$) increased the binding of ^{14}C-dihydroartemisinin to Nb2a cell proteins at ^{14}C-dihydroartemisinin concentrations of 3.75 μM and above (N = 4) (figure 3.29). In the high affinity / low capacity phase of binding, haemin significantly ($p< 0.001$ & 0.05 respectively) increased the B_{max} and k_D values (table 3.7). However, in the low affinity / high capacity binding phase, contrary to its effects on rat cortex homogenate, in Nb2a cells haemin had no effect on the k_D value, but significantly ($p< 0.001$) increased the B_{max} (table 3.7). Dihydroartemisinin also has a significantly ($p< 0.001$) greater affinity (lower k_D values) for both the high and low affinity binding sites of Nb2a cells than those of either C6 cells or cortex homogenate (table 3.7), dihydroartemisinin k_D values for Nb2a cells being 0.47 μM and 107 μM compared to 1.97 μM and 264 μM for rat cortex homogenate and 2.23 μM and 545 μM for C6 cells.

C6 cells. Haemin significantly ($p< 0.01$ & $p< 0.001$) increased the total binding of ^{14}C-dihydroartemisinin to C6 cells at all concentrations of dihydroartemisinin used (N = 4) (figure 3.30). Binding parameters calculated from these data revealed that in

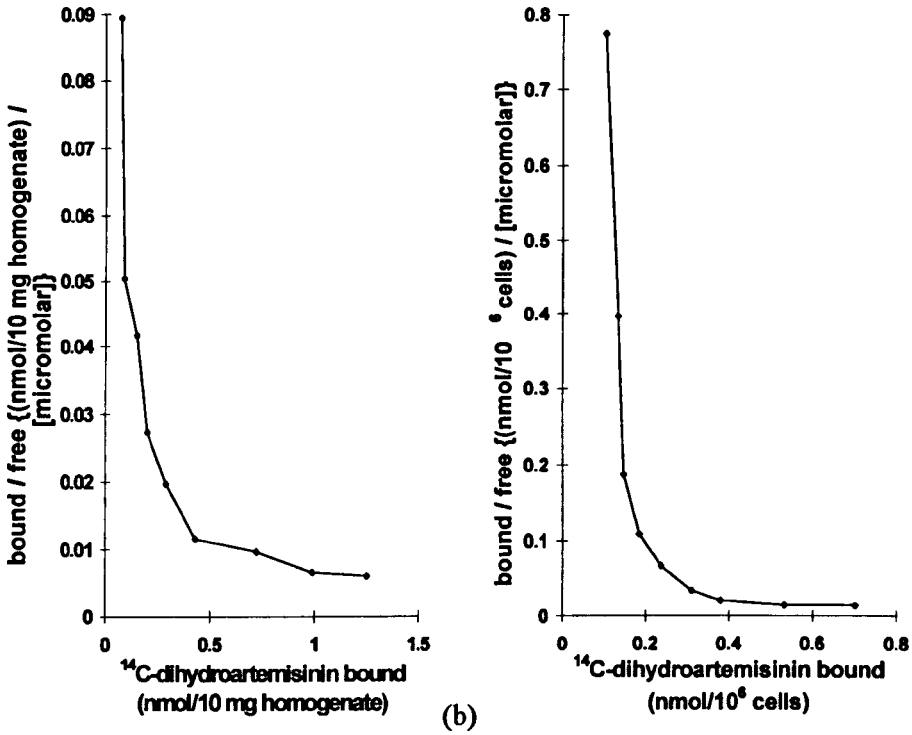
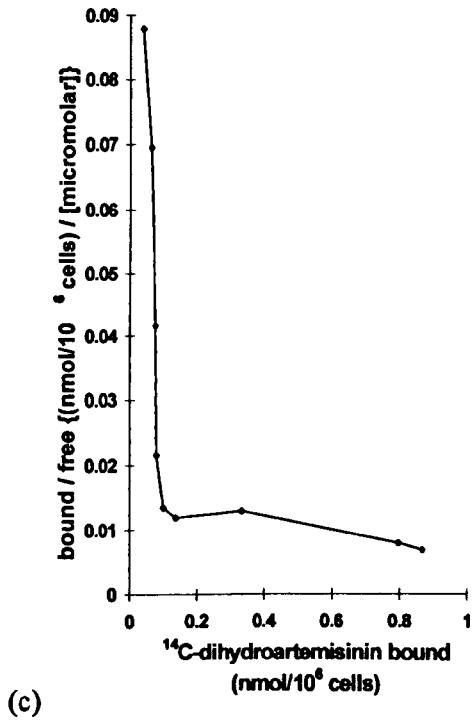


Figure 3.27. Rosenthal plots of **(a)** rat cortex homogenate **(b)** Nb2a cells **(c)** C6 cells showing the effects of increasing ^{14}C-dihydroartemisinin concentration on drug-protein binding. Binding was assessed as was determined as outlined in figures 3.28, 3.29 and 3.30. N = 4. The curvature of each plot is very pronounced, suggesting two discrete populations of binding sites, with affinity and capacity for dihydroartemisinin that differ greatly.



(c)

	CONTROL - NO HAEMIN		+ 2μM HAEMIN	
RAT CORTEX HOMOGENATE BINDING SITES	K_D [μM]	B_{MAX} (NMOLES / 10 MG HOMOGENATE)	K_D [μM]	B_{MAX} (NMOLES / 10 MG HOMOGENATE)
HIGH AFFINITY, LOW CAPACITY	1.97±0.19	0.23±0.02	2.19±0.09	1.59±0.07
LOW AFFINITY, HIGH CAPACITY	264±96.2	4.24±1.23	51.5±3.50	4.43±0.30
NB2A NEUROBLASTOMA CELL BINDING SITES	K_D [μM]	B_{MAX} (NMOLES / 10^6 CELLS)	K_D [μM]	B_{MAX} (NMOLES / 10^6 CELLS)
HIGH AFFINITY, LOW CAPACITY	0.47±0.13	0.18±0.01	1.11±0.36	0.56±0.05
LOW AFFINITY, HIGH CAPACITY	107±24.7	0.93±0.05	121±21.5	1.51±0.10
C6 GLIOMA CELL BINDING SITES	K_D [μM]	B_{MAX} (NMOLES / 10^6 CELLS)	K_D [μM]	B_{MAX} (NMOLES / 10^6 CELLS)
HIGH AFFINITY, LOW CAPACITY	2.23±0.66	0.12±0.01	2.46±0.10	0.24±0.02
LOW AFFINITY, HIGH CAPACITY	545±222	2.84±1.25	292±43.0	3.86±0.57

Table 3.7. The effects of haemin on the binding parameters of ^{14}C-dihydroartemisinin and rat cortex homogenate / Nb2a cells / C6 cells. Binding was determined as outlined in figures 3.28, 3.29 and 3.30. Data represents binding per million cells or 10 mg wet weight homogenate. N = 4, values presented as mean ± standard deviation.

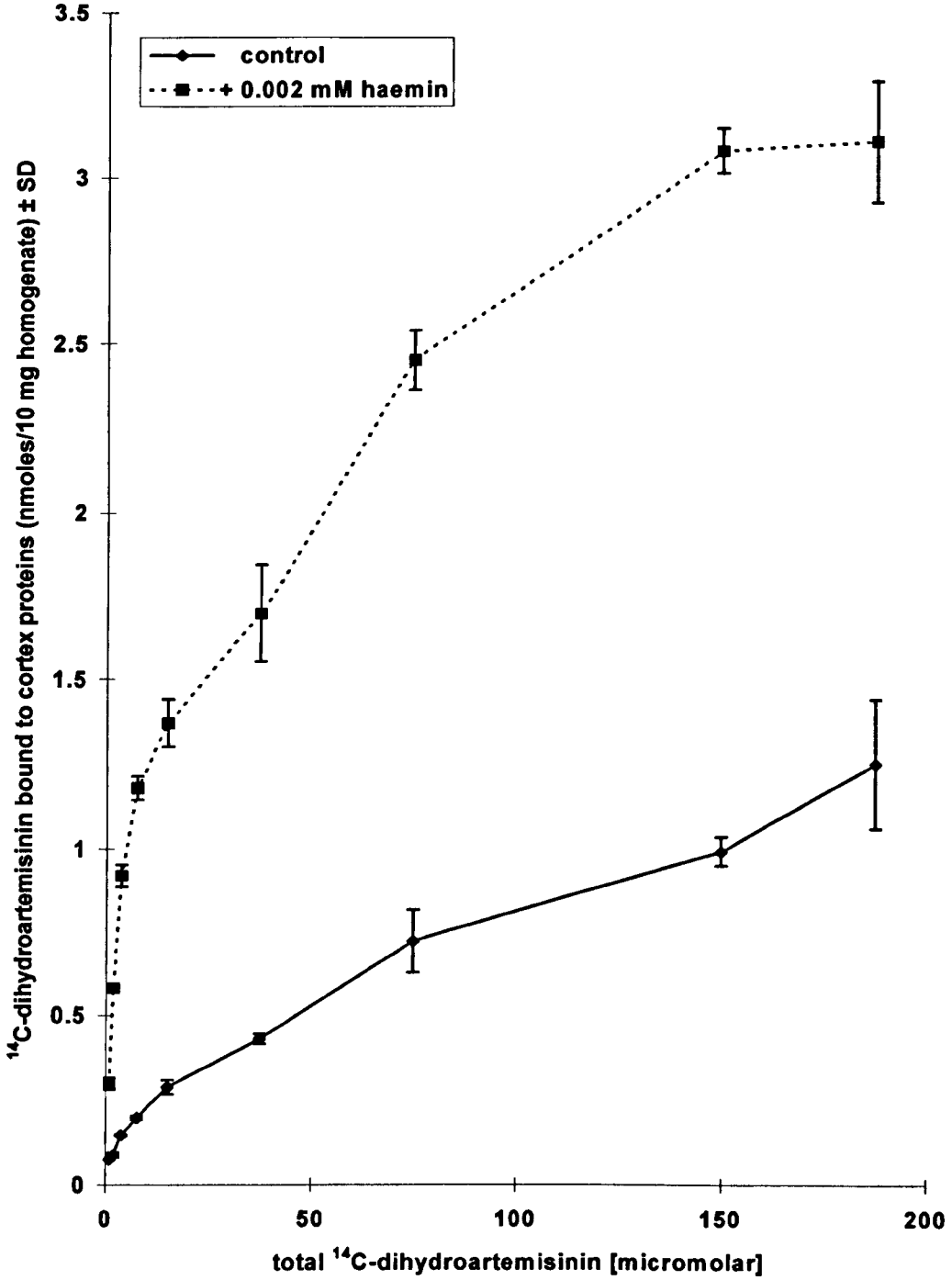


Figure 3.28. The effect of haemin on ^{14}C-dihydroartemisinin binding to rat cortex proteins. Rat cortex homogenate was incubated for 24 h with ^{14}C-dihydroartemisinin (0.94 μM to 187 μM) with or without haemin (2 μM). After 24 h the cortex homogenate was washed twice in PIPES buffer to remove free ^{14}C-dihydroartemisinin and haemin. The cortex proteins were then precipitated from resuspended cortex homogenate in acetone at -20°C. The protein was then resuspended in PIPES buffer and aliquots used to determine the ^{14}C-dihydroartemisinin binding by liquid scintillation counting. At all ^{14}C-dihydroartemisinin concentrations, haemin significantly ($p < 0.001$) increased the total binding. N = 4. Error bars represent standard deviations.

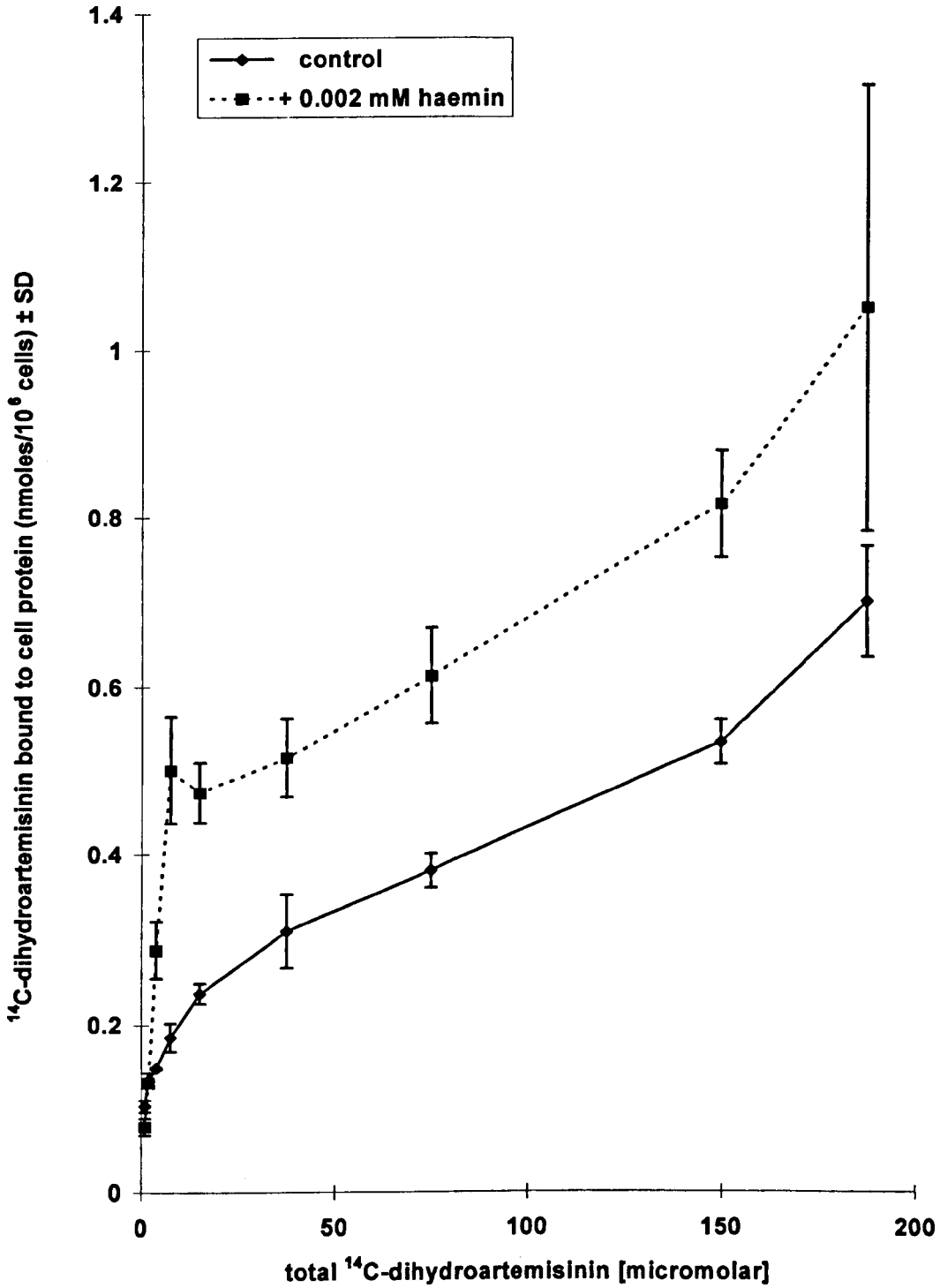


Figure 3.29. The effects of haemin on ^{14}C-dihydroartemisinin binding to Nb2a cells. Binding was determined as outlined in figure 3.28, except that ^{14}C-dihydroartemisinin and haemin were incubated with the cells in 0.5 mM dbcAMP in serum-free medium rather than PIPES buffer, to induce cell differentiation. At ^{14}C-dihydroartemisinin concentrations of 3.75 μM and above haemin significantly ($p< 0.001$ or $p< 0.01$) increased binding. N = 4. Error bars represent standard deviations.

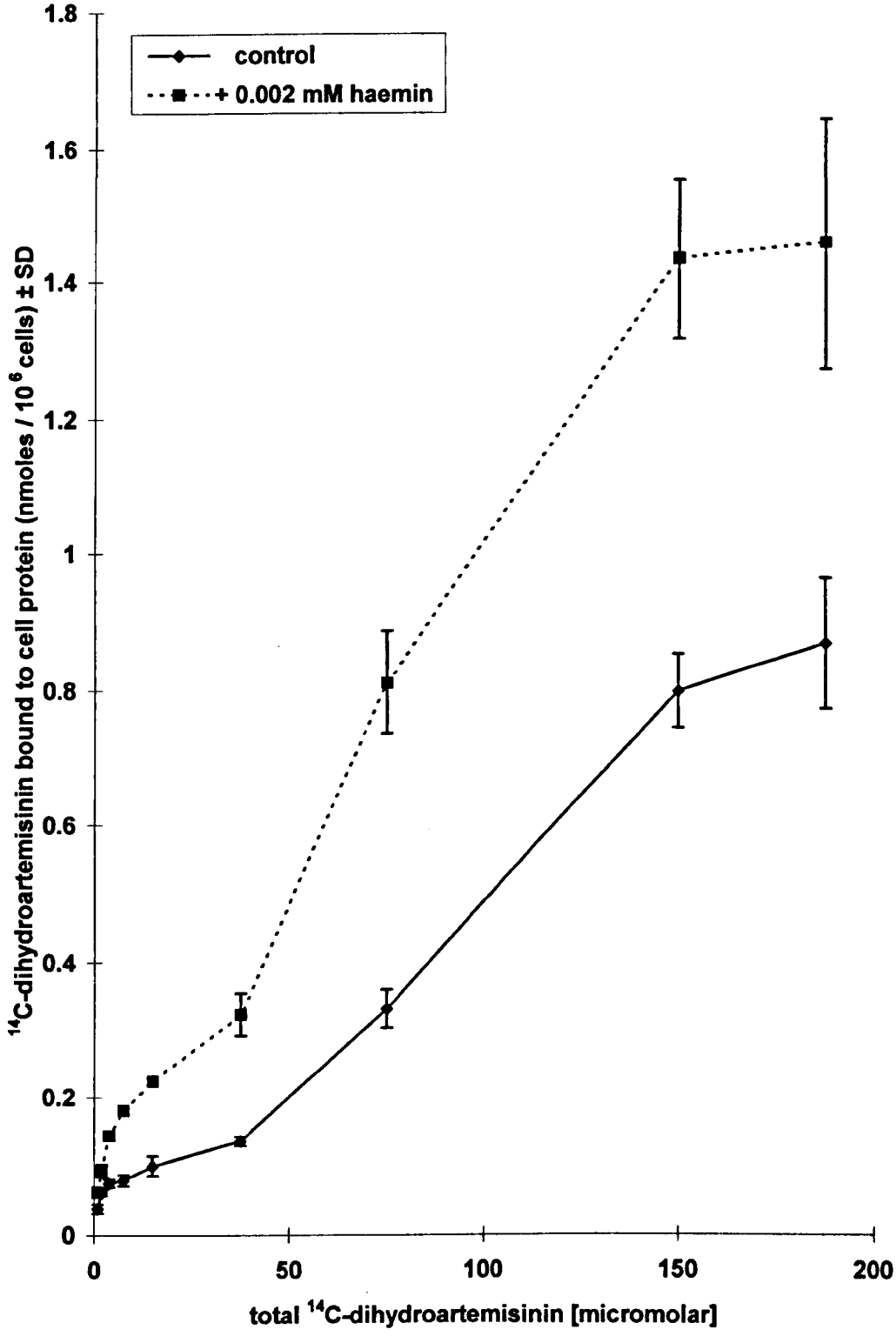


Figure 3.30. The effects of haemin on ^{14}C-dihydroartemisinin binding to C6 cells. Binding was determined as outlined in figure 3.29. Haemin significantly ($p< 0.01$) increased the total binding at all concentrations of ^{14}C-dihydroartemisinin used. N = 4. Error bars represent standard deviations.

the high affinity / low capacity binding sites the effects of haemin were the same as on rat cortex *i.e.* it significantly ($p< 0.001$) increased the B_{max} value of the high affinity / low capacity binding sites but had no significant effect on their k_D value (table 3.7). Haemin did not significantly affect the binding parameters of the low affinity / high capacity binding sites (table 3.7).

3.4.4. Effects of thiol and amine group blocking reagents

To further characterise the interaction between the artemisinin derivatives and their binding sites cortex homogenate was pre-incubated with the thiol and amine group blocking agents iodoacetamide and sodium cyanate in an attempt to determine which protein moieties have an important role in ^{14}C-dihydroartemisinin binding.

Pre-incubation of rat cortex homogenate with iodoacetamide or iodoacetamide and sodium cyanate significantly ($p< 0.001$) reduced binding of ^{14}C-dihydroartemisinin to homogenate proteins at all concentrations of ^{14}C-dihydroartemisinin used (N = 4). It reduced total binding by approximately 60-70% (figure 3.31). There was little difference between the inhibition caused by iodoacetamide / sodium cyanate and iodoacetamide alone suggesting that the majority of the inhibition was the inhibition of dihydroartemisinin binding to protein thiol groups. The blocking reagents significantly reduced the B_{max} of the cortex proteins for ^{14}C-dihydroartemisinin in both the high affinity / low capacity and low affinity / high capacity binding sites (both $p< 0.001$) (table 3.8), but had no effect on

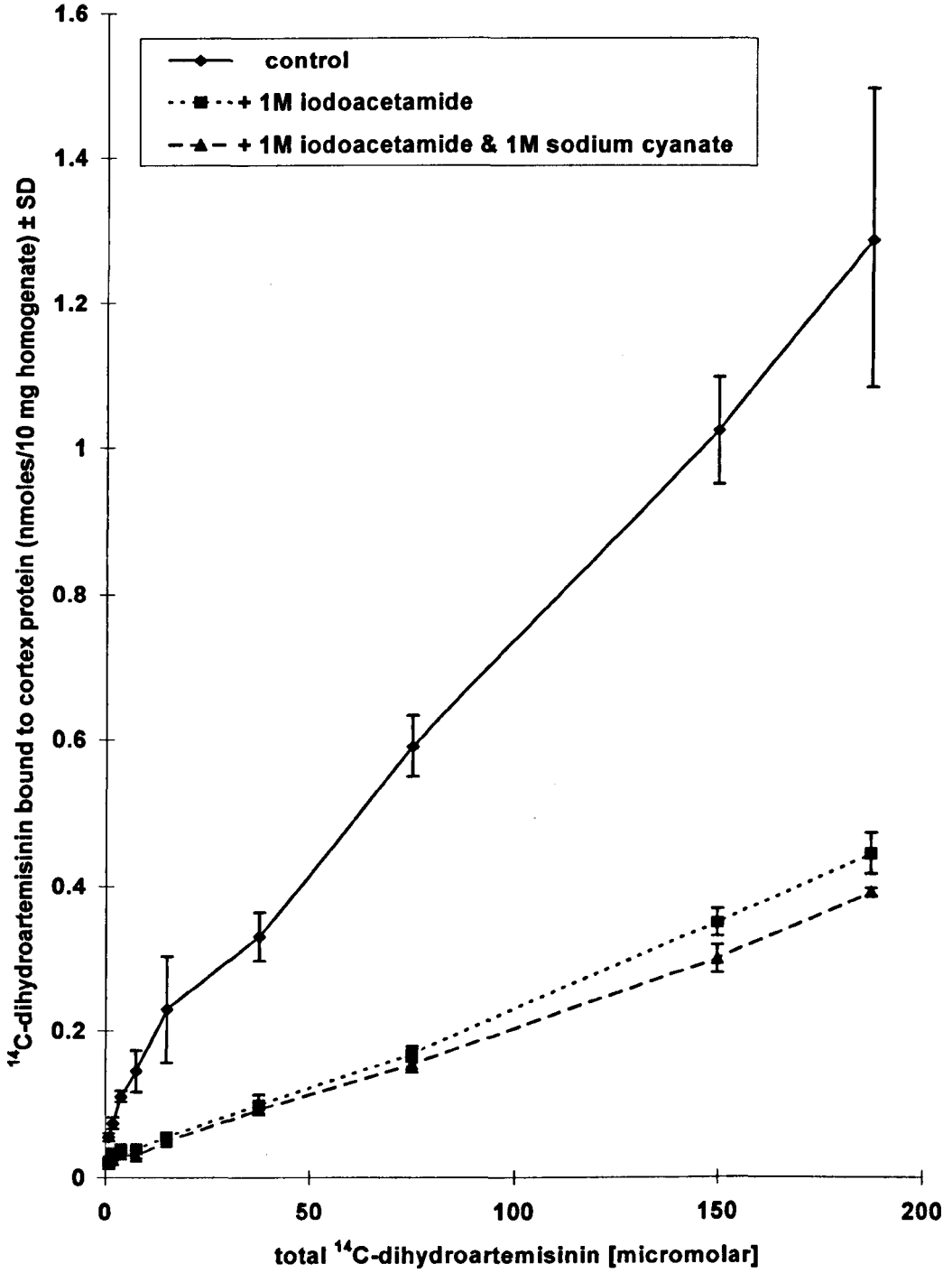


Figure 3.31. The effects of iodoacetamide and iodoacetamide plus sodium cyanate on ^{14}C-dihydroartemisinin binding to rat cortex protein. Binding was determined as outlined in figure 3.28 except the rat cortex homogenate was pre-incubated with either 1 M iodoacetamide or 1 M iodoacetamide and 1 M sodium cyanate before the binding assay began. ^{14}C-dihydroartemisinin binding was significantly ($p < 0.001$) inhibited at all concentrations of blocking reagent used. N = 4. Error bars represent standard deviations.

	PARAMETER	
CONTROL BINDING SITES	K_D [μM]	B_{MAX} (NMOLES / 10 MG HOMOGENATE)
HIGH AFFINITY, LOW CAPACITY	2.10±0.25	0.18±0.01
LOW AFFINITY, HIGH CAPACITY	520±148	3.60±0.97
IODOACETAMIDE -BLOCKED BINDING SITES	K_D [μM]	B_{MAX} (NMOLES / 10 MG HOMOGENATE)
HIGH AFFINITY, LOW CAPACITY	1.55±0.22	0.06±0.01
LOW AFFINITY, HIGH CAPACITY	835±223	2.09±0.56
IODOACETAMIDE & SODIUM CYANATE-BLOCKED BINDING SITES	K_D [μM]	B_{MAX} (NMOLES / 10 MG HOMOGENATE)
HIGH AFFINITY, LOW CAPACITY	1.79±0.24	0.05±0.01
LOW AFFINITY, HIGH CAPACITY	562±162	2.18±0.28

Table 3.8. The effects of iodoacetamide and sodium cyanate pre-incubation on the binding parameters of ^{14}C-dihydroartemisinin and rat cortex homogenate. Binding was assessed as outlined in figure 3.31. Data represents binding per 10 mg wet weight homogenate. N = 4. Values are presented as mean ± standard deviation.

the k_D values of either the low or high affinity binding sites. Iodoacetamide plus sodium cyanate inhibited ^{14}C-dihydroartemisinin binding to a significantly ($p< 0.05$) greater degree than iodoacetamide alone at ^{14}C-dihydroartemisinin concentrations up to 7.5 μM, although there were no significant differences between the binding parameters derived from cortex pre-incubated with iodoacetamide and the binding parameters derived from iodoacetamide and sodium cyanate pre-incubation (table 3.8). This effect of pre-incubation of sodium cyanate was very small, and whilst statistically significant, is probably not of any "physiological" significance to binding.

3.4.5. Effects of arteether and desoxyartemisinin on ^{14}C-dihydroartemisinin-protein binding

The relationship between the toxicity of the artemisinin derivatives and protein binding was examined by investigating the effects of arteether, an artemisinin derivative that is toxic to rats and cultured neural cells, and desoxyartemisinin, a non-toxic metabolite, on ^{14}C-dihydroartemisinin binding to cortex proteins. Incubation of rat cortex homogenate with 37.5 μM ^{14}C-dihydroartemisinin in the presence of 37.5 μM arteether led to a significant ($p< 0.001$) 42% decrease in ^{14}C-dihydroartemisinin binding ($N = 4$). Incubation of rat cortex homogenate with 37.5 μM ^{14}C-dihydroartemisinin in the presence of 37.5 μM desoxyartemisinin did not significantly ($p> 0.05$) reduce the binding of ^{14}C-dihydroartemisinin to cortex proteins (figure 3.32). These results suggest that the

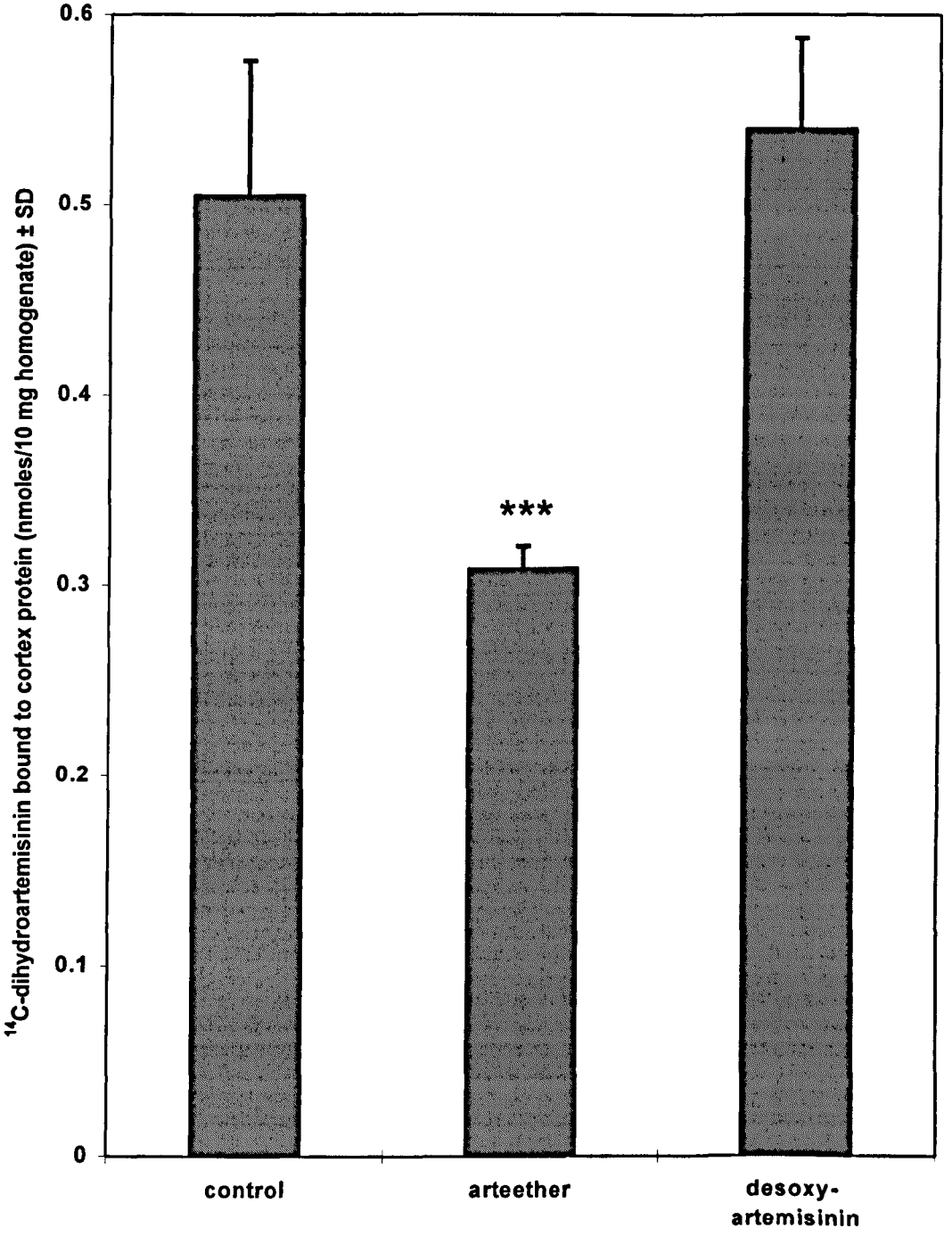


Figure 3.32. The effects of arteether and desoxyartemisinin on ^{14}C-dihydroartemisinin binding to rat cortex proteins. Rat cortex homogenate was incubated for 24 h at 37°C with ^{14}C-dihydroartemisinin (37.5 μM) alone, or ^{14}C-dihydroartemisinin in the presence of either arteether (37.5 μM) or desoxyartemisinin (37.5 μM). Protein was washed, extracted and ^{14}C-dihydroartemisinin binding to protein was assessed as outlined in figure 3.28. Arteether significantly ($p< 0.001$) reduced ^{14}C-dihydroartemisinin binding to rat cortex protein, desoxyartemisinin did not ($p> 0.05$). N= 4. Data represents nmoles bound per 10 mg wet weight homogenate, *** indicates a significant difference ($p< 0.001$) from control values. Error bars represent standard deviations.

artemisinin drugs compete for the same binding sites and, as was seen in the haemin incubations, a relationship exists between the ability to bind to proteins and exert neurotoxic effects *in vitro*.

3.5. SUMMARY OF RESULTS.

The effects of artemisinin and its derivatives on the proliferation of cultured Nb2a mouse neuroblastoma and C6 rat glioma cells were examined along with their effects on neurite outgrowth from Nb2a cells differentiated by the removal of serum and addition of dbcAMP. In cultured Nb2a and C6 cells, all drugs except desoxyartemisinin significantly inhibited cell proliferation in a dose-dependent manner with the lowest effective concentration being that of artemisinin at 0.1 μM. Artemether, arteether, artemisinin, and dihydroartemisinin also caused a dose-related decrease in the number of neurites / extensions produced by differentiating Nb2a cells, the lowest effective concentration being 1 nM dihydroartemisinin. Desoxyartemisinin had no effect on neurite outgrowth.

The ultrastructural damage caused by the artemisinin derivative dihydroartemisinin in differentiated Nb2a neuroblastoma was studied by transmission and scanning electron microscopy, Western blotting and immunocytochemistry, silver- and Coomassie Blue- staining of polyacrylamide gels and autoradiography. Stained polyacrylamide gels of Nb2a cells or rat cortex incubated with dihydroartemisinin with or without haemin did not reveal any drug-induced aggregation or degradation of proteins. Autoradiography of polyacrylamide gels of rat cortex incubated with ^{14}C-dihydroartemisinin revealed radiolabelling of proteins of molecular weights of approximately: 84 kilodaltons (kDa), 32 kDa and 24 kDa. Western blotting with monoclonal antibodies directed against common

targets for neurotoxic agents failed to detect any specific changes to these cytoskeletal proteins, nor were there any changes detected by immunocytochemistry. This was consistent with electron microscopy of cell neurites. However, transmission electron microscopy revealed that dihydroartemisinin damaged Nb2a cell mitochondrial cristae and endoplasmic reticulum. Scanning electron microscopy revealed that dihydroartemisinin severely damaged the microspike processes projecting from the surface of the cell body and neurites.

A study of Nb2a cells as a suitable model for the study of artemisinin neurotoxicity revealed that proliferating Nb2a cells did not possess significantly greater sensitivity to dihydroartemisinin toxicity than non-neuronal cell lines. However, incubation of differentiating and proliferating Nb2a cells with neurotoxic agents and non-specific toxic compounds demonstrated that neurotoxins had a significantly greater effect on neurite outgrowth from differentiating cells than on proliferation of undifferentiated cells and that non-specific toxins did not. The effects of dihydroartemisinin on neurite outgrowth were compared with that of a range of neurotoxic agents; the dihydroartemisinin IC_{50} for neurite outgrowth inhibition was of a similar magnitude to those of the neurotoxins, however transmission and scanning electron microscopy of Nb2a cells incubated with colchicine or 2,5-hexanedione did not reveal damage similar to that caused by dihydroartemisinin.

Incubation of rat cerebellum, cortex and brain stem with ^{14}C-dihydroartemisinin for 2 h and 24 h demonstrated that there was no significant difference in the amount of binding to these three areas of the brain and that binding

increased significantly between 2 h and 24 h; it also demonstrated that binding to protein was significantly greater than binding to other cell material after 24 h. Consequently, the binding characteristics of dihydroartemisinin and neural proteins and cells in culture were studied by incubating differentiating C6 and Nb2a cells and rat cortex homogenate with ^{14}C-labelled dihydroartemisinin with or without haemin. The role of protein thiol and amine groups in dihydroartemisinin binding was assessed by pre-incubation of cortex homogenate with sodium cyanate and / or iodoacetamide. The relationship between the toxicity of the artemisinin derivatives and protein binding was examined by co-incubating ^{14}C-dihydroartemisinin with arteether or desoxyartemisinin. Rosenthal plots of binding data demonstrated that there were two discrete phases of dihydroartemisinin binding. Haemin significantly increased the total binding of dihydroartemisinin to both cortex and cell proteins. In each preparation haemin significantly increased the B_{max} of the high affinity binding sites and also significantly increased the k_D of Nb2a high affinity binding sites and decreased the k_D of cortex low affinity binding sites. The effects of haemin on the binding parameters of cortex bore a greater resemblance to its effects on C6 cells than Nb2a cells. Nb2a high and low affinity binding sites had a significantly greater affinity for dihydroartemisinin than those of either rat cortex or C6 cells. Iodoacetamide and sodium cyanate reduced binding to cortex proteins significantly, by approximately 70%. Co-incubation of ^{14}C-labelled dihydroartemisinin with arteether significantly reduced ^{14}C-dihydroartemisinin binding to cortex proteins, co-incubation of ^{14}C-labelled dihydroartemisinin with the inactive desoxyartemisinin did not.

CHAPTER FOUR

DISCUSSION

PART ONE

4.1. THE EFFECTS OF THE ARTEMISININ DERIVATIVES ON CULTURED NEURAL CELLS

Part one of this thesis examined the effects of the artemisinin derivatives on cultured neuronal cells. The proliferation of undifferentiated Nb2a and C6 cells and neurite outgrowth from differentiating Nb2a cells were significantly inhibited by artemisinin, its recently developed analogues and metabolites in a dose-related manner. Desoxyartemisinin lacking the endoperoxide bridge, had no effect on proliferation or neurite outgrowth. In Nb2a and C6 cell cultures there was significant inhibition of proliferation at concentrations of the artemisinin derivatives as low as 0.1 μM, and Nb2a neurite outgrowth was significantly inhibited by dihydroartemisinin at a concentration as low as 1 nM.

These data have implications for the clinical use of the artemisinin derivatives. The IC_{50} values for both the inhibition of Nb2a and C6 proliferation and Nb2a neurite outgrowth were in the high nanomolar to low micromolar range (table 3.1). This is a cause for concern as clinical and pharmacokinetic studies have demonstrated that plasma concentrations of the artemisinin derivatives reach the same concentrations during treatment.

For example, a single oral 500 mg dose of artemisinin in healthy subjects has a maximum plasma concentration of ~ 1.5 μM and remains above 100 nM for over 12 h (Duc *et al.*, 1994), and in cases of uncomplicated *falciparum* malaria and in

healthy volunteers, a single oral 100 mg dose of sodium artelinate is rapidly converted to dihydroartemisinin which then reaches plasma concentrations of 2-3 μM (Teja-Isavadharm *et al.*, 1996b). Not only are such concentrations higher than a lot of the IC_{50} values for Nb2a and C6 cell proliferation and neurite outgrowth, but artemisinin derivatives also produced toxic effects in these cultured neuronal cells at concentrations significantly lower, especially to neurite outgrowth from differentiated Nb2a cells where all the pharmacologically active compounds tested in this study produced significant inhibition at concentrations ranging from 1-100 nM. These levels of the drugs must be present in the plasma of patients for a considerable length of time, meaning that they are routinely exposed to doses of the drugs at which potentially dangerous neurotoxic effects could occur. The results of parasite culture studies (Janse *et al.*, 1994; Hassan Alin *et al.*, 1995) using *P. berghei* and *P. falciparum* parasites incubated with artemisinin, artemether, arteether and sodium artelinate, suggest that *in vivo* concentrations of approximately 10-100 nM are required for effective treatment of malaria with these drugs. Consequently, reducing the amount of drug administered to patients to avoid any potential neurotoxicity may compromise the effectiveness of treatment and a change in the dosing regimen, administering smaller doses with greater frequency, to reduce peak plasma concentrations would not be sufficient to avoid exposing the patient to potentially neurotoxic levels of the drugs.

The artemisinin derivatives have been in use for over 20 years and remarkably little toxicity has been recorded in a clinical setting (see introduction). However, there have recently been some reports that suggest that treatment of

P. falciparum with artemisinin derivatives is associated with neurological sequelae (van Hensbroek *et al.*, 1996; Miller & Panosian, 1997). The tissue concentrations of the artemisinin analogues, or as frequently seems to be the case that of their common metabolite dihydroartemisinin, are determined from plasma samples and these may not necessarily reflect their levels in cerebrospinal fluid, which have not been measured. Pharmacokinetic factors determine the relative concentrations of the drugs at potential sites of action in body tissues and fluids and it may be that the drugs occasionally, in certain circumstances, *e.g.* in children or cases of cerebral malaria, reach concentrations approaching those in plasma which appear to be sufficient to produce neurotoxic effects. Consequently, it would be beneficial to determine the levels of the drugs in cerebrospinal fluid of patients relative to plasma, especially those patients that subsequently suffer neurological sequelae following their recovery from malaria.

Recent work in this laboratory (Smith *et al.*, 1997 and unpublished), using a different batch of compounds, has suggested that the artemisinin derivatives used alone are not as toxic to Nb2a neurite outgrowth at the very low concentrations reported in this study and that arteether and artemether in particular are generally less toxic to the cells than reported here and in other studies of artemisinin neurotoxicity using Nb2a cells and other neuronal cell lines (Wesche *et al.*, 1994). However, there is no disagreement on the neurite outgrowth IC_{50} values for dihydroartemisinin and desoxyartemisinin, the two compounds used in all subsequent studies.

Although the IC_{50} values for the inhibition of proliferation of Nb2a neuroblastoma and C6 glioma cells by the artemisinin derivatives are in the 10^{-6} M range there were differences in the toxicity of individual artemisinin derivatives toward proliferating Nb2a neuroblastoma and C6 glioma cells. Dihydroartemisinin was for instance less toxic in C6 cells than in Nb2a cells and artemether and arteether were notably more toxic in C6 cells than Nb2a cells.

A number of factors may contribute to these differences in toxicity. Differences in phenotype between neuronal and glial cells, or perhaps the different lengths of incubation time of the drugs with C6 cells and Nb2a cells may both influence toxicity. Although proliferating Nb2a neuroblastoma and C6 glioma cells are in an undifferentiated state they still show many characteristics of their mature phenotypes. Neuroblastoma is a tumour of the sympathetic nervous system, arising from neural crest precursors that are arrested in their normal development (Tonini *et al.*, 1991; Gaetano *et al.*, 1995; Hedborg *et al.*, 1995). Nb2a cells are a mouse neuroblastoma cell line, originally derived from another mouse neuroblastoma cell line C-1300, taken from a spontaneous tumour of the spinal cord (Augusti-Tocco & Sato, 1969; Walum & Peterson, 1982). They contain sizeable cytosolic pools of many specifically neuronal cytoskeletal proteins, *e.g.* GAP-43 and unphosphorylated neurofilament subunits and polymers, possess many specifically neuronal enzymes such as tyrosine hydroxylase, acetylcholine esterase, choline acetylase transferase, albeit at lower levels than differentiated cells (Prasad, 1991), and contain an acetylcholine receptor and signalling receptor system, (Augusti-Tocco & Sato, 1969). Moreover, undifferentiated Nb2a cells constantly extend and

retract very short neurite-like filopodia that possess the actin microfilament structures of neurite growth cones (Shea *et al.*, 1991a).

C6 rat glioma were originally derived from a chemically induced tumour (Benda *et al.*, 1968) and resemble fibroblast cells when in an undifferentiated state. They possess many characteristics of differentiated glial cells *e.g.* enzymes involved in the synthesis and modification of neurotransmitters: acetylcholine esterase, choline acetyl transferase, glutamic acid decarboxylase, monoamine oxidase, catechol-O-methyltransferase and histamine-N-methyltransferase (Fedoroff & Hertz, 1977). They also possess both specifically astrocytic and oligodendrocytic glial properties as shown by the presence of specific enzyme activities, glutamine synthetase for astrocytes and cyclic nucleotide phosphohydrolase for oligodendrocytes. They also stain with markers that indicate astrocytes: glial fibrillary acidic protein, and oligodendrocytes: galactocereboside (Parker *et al.*, 1980; Lee *et al.*, 1992). These characteristics suggest that the undifferentiated Nb2a and C6 cells express their mature phenotypes sufficiently to be viable models for neurotoxicity studies and that these differences in phenotype could account for differences in toxicity.

The artemisinin derivatives were incubated with Nb2a and C6 cells for different durations as C6 cells proliferate at a much slower rate than Nb2a cells. Consequently, to ensure that a measurable degree of C6 cell proliferation took place the cells had to be incubated for a longer period. Using a higher C6 cell density would have led to cell death due to overcrowding, as would the alternative approach, *viz.* performing Nb2a proliferation assays over 5 days - this could not be rectified by

reducing the Nb2a cell density as this would have made adding accurate numbers of cells to plates difficult and led to unacceptable variations in data from experiment to experiment. The longer incubation period of the C6 assays may have increased the metabolism, or reduced the stability of, pharmacologically active drugs; alternatively it could have resulted in an overall increase in conversion of some drugs to more reactive products. In these assays that could mean that the longer C6 cell incubation period led to a steady conversion of artemether and arteether into reactive products throughout the five day period resulting in a higher concentration of toxic products overall than would occur in a 24 h incubation; this is supported by other work in this laboratory (Smith, unpublished) in which the effects of dihydroartemisinin and artemether on Nb2a neurite outgrowth over 1 day and 5 days were compared: the IC_{50} value for dihydroartemisinin toxicity after 5 days was the same as after 1 day, whereas artemether toxicity has increased significantly. Therefore, differences in the lengths of the incubation periods in Nb2a and C6 cells could also influence the apparent differences in toxicity of the artemisinin derivatives.

As discussed previously, proliferating neuroblastoma and glioma cells express some of the characteristics of cells of the mature nervous system and consequently are frequently used for the *in vitro* study of neurotoxicity. However, differentiated Nb2a cells more closely resemble neuronal cells in terms of cell shape and neurite maintenance and so measurement of drug effects on neurite outgrowth may be a more sensitive and reliable indicator of neurotoxicity. This method has been validated by other researchers (Abdulla & Campbell, 1993) and is achieving wide use for measurement of neurotoxicity (Henschler *et al.*, 1992; Brat &

Brimijoin, 1993; Flaskos *et al.*, 1994). Neurite outgrowth as a parameter useful in the measurement of neurotoxicity is discussed in depth in section 4.3 below.

The IC_{50} values for the inhibition of the proliferation of undifferentiated Nb2a and C6 cells may not as a whole have differed significantly, but each derivative significantly inhibited neurite outgrowth from differentiating Nb2a cells at concentrations lower than those at which it inhibited proliferation of undifferentiated cells, although the cells were exposed to the drugs for twice as long in proliferation assays as in neurite outgrowth assays. At the low concentrations at which no significant inhibition of proliferation took place the effects of the drugs were apparent on neurite outgrowth.

The sensitivity of neurite outgrowth to the toxic effects of these compounds appears to be a genuine measure of neurotoxicity instead of a representation of the general condition of the cell. The effects of the artemisinin derivatives on the general metabolism of the differentiated cell, as measured by conversion of MTT to a coloured product, were compared with its effects on neurite outgrowth. The IC_{50} values for inhibition of general cell metabolism, like those of proliferation, were significantly higher than those of neurite outgrowth (Smith, unpublished work in this laboratory), furthermore, combination of the drug with haemin has a far greater effect on neurite outgrowth than on MTT metabolism (Smith *et al.*, 1997). Consequently, compounds that inhibit neurite outgrowth to a significantly greater degree than they inhibit proliferation or general metabolism probably have neurotoxic effects on the cells. A more thorough evaluation of the proliferating and differentiating Nb2a cell as an *in vitro* model suitable for the study of mechanisms of

neurotoxicity and as a screening tool formed the basis of part three of this thesis and is discussed in detail in section 4.3.

C6 cells can also be made to differentiate using a similar method to that used with Nb2a cells. The cells are induced to differentiate by adding dibutyryl cyclic AMP (Tas & Koschel, 1990) and withdrawing serum, which contains lysophosphatidic acid, the factor which suppresses differentiation (Koschel & Tas, 1993). However, the cells differentiate into a mixed population of cells (Parker *et al.*, 1980; Lee *et al.*, 1992) and the neuritic material expressed by the two cell types is strikingly different. In my experiments, differentiation into one cell type led to the formation of lamellopodia of neuritic material surrounding the cell body while the other differentiated cell type expressed a few neurites projecting away from the cell body in a manner similar to that of differentiated Nb2a cells (figure 4.1). Identification of the two cell types is difficult, but it may be that the cells that produced lamellopodia were the astrocytic cell type while the cells that expressed neurites were the oligodendrocytic cell type. Furthermore, the relative proportions of the two cell types varied from experiment to experiment. This represented a problem in assessing neurotoxicity to differentiated C6 glial cells. Quantifying drug effects on a mixed population of two different types of cells which express different amounts, and types of neurites which may have different sensitivities to the drug was very difficult. It was not amenable to the methods of analysis used in the evaluation of the toxicity of the artemisinin derivatives and other toxic compounds toward Nb2a neurite outgrowth. Consequently, the effects of the artemisinin derivatives on differentiating C6 cells were not assessed.

Figure 4.1. Differentiated C6 cells

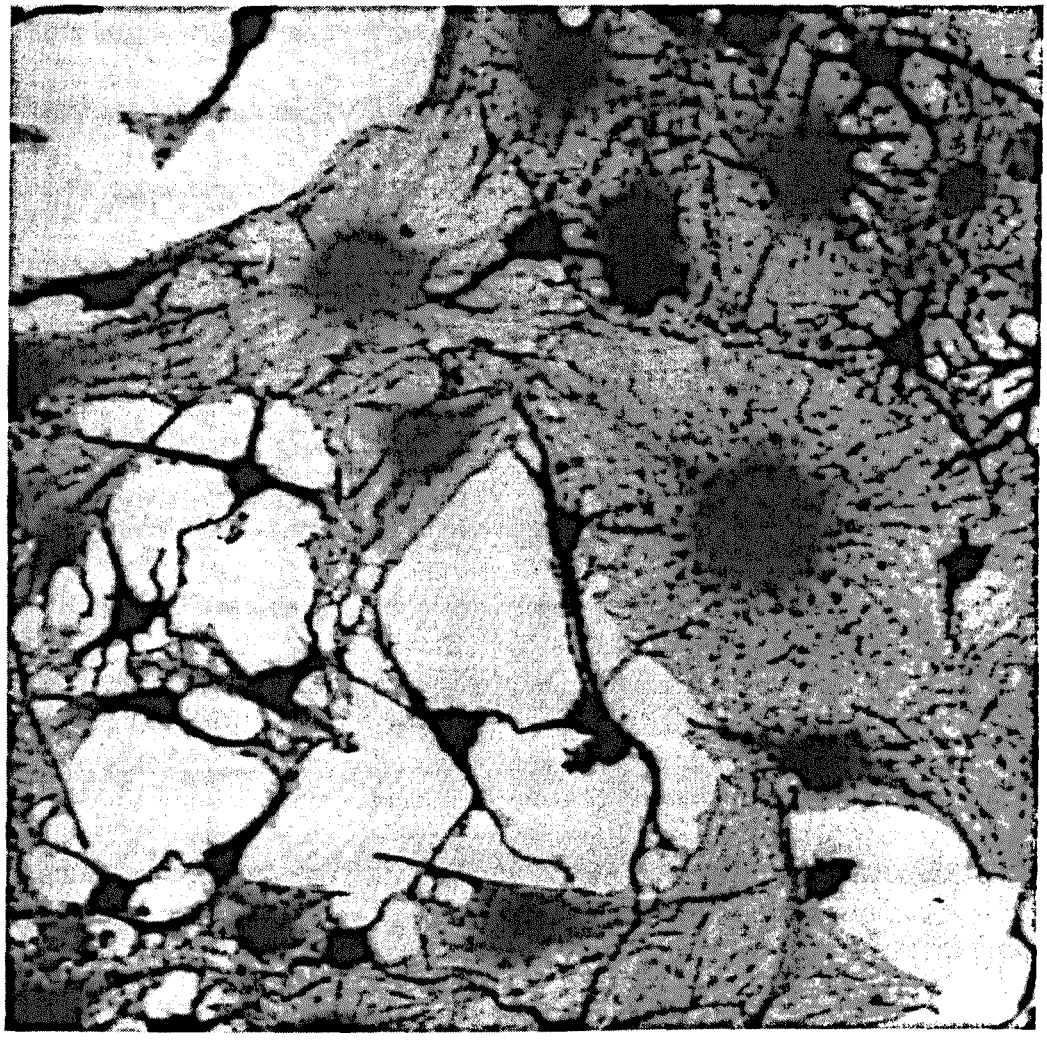


Figure 4.1. C6 cells incubated for 5 days in serum free medium containing 500 μM dibutyryl cyclic AMP. The cells differentiated into two distinct cell types. Some flatten and spread over the extracellular matrix and lamellopodia formed around the cell edge, others extended "neurites" in a manner similar to neuronal cell types. The identity of these cell types in uncertain but undifferentiated C6 cells possess both astrocytic and oligodendrocytic characteristics and it may be that the flattened cells are astrocytes and the cells that extend "neurites" are oligodendrocytes.

All the artemisinin derivatives that possessed an endoperoxide moiety caused significant inhibition of cell proliferation and neurite outgrowth. This unusual endoperoxide linkage of sesquiterpene lactones is known to be necessary for their pharmacological activity (Tani *et al.,* 1985; Imakura *et al.*, 1988; Butler & Wu, 1992). Desoxyartemisinin and desoxyarteether, which do not possess this group, were therefore included in this study to determine if the pharmacophore of the artemisinin derivatives was also necessary for toxicity. Desoxyartemisinin lacked any significant effects on neurite outgrowth and desoxyartemisinin and desoxyarteether lacked significant anti-proliferative effects, although at a concentration of 100 μM, desoxyarteether inhibited proliferation of Nb2a cells. At the highest concentrations there was a trend among the desoxy- derivatives toward inhibition of proliferation / neurite outgrowth, but this may not be a genuine indication of neurotoxicity caused by compounds that do not contain an endoperoxide bridge. It is possible that at concentrations of 50-100 μM small impurities in the sample, related compounds containing an endoperoxide moiety, may be present in sufficient amounts to begin to exert an effect.

The endoperoxide bridge is a relatively weak bond, and breaking this bond and opening up the lactone ring followed by a series of rapid rearrangements, results in highly reactive carbon-centred free radicals (see introduction) and other reactive products including an electrophilic epoxide and at least one diketone (Meshnick *et al.*, 1991, 1993; Posner *et al.*, 1992, 1996; Zhang *et al.*, 1992; Baker *et al.*, 1993). The postulated pharmacological mechanism of action of these compounds is that these reactive products and others like them bind to specific malarial proteins,

causing disorganisation and death of the parasite within hours of administration of the drug (Ellis *et al.*, 1985; Maeno *et al.*, 1993; Kamchonwongpaisan *et al.*, 1997) (see introduction). Similar processes in neural cells may be responsible for the toxic effects of these drugs. Carbon-centred free radicals produced by decomposition of the endoperoxide bridge could cause alkylation, lipid peroxidation and protein oxidation of differentiating and proliferating Nb2a cells, and diketones, which are known to be neurotoxic, can crosslink and aggregate cytoskeletal proteins. The results of this study suggest that the endoperoxide moiety is necessary for most if not all of the toxicity of the artemisinin derivatives to proliferating and differentiating Nb2a and C6 cells.

It appears therefore that the presence or absence of an endoperoxide moiety is the single most important factor in determining if the artemisinin derivatives have pharmacological activity / neurotoxicity. Nevertheless, the endoperoxide bridge may not be the only part of the artemisinin molecule necessary for neurotoxicity. A tetraoxane compound possessing an endoperoxide moiety but not the artemisinin ring structure has no toxicity towards proliferating Nb2a neuroblastoma cells (Wesche *et al.*, 1994). It may be that other parts of the artemisinin molecule possess some form of neurotoxic effect, or perhaps facilitate the delivery to or binding of the artemisinin molecule to its target sites. Other sesquiterpene lactones, including cynaropicrin, solistitalin and repin, although lacking an endoperoxide moiety, are still neurotoxic *in vivo* and to cultured neuronal cells (Wang *et al.*, 1991; Mullin *et al.*, 1991; Butterfield *et al.*, 1993). Therefore some element of the neurotoxicity of artemisinin may be attributable to other parts of the molecular structure. Moreover,

since there are also differences in the relative toxicity's of all the active derivatives that possess the endoperoxide group and the basic artemisinin structure, other factors must also be involved in determining the level of toxicity.

The only structural divergence between the active artemisinin derivatives used in this study are the different substituents at the C-10 position of artemisinin (the carbonyl group is not itself necessary for neurotoxicity): dihydroartemisinin has a hydroxy- group in this position, artemether a methyl ether group, arteether an ethyl ether group, and artelinate an carboxybenzyl ether (see figure 2.1 for structures). There are a number of ways in which these substitutions could affect the physico-chemical properties, and so the toxicity, of these drugs.

The substitutions at the C-10 position are probably too remote from the endoperoxide bridge to exert significant direct electronic effects on the reactivity of this moiety. Instead the C-10 substituents may modulate the reactivity and toxicity of the derivatives by controlling the rate at which they are converted into dihydroartemisinin. Dihydroartemisinin is the most neurotoxic compound *in vivo* and the most potent inhibitor of Nb2a neurite outgrowth. Clinical studies and *in vitro* metabolism studies have demonstrated that all the artemisinin derivatives are converted to dihydroartemisinin to a greater or lesser extent (see introduction) and consequently it makes a substantial contribution to the activities of all these compounds. Sodium artesunate undergoes extremely rapid hydrolysis to dihydroartemisinin while artemether and arteether possess an ether linkage which is resistant to hydrolysis and are instead enzymatically converted to dihydroartemisinin (Lin & Miller, 1995). The desoxy- metabolites cannot, of course, undergo

conversion to dihydroartemisinin. The contribution of dihydroartemisinin to the neurotoxicity of the artemisinin derivatives in Nb2a and C6 cells is not known as the metabolism of these compounds in cultured neural cells has not been elucidated. The relative toxicities of the compounds based on conversion to dihydroartemisinin would be: dihydroartemisinin > artesunate > artelinate, artemether and arteether > artemisinin (which is not converted to dihydroartemisinin). This does not correlate *exactly* with the rank order of IC_{50} values for the inhibition of Nb2a neurite outgrowth, Nb2a proliferation or C6 cell proliferation (see sections 3.1.2-3.1.4), nor with *in vivo* neurotoxicity studies (Brewer *et al.*, 1993), although there is broad agreement in each case. This suggests that conversion of the artemisinin derivatives to dihydroartemisinin, or a similar conversion to other toxic metabolites, does occur and is a significant influence on *in vitro* neurotoxicity. It also implies that another factor, or combination of factors, also has an important influence on the neurotoxicity of the drugs.

The non-polar hydrocarbon skeleton of artemisinin makes it a lipophilic compound. Substitution of the carbonyl group will alter this lipophilicity; the ethyl and methyl substituents of arteether and artemether make them more lipophilic, enabling more effective penetration of the cell membranes and increasing their concentration at the target sites. In contrast, the substitutents on dihydroartemisinin and sodium artelinate make them more hydrophilic, increasing their water solubility. Studies of the effects of dihydroartemisinin and artemisinin (Ellis *et al.*, 1985; Maeno *et al.*, 1993, respectively) on malaria parasite cultures demonstrate that the majority of the drug partitions into parasite membranes and that many of these

membranes are severely damaged by the drug. Lipophilicity is an important factor in determining to what extent a compound will partition into cell membranes: the greater the lipophilicity of a compound the more it will partition into these structures. Consequently, differences in lipophilicity could be an important factor in determining the drug's relative toxicity if neurotoxicity and antimalarial action have a similar mechanism of action. Indeed, increasing cytotoxicity has been reported with increasing lipophilicity with several other sesquiterpene lactones (Kupchan *et al.*, 1971). Lipophilicity may be an important factor for determining drug toxicity towards proliferating C6 glioma cells as the rank order of toxicity (which is: arteether > artemether > dihydroartemisinin) corresponds with increasing lipophilicity (Avery *et al.*, 1995). However in proliferating and differentiating Nb2a cells, and in *in vivo* models for neurotoxicity (Brewer *et al.*, 1993), they do not correlate and other factors must have a greater influence on toxicity.

The binding relationship between the drug and its target site, or possibly between the drug and the haemin / iron molecule that catalyses the formation of reactive products, will also be important factors in determining the relative toxicity of the artemisinin derivatives. The substitutions at the C-10 position may physically either help or hinder the interaction of the drug with its binding sites or with haem (if haem is involved), affecting the degree of drug binding or conversion of the drugs into reactive products respectively, thus influencing toxicity. As the target sites for the drug are not known, the effects these substitutions will exert on binding cannot be established. This problem makes drawing unequivocal conclusions about the structure-activity relationships of these drugs, other than the necessity of the

endoperoxide bridge, difficult. This in turn hinders attempts to comprehend the mechanism of neurotoxicity. As the specific targets are not known an alternative approach to improving our understanding of the mechanism of action is by comparing the relative toxicity's of the drugs with their potencies as antimalarial agents and their *in vivo* neurotoxicity to determine if any similarities exist between them.

Further elucidation of the mechanism of neurotoxicity by comparison of the relative orders of the *in vitro* neurotoxicity of the artemisinin derivatives with *in vivo* clinical studies is difficult; many different routes of administration, drug preparations, combinations with other antimalarial drugs and dosing regimens are used. However, it is possible to compare *in vitro* neurotoxicity with the relative orders of toxicity in cultured malaria parasites to discover if the same factors, whatever they may be, also modulate pharmacological activity. Comparisons can also be made with animal neurotoxicity studies to determine if *in vitro* neurotoxicity reflects the *in vivo* situation and also help validate the use of Nb2a cells as models for neurotoxicity.

Neurite outgrowth from differentiating Nb2a cells is the most accurate and sensitive measure of neurotoxicity of the three parameters measured in these cell culture studies (see part three of this thesis). The rank order of neurotoxicity of these compounds as represented by IC_{50} values for the inhibition of neurite outgrowth correlate well with the IC_{50} values from *in vitro* parasite studies. The rank order of IC_{50} values for neurite outgrowth is dihydroartemisinin > artemether > arteether > artemisinin (table 3.2) and is in agreement with *in vitro* parasite studies. The effects

of several artemisinin derivatives on the W2 and D3 clones of *P. falciparum in vitro* have been compared and it has been established that dihydroartemisinin is the most toxic followed by artemether and arteether then artemisinin (Trigg, 1989). Dihydroartemisinin is more toxic *in vitro* to *P. berghei* than artemisinin (Janse *et al.*, 1994) and also *in vivo* using *P. berghei*-infected mice. Another study (Basco & Le Bras, 1994) reports that arteether and artemether have greater activity than artemisinin *in vitro* against Cambodian isolates of *P. falciparum*. Finally, dihydroartemisinin possesses greater activity against Vietnamese isolates of *P. falciparum* than artemisinin (Wongsrichanalai *et al.*, 1997). These similarities suggest that the factors that influence the precise degree of neurotoxicity also modulate the pharmacological activity of the drugs in a similar manner. Along with the influence of the endoperoxide moiety and haemin, this strongly suggests that the same transformations of the drugs, and the toxic products that result from these, are involved in both neurotoxicity and antimalarial activity and that it is possible there are further similarities between their mechanisms of action.

The *in vivo* neurotoxicity studies used a wide range of animals and derivatives of artemisinin. Unfortunately, most studies only used one of the drugs, usually artemether or arteether (Petras *et al.*, 1993a, 1993b, 1994; Kamchonwongpaisan *et al.*, 1997), and when a variety of derivatives were used (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982d) different dosing regimens were also employed making direct comparison of relative toxicity's impossible. Brewer and co-workers (1993) investigated the neurotoxicity of a number of artemisinin derivatives to dogs and rats

and reported that dihydroartemisinin is more toxic than artemether and arteether which were themselves more toxic than artesunate and artelinate. This is in agreement with the results of both the Nb2a neurite outgrowth and proliferation studies, suggesting that Nb2a cells are affected by the drugs in a similar manner to the brains of animals that are vulnerable to artemisinin neurotoxicity. This provides support for the use of Nb2a cells as a model with which to study the neurotoxicity of the artemisinin derivatives *in vitro*.

There is one curious difference between the results of the Nb2a assays and the results of *in vitro* parasite and *in vivo* neurotoxicity studies. *In vivo*, and in parasite studies, artemether and arteether have similar activities / toxicity's, yet their toxicity towards Nb2a cell proliferation and neurite outgrowth and C6 proliferation differ. This is not an isolated occurrence as other *in vitro* neurotoxicity studies (Wesche *et al.*, 1994) have also reported differences in artemether and arteether toxicity in a number of primary and secondary neuronal cell cultures. It is difficult to offer an explanation for this anomaly other than to say that they may not both undergo metabolism to the same degree in cultured neuronal / glial cells as they do *in vivo* or by parasites

Despite the high degree of agreement about the relative toxicity's of these half-dozen commonly used derivatives in both *in vivo* and *in vitro* models for artemisinin neurotoxicity (and *in vitro* parasite studies), the use of neuronal cell culture alone is not sufficient to clarify the secondary structure-activity relationships of the artemisinin derivatives. The role of chemical structure in the neurotoxicity and pharmacological activity of the artemisinin derivatives is unusually complex as it

appears to be multi-layered. The absolute necessity of the endoperoxide linkage for any activity is obvious. In addition, all the derivatives used in these assays have different moieties in the C-10 position (figure 1.1) which will modulate the degree of conversion to dihydroartemisinin, the most toxic artemisinin derivative, and perhaps other toxic metabolites. The production of toxic molecules involves the interaction of the drug with a catalyst, possibly free iron but more likely to be the large iron-containing molecule haem, and C-10 substitutions could influence this interaction. Subsequently, the products of this reaction then bind to their target sites and the structural variations of the derivatives could influence toxicity at this point also. Difficulties in interpreting the precise effects of the substitutions arise because a substituent that makes the molecule more susceptible to conversion to dihydroartemisinin may also hinder the interaction of unchanged drug with the haem catalyst or the target proteins or *vice versa*. In other words, C-10 substitutions clearly influence activity but why they do so is less obvious. The relative neurotoxicity's of the artemisinin derivatives in these studies are a complex composite of these factors in addition to more conventional influences on drug activity such as lipophilicity.

4.2. MORPHOLOGICAL AND IMMUNOCYTOCHEMICAL EFFECTS OF DIHYDROARTEMISININ ON DIFFERENTIATING NB2A NEUROBLASTOMA CELLS

Part one of this thesis demonstrated that artemisinin derivatives are toxic to Nb2a cells and that they are significantly more toxic to neurite formation by differentiated Nb2a cells than to proliferation of undifferentiated Nb2a cells. In their differentiated form Nb2a cells more closely resemble the phenotype of cells of the mature nervous system, producing axon-like neurites and ceasing to proliferate. This suggested that in differentiated Nb2a cells the artemisinin derivatives exerted neurotoxic effects as well a more general toxicity observed in proliferating, undifferentiating cells. These neurotoxic effects could be mediated in a number of ways. The differentiated Nb2a cells could express proteins that act as targets for the neurotoxicity of artemisinin derivatives. Neuronal cells often contain high levels of iron, in various forms, and so are relatively sensitive to oxidant stress; consequently the artemisinin derivatives may exert their neurotoxic effects through conversion of the drug into free radicals which then bind to neuronal proteins in a similar manner to which they selectively alkylate parasite proteins. Other characteristic features of neuronal cells are their large energy requirements and high levels of protein synthesis; both of which are very important for the proper functioning of the axonal transport of vital metabolic and structural elements and therefore may also be targets

for the neurotoxic effects of the artemisinin derivatives. Consequently, the ultrastructural damage caused by the artemisinin derivative dihydroartemisinin in differentiated Nb2a neuroblastoma cells was studied by transmission and scanning electron microscopy and immunocytochemistry. In addition possible drug effects on cell proteins were investigated by autoradiography, silver- and Coomassie Blue-staining, and Western blotting of cell proteins.

The results of sections 3.4.1 - 3.4.3 demonstrated that substantial drug-protein binding took place and that as haemin increased both binding and toxicity (Smith *et al.*, 1997) this binding is implicated in drug toxicity. Autoradiographs of polyacrylamide gels of rat brain incubated with ^{14}C-dihydroartemisinin revealed that the drug bound to several neuronal proteins (section 3.2.4 & figure 3.10). The identity or function of these proteins is not known. The stock ^{14}C-dihydroartemisinin was not of a high specific activity, and consequently high concentrations of drug had to be used to obtain sufficiently strong radiolabelling of the target proteins. Unfortunately, high concentrations of drug tend to increase non-specific binding and this may obscure specific binding. However, only three or four proteins were radiolabelled and did not correlate with the most abundant cell proteins on the Coomassie- and silver-stained gels (figures 3.5 & 3.6). Furthermore, non-specific binding between the artemisinin derivatives and erythrocyte membrane proteins is severed by treatment with mercaptoethanol or urea, implying non-covalent binding (Asawamahasakda *et al.*, 1994a), whereas this binding to rat brain proteins, and binding to Nb2a cell and parasite proteins (Asawamahasakda *et al.*, 1994b; Kamchonwongpaisan *et al.*, 1997), is not severed by this treatment. Consequently,

the drug-rat brain protein binding may be specific and one or more of these proteins may be the target through which the neurotoxicity of the artemisinin derivatives is mediated. The involvement of specific labelling of proteins in the neurotoxicity of artemisinin is also supported by the specific alkylation of the proteins of Nb2a cells when they are incubated with ^{3}H-dihydroartemisinin (Kamchonwongpaisan *et al.*, 1997). Indeed there are similarities between the masses of the proteins labelled by dihydroartemisinin in rat brain cells and those labelled in Nb2a cells. The approximate molecular weights of the labelled proteins in rat brain were 24 kDa, 32 kDa and 84 kDa, while many proteins were labelled in Nb2a cells with the most likely candidates for specific alkylation being of molecular weights 27 kDa, 32 kDa, 40 kDa and 81 kDa. Furthermore, *P. falciparum* malaria-specific proteins of molecular weights 22 kDa, 25 kDa, 32 kDa, 42 kDa, 50 kDa, 65 kDa and 200 kDa+ also bind the artemisinin derivatives (Kamchonwongpaisan *et al.*, 1997). These similarities between the masses of the proteins suggests that the target(s) for the drugs in neuronal cells and parasites may be related.

Silver- and Coomassie Blue- staining gels of differentiated Nb2a cells and rat brain incubated with dihydroartemisinin failed to reveal any changes in the cell proteins visible on polyacrylamide gels. Consequently, although dihydroartemisinin certainly binds to neuronal proteins (sections 3.2.4 and 3.4.1; Smith *et al.*, 1997) its toxic effects do not appear to manifest themselves through gross changes in protein synthesis nor through aggregation of proteins such as neurofilaments / microtubules (a common cause of neurotoxicity) or degradation of proteins into smaller fragments. The results of the Western blotting of differentiated Nb2a cells, which

suggested that the common cytoskeletal proteins spectrin, neurofilaments and microtubules are not the targets for neurotoxicity of dihydroartemisinin, support this idea.

Haemin significantly increased the binding of dihydroartemisinin to cell proteins (section 3.4.3), and increased the toxicity of the drugs to Nb2a cells (Smith *et al.*, 1997). It was hoped that by using haemin to catalyse additional free radical production the resulting enlargement in binding / toxicity might increase the size of any drug effects on the proteins that had not previously been visible on the gels to a point where they became detectable. However even when co-incubated with haemin no drug effects on the proteins were visible. This suggests that if gross changes in cell proteins such as crosslinking have taken place then the proteins involved must be either present in the cell in small amounts or not affected by the addition of haemin to the incubations. Obviously, if the effects of the drugs on cell proteins do not take the form of changes detectable on polyacrylamide gels then a haemin-induced increase in drug binding will not be observed.

Changes in the distribution of proteins within Nb2a cells will not be detected by Western blotting or on stained gels but will be detected by indirect immunofluorescence of fixed cells. In these studies no drug-induced changes in the distribution of spectrin, acetylated or tyrosinated α-tubulin or NF-H were detected in fixed Nb2a cells. Changes of this kind may still have occurred but there are hundreds of possible target proteins that could be affected, and practical considerations mean that no more than a few of the most likely targets could be investigated.

Silver- and Coomassie Blue- staining of cell proteins, Western blotting and immunofluorescent microscopy are limited to the detection of changes in proteins such as crosslinking of neurofilaments and microtubules, gross alterations in the abundance of a particular protein or changes in protein distribution, and as mentioned previously, it is possible that the changes occurring within the cell are of a functional nature and they may not be detectable using these methods. Binding to key receptors, enzymes, ion channels *etc.* would make little difference to the mass of such macromolecules, but could have significant effects on cell function.

Transmission electron micrographs revealed that dihydroartemisinin affected the RER and mitochondria of both cell bodies and neurites but there was no obvious disorganisation of the cytoskeletons of neurites. This is in agreement with the results of Western blotting and immunofluoresence of Nb2a cells in which spectrin, microtubules and neurofilaments were unaffected by the drug.

Artemisinin derivatives produce free radicals which cause lipid peroxidation of intact erythrocyte membranes and thiol oxidation in isolated erythrocyte membranes (Meshnick *et al.*, 1989, 1991). TEM and autoradiography reveal that in cultured malaria parasites artemisinin and dihydroartemisinin are largely localised in the membranes of food vacuoles, mitochondria and the perinuclear membranes (Maeno *et al.*, 1993, Ellis *et al.*, 1985). The artemisinin derivatives act on the mitochondria, food vacuoles and the perinuclear membranes and ER of *P. falciparum* causing them to swell and then degenerate as the parasite becomes completely disorganised (Maeno *et al.*, 1993).

Artemisinin has also been reported to cause swelling and

vacuolar degeneration of mitochondria, including degeneration of cristae, and dilatation of ER in the cardiac cells of Rhesus monkeys, although this damage, even at very high doses, is transient (Wang & Liu, 1983).

Changes in some of these organelles were seen in drug-treated Nb2a cells. In our study the cristae of the mitochondria were deformed or absent and the amount of RER was reduced (this may be due, in part, to a dissociation of ribosomes from ER membrane hindering identification) and the RER which remained was often reduced in length and slightly dilated, although no changes in nuclear membranes of Nb2a cells were observed.

None of the changes to cell organelles seen on transmission electron micrographs therefore offers a clearcut explanation for the specifically neuronal effects seen in studies of neurite outgrowth in part one of this thesis, although damage to both mitochondria and ER observed on the TEM's may contribute. As mentioned previously, neurones have high energy requirements; mitochondria produce the energy for the cell, converting adenosine diphosphate to adenosine triphosphate (ATP) through oxidative metabolism of glucose and oxygen to water and carbon dioxide (*i.e.* respiration). Damage to, or loss of, mitochondria would reduce ATP production, hampering a wide range of general cell processes, such as metabolism and biosynthesis, having a damaging effect on the cell. This may include more specifically neurotoxic effects as extension of neurites, the synthesis and transport of cytoskeletal components, neurotransmitter vesicles and other organelles down the axon, and the maintenance of axonal ion gradients for the generation of action potentials require a great deal of energy. Indeed, the mitochondria present in

the neurites were as badly damaged by dihydroartemisinin as mitochondria throughout the cell.

Rough endoplasmic reticulum, ER with ribosomes attached, is responsible for the synthesis of membrane glycoproteins, secreted proteins and extrinsic membrane proteins located on the exoplasmic face of cells *e.g.* laminin, and collagen. A corresponding reduction in protein synthesis happens when damage to the ER occurs in parasites treated with artemisinin derivatives (Ellis *et al.*, 1985). Again, a high level of protein synthesis is a characteristic feature of neurones, a significant amount of this protein synthesis being responsible for the particular high energy requirements of the neuronal cells listed above. Disruption of RER function would reduce protein synthesis and have detrimental effects on the composition of cell membranes, cell attachment to the growth substrate and communication with other cells, which may have a bearing on neurite outgrowth. Therefore, the deleterious effects of dihydroartemisinin on the RER of Nb2a cells may also contribute to the neurotoxicity. Unattached ribosomes produce proteins localised on the cytoplasmic face of the membrane: spectrin, ankyrin *etc.* (Darnell *et al.*, 1990) and there is no evidence from this study that dihydroartemisinin affects them; this is consistent with the results of Western blotting and immunofluoresence of fixed cells.

It is possible that the effects on mitochondria and RER are linked in some way. Mitochondria may be induced to swell and become damaged when exposed to elevated calcium levels (Rapoport *et al.*, 1985) and the lumen of ER is one of the main stores of calcium within the cell. However, the sequence of events within the cell is not known and intracellular calcium levels tend to rise generally in damaged

(neural) cells, not just specifically as a result of damage to ER. Alternatively, drug-induced damage to the mitochondria may be the initial cause of drug toxicity, and by reducing energy levels in the cell, could in turn cause a reduction of protein synthesis by the RER. Damage to mitochondria has also been proposed as a primary cause of the drug's toxicity towards malaria parasites (Maeno *et al.*, 1993). Mitochondria may be particularly vulnerable to the toxic effects of the artemisinin derivatives as they contain high levels of iron (Darnell *et al.*, 1990), and iron is an essential feature of mitochondrial enzymes such as cytochrome oxidase (Morris *et al.*, 1992a), the activity of which is inhibited by sodium artesunate (Zhao *et al.*, 1986). Overall, damage to mitochondria and RER will result in a general and wide ranging impairment of cell function which would presumably include some specifically neuronal aspects of the cell. The more generalised disruption of normal function may manifest itself as the non-specific toxic effects of dihydroartemisinin that inhibit the proliferation of neuronal and non-neuronal cell lines rather than being directly involved in neurotoxicity and the inhibition of neurite outgrowth from differentiating Nb2a cells.

The dihydroartemisinin-induced damage to the cell membranes visible on scanning electron micrographs, where the surface protrusions on the soma and neurites of control cells were badly damaged or completely absent, may be of greater significance to drug toxicity and inhibition of neurite outgrowth. The structures protruding from the surface of control cells are commonly known as microspikes. these contain a bundle of actin microfilaments and several associated proteins such as moesin, fascin and myosin (Bray & Chapman, 1985; Condeelis, 1993; Amieva &

Furthmayr, 1995; Yamakita *et al.*, 1996) and are thought to have a sensory function (Schwartz-Albiez *et al.*, 1995). Microspikes are not unique to neuronal cells but are a common feature of many cultured cells *e.g.* 3T3 cells (Kozma *et al.*, 1995) for which it is thought they perform a similar function. Basal microspikes, in addition to performing a sensory function anchor the cell to the extracellular matrix (Pietrasanta *et al.*, 1994) and are often referred to as filopodia. The filopodial microspikes of neuronal cells differ significantly from those of other cell types as they are crucial for neuritogenesis (Smith, 1994b; Jackson *et al.*, 1996). In undifferentiated Nb2a cells, the filopodial microspikes around the base of the cell take the form of very short neurites, constantly being extended and retracted, projecting from the cell membrane along the extracellular growth matrix in a filopodia-like manner, but this limited neurite outgrowth is suppressed by factors present in serum (Shea *et al.*, 1991a). When provided with the correct stimulus neuronal cells, *e.g.* for Nb2a cells when serum is withdrawn and dibutyryl cyclic AMP added, neurite outgrowth can begin and the neurite-like microspikes in contact with the culture substrate are invaded by the cytoplasm of the cell and develop into mature neurites (Monard, 1985; Smith, 1994a, 1994b; Jackson *et al.*, 1996). Consequently, damage to basal microspikes / filopodia during this process will prevent the transformation of these putative neurites into mature neurites. This suggests that neuritogenesis rather than neurite extension or maintenance may be the stage at which the artemisinin derivatives exert their neurotoxic effects.

Neurofilaments are synthesised when neurite outgrowth has already begun and may not participate directly in neurite outgrowth (Shea & Beermann, 1994;

Shea, 1995). Microtubules and their associated proteins and the post-translational modification of proteins such as phosphorylation of neurofilaments or tyrosination of tubulin may be of greater importance to the elongation and stabilisation of the neurite (Shea *et al.*, 1991a; Shea & Beermann, 1994; Shea, 1995) which follow neuritogenesis. Indeed, incubation with a microtubule inhibitor did not appear to inhibit the initiation of neurite outgrowth but instead seemed to prevent further extension of the neurite (figure 3.23; Smith, 1994a). Moreover, Western blotting and immunofluoresence of fixed Nb2a cells suggested that these proteins were not the targets for the artemisinin derivatives and the apparent toxicity of the drugs towards the filopodial microspikes, inchoate neurites, early in neurite formation is consistent with this idea.

If the loss of basal microspikes / filopodia can be linked with the inhibition of neuritogenesis then the effect of the loss of the apical microspikes, which do not participate in neurite outgrowth, is not clear. It has been proposed that apical microspikes have a sensory function. Alternatively, they may have a role in cell metabolism analogous to that of microvilli, as which the longer processes are sometimes referred, although if this is the case then their transient, dynamic and fragile nature makes them distinct from brush border microvilli. These structures decorate both the soma and the neurites of differentiated cells (figure 3.15b) and both were greatly affected by the drug. Furthermore, their number increases significantly following the development of a neuronal phenotype by cultured neuronal cells (Amieva & Furthmayr, 1995; Brinton *et al.*, 1994). Whether this a specifically neuronal development, or a feature common to differentiated cells in

general, it is part of the expression of a differentiated phenotype and the dramatic effects of the drug on these structures must have some significance.

Non-specific free radical damage rather than binding to specific targets on or in microspikes may instead be responsible for the damage, causing indiscriminate damage to membrane phospholipids and proteins, *e.g.* by lipid peroxidation and protein alkylation (Meshnick *et al.*, 1989, 1991). The consequent generalised damage to the membrane and impairment of normal membrane functions may debilitate microspikes, and make it difficult or impossible for basal microspikes to respond to whatever internal or external signals initiate neurite outgrowth, resulting in inhibition of neuritogenesis.

The damage to microspikes may also be a reflection of events within the cell rather be the primary targets for artemisinin toxicity. These surface projections are transient, dynamic and unstable and may be vulnerable to metabolic changes in the cell. Disruption of metabolism, such as might be caused by damage to mitochondria and RER for instance, could induce microspike retraction and prevent neurite outgrowth. However, microspikes were often still present on the surface of the drug-treated cells but in a flaccid, badly injured form (figure 3.15c) and appeared to have themselves been directly affected by the drug.

Microspikes consist predominately of F-actin filaments. Actin is a common cytoskeletal protein that is known to be the target of some toxic agents, *e.g.* phalloidin and cytochalasin B (Paves *et al.*, 1990). In addition to being the main component of microspikes, actin is located beneath the membrane where it is thought to be involved in connecting the cytoplasmic cytoskeleton to the membrane

and, more importantly, it is present in large amounts in the neurite growth cone. As neuritogenesis appears to be the stage at which neurite outgrowth is inhibited, its presence in the filopodia that become neurites suggests this may be a target for the drugs. However, scanning electron micrographs of the growth cones of cells treated with dihydroartemisinin, where the presence of actin is critical for their viability, failed to reveal any of the damage or deformation (figure 3.16) which would be anticipated if actin were the target. Alternatively, the toxicity of dihydroartemisinin could mean that any neurites that the cells managed to produce would be exceptional and unrepresentative of effects of the drugs so no damage to the growth cones of these surviving neurites would be expected. However, transmission electron micrographs of the neurites of drug-treated cells (not pictured in this thesis) revealed that although the neurite cytoskeleton was unaffected by the drug there was damage to mitochondria present in the neurites. Therefore these neurites were affected by the drug and, unless the mitochondria are more sensitive to the drugs than actin microfilaments, actin does not appear to be targeted by the drug.

Many other proteins are also involved in the initial stages of neurite outgrowth and could be targets for the drugs. Enzymes, in particular a large variety of kinases and proteases *e.g.* calcium / calmodulin-dependent protein kinase II (Goshima *et al.*, 1993), are particularly important. G-proteins may also participate in neuritogenesis (Prasad, 1991). There are also a number of proteins that are associated with actin in microspikes, including myosin, fascin, and moesin (Condeelis, 1993; Yamakita *et al.*, 1996; Schwartz-Albiez *et al.*, 1995).

Alternatively, the artemisinin derivatives could bind to membrane receptors (Wesche *et al.*, 1996), block ion channels or interfere with vital stimuli to neuritogenesis.

Focusing on the effects of the drugs on filopodia / neuritogenesis may be misleading. Electron microscopy has revealed that the toxicity of dihydroartemisinin on the cells is wide ranging. It is more far-reaching than just the inhibition of neurite outgrowth that was apparent by light microscopy, but these other effects may have a profound influence on this process. A variety of cell functions, not just those directly associated with neurite outgrowth, are involved in the initiation of, or are subsequently affected by, the neurotoxicity of the artemisinin derivatives.

PART THREE

4.3. VALIDATION OF NB2A CELLS AS A MODEL SUITABLE FOR THE ASSESSMENT OF NEUROTOXICITY

A large proportion of the work in this thesis used the proliferating and differentiating Nb2a cell as the models with which the neurotoxicity of the artemisinin derivatives was examined. Part three of this thesis attempted to validate the use of the cells for this purpose.

Why use cultured neuronal cells as a test system at all ? What advantages do they possess over other test systems and techniques for quantifying toxicity and elucidating mechanisms of action ? Testing for neurotoxicity *in vivo* is not always completely reliable, *e.g.* the neurotoxicity of clioquinol and bupropion led to their withdrawal from the market (Grahame-Smith & Aronson, 1992), and often the neurotoxicity of many compounds is only discovered after accidental exposure of humans e.g. n-hexane (Herskowitz *et al.*, 1971) and β-*N*-methylamino-L-alanine (Spencer *et al.*, 1987). Moreover, the complexity of the human nervous system and the corresponding complexity of the mechanism of action of many neurotoxicants means that elucidation of the neurotoxic effects *in vivo* can be very difficult. Consequently, there is a role in risk assessment and elucidation of the underlying mechanisms of neurotoxicity for a simple *in vitro* test system. In these simplified test systems the environment of the cells can be manipulated by the investigator, the

concentrations of the neurotoxicant controlled with precision, the type of target cell is well defined and the changes in the cell following treatment more easily examined.

Primary cell cultures of nervous tissue have a number of technical disadvantages: they are post-mitotic and cannot be serially passaged, they are difficult to grow compared to other cell cultures, they are difficult to standardise and they contain a multitude of different cell types including non-neural material that is often necessary for neural cell survival and may complicate efforts to interpret toxic effects. Consequently, continuous cell lines may be more useful for assessment of neurotoxicity. The Nb2a neuroblastoma is a transformed cell line originally derived from the neuroblast precursor cells of a mouse tumour and is widely used as a model for the study of neurotoxicity; proliferating Nb2a cells have been demonstrated to respond to the artemisinin derivatives in a similar manner to primary cultures of neuronal cells (Wesche *et al.*, 1994). The neuronal properties of proliferating Nb2a cells were described in section 4.1. Having in part one and part two of this thesis examined the effects of artemisinin derivatives on Nb2a cell proliferation, neurite outgrowth and cell ultrastructure it was necessary to confirm that the effects of the artemisinin derivatives on these parameters were at least in part neurotoxic. Additionally, the usefulness of proliferating and differentiating Nb2a cells as a test system for the neurotoxicity of the artemisinin derivatives also depends upon their ability to respond in a predictable manner to known neurotoxic compounds.

Comparison of the effects of dihydroartemisinin on the proliferation of undifferentiated Nb2a cells with that of two non-neuronal cell lines, HeLa B and

CRFK cells, demonstrated that although Nb2a cells were marginally more sensitive to the toxic effects of dihydroartemisinin, (figure 3.19 and table 3.3) the difference between the sensitivity of the proliferating, undifferentiated, Nb2a cells and the non-neuronal cells to the toxic effects of the compound was not significant. Using IC_{50} values as measure of toxicity, CRFK cells were significantly less susceptible to dihydroartemisinin toxicity than the other cell lines, although taking the incubation as a whole the difference is not noteworthy; consequently all three proliferating cell lines, neuronal and non-neuronal, are similarly vulnerable to the toxicity of dihydroartemisinin. HeLa B cells are derived from an epitheloid adenocarcinoma of the human uterine cervix and resemble epithelial cells when in culture (Iotsova & Stehelin, 1995) and CRFK cells, also known as Crandell cells, are a feline kidney fibroblast cell line (Crandell *et al.*, 1973). These cells, like Nb2a cells, are immortalised tumour cell lines whose shared characteristics as cancer cells may outweigh any differences of phenotype. For example, one common feature of cancer cells is that they express higher membrane concentrations of transferrin receptors than non-transformed cells; they also have a higher rate of iron flux via those transferrin receptors (Lai & Singh, 1995). This may go some way toward explaining the similar vulnerabilities of the three (four, if C6 rat glial cells are included) cell lines to dihydroartemisinin toxicity as iron or haemin plays such a prominent role in mediating this toxicity.

Proliferating immortalised tumour cells are undifferentiated and this means that they do not express important parts of the phenotype of their particular differentiated cell type. Nb2a cells express many of the biochemical markers of

mature neurones but of course do not possess certain characteristic features of those cells *e.g.* axons. Therefore study of some types of neurotoxicity may not be feasible using undifferentiated Nb2a cells, as the employment of a model system to study the toxicity of a compound is only useful if that model expresses the targets for the toxicity of that compound. Undifferentiated Nb2a cells may not possess the features that make mature neuronal cells particularly vulnerable to the toxicity of the artemisinin derivatives. As well as certain neuronal properties undifferentiated neuroblastoma cells also possess structural proteins, metabolic cycles, enzymes *etc.* common to all cells and the inhibition of proliferation caused by dihydroartemisinin may be as a result of damage to one or more of these general cell features. Consequently, although undifferentiated Nb2a cells may be a viable model for the neurotoxicity of many compounds, especially those that do not act on axons or interact with iron or haem, and other groups (Wesche *et al.*, 1994) consider them good indicators of *in vivo* neurotoxicity and artemisinin neurotoxicity *in vitro*, our studies suggest that Nb2a cell proliferation may not be the best model for assessing artemisinin neurotoxicity *in vitro.* A preferable approach is the measurement of drug effects on some characteristic of neuronal cells such as the production and maintenance of neurites from differentiating cells, as the results of section 3.1 suggest. A direct comparison between cell lines based on neurite outgrowth was not possible of course, as proliferating CRFK and HeLa B cells cannot be made to express neurites and differentiated Nb2a cells cannot be made to proliferate. In other words we cannot make the same measurements on the same cells in a differentiated and undifferentiated state.

Observations of the comparative effects of neurotoxic and non-specific toxic agents on Nb2a cell neurite outgrowth and cell proliferation were a logical second step in the testing of Nb2a cell suitability. The ability of the differentiated cells to respond to neurotoxic agents in a predictable manner is important in establishing the credibility of neurite outgrowth as model for neurotoxicity. Undifferentiated Nb2a cells can be induced to express the phenotype of cells of the mature nervous system and extend axon-like neurites. Axogenesis / neuritogenesis is unquestionably a very complex process, involving the action of multiple enzymes and proteins influencing the expression and modification of numerous axoskeletal components. However many aspects of this process have now been elucidated and demonstrate that the process of neurite outgrowth is a useful model system for the study of artemisinin neurotoxicity and of neurotoxicants in general.

Serum contains calpain- and thrombin-like factors that act on undifferentiated cells to suppress neurite outgrowth; to stop proliferation and induce differentiation serum is withdrawn from culture medium (Shea *et al.*, 1991a). Initial neurite outgrowth is protein synthesis-independent; undifferentiated cells have a pool of cytoskeletal proteins sufficient for the initial stages of neurite outgrowth (Shea *et al.*, 1991a). It is thought that calpain or calpain-like molecules modulate the size of these pools and when it is withdrawn, the size of the pools of cytoskeletal components increases and the cell awaits an exogenous signal for outgrowth to begin (Shea *et al.*, 1991a). Dibutyryl cyclic AMP, an analogue of the important cell second messenger cyclic AMP is added to the medium when serum is withdrawn and neuritogenesis is triggered by an influx of calcium ions (and an efflux of sodium

ions) and disruption of the actin network submembrane (Littauer *et al.*, 1979; Shea *et al.*, 1991b). Undifferentiated cells continuously extend short neurites / filopodia and it has been demonstrated in several neuronal cell types that as neurites grow during differentiation they appear to do so by stabilising these short neurites and promoting their continuing growth (Monard, 1985; Shea *et al.*, 1991a; Smith, 1994b; Jackson *et al.*, 1996). These filopodia, which consist of actin microfilaments and several associated proteins including myosin, fascin, and moesin (Condeelis, 1993; Yamakita *et al.*, 1996; Schwartz-Albiez *et al.*, 1995), become the growth cones of the extending neurites (Monard, 1985; Abdulla & Campbell, 1993; Condeelis, 1993; Smith, 1994b). As the neurite extends, growth-associated protein 43 (GAP-43) translocates from the cell body to the neurite and greatly increases the rate at which new membrane is added to the growth cone. The cytoskeleton of the newly formed neurite consists primarily of rapidly assembled microtubules and their associated proteins *e.g.* microtubule-associated protein 1B and tau proteins; this microtubule assembly is necessary for elongation and maintenance of axons (Shea *et al.*, 1991b; Abdulla & Campbell, 1993; Shea & Beermann, 1994; Shea & Benowitz, 1995). These neurites are subsequently stabilised by the synthesis and incorporation of neurofilaments into the neurite cytoskeleton. Neurofilaments do not appear to participate directly in neurite extension, but are incorporated into the cytoskeleton, which they stabilise by interacting with microtubules and microtubule-associated proteins. These interactions between neurofilaments and other proteins are governed by selective phosphorylation of the neurofilament subunits by various kinases (Shea *et al.*, 1988, 1989, 1990; Shea & Beermann, 1994; Shea, 1995). Indeed kinases and

proteases have been shown to regulate the initiation, elongation and stabilisation of neurites (Shea *et al.*, 1991a; Goshima *et al.*, 1993; Shea, 1994). Vimentin and spectrin, which co-localise with actin and intermediate filaments are also found in the neurites of differentiated Nb2a cells (Shea *et al.*, 1988; Condeelis, 1993; Takemura *et al.*, 1993). Differentiation of neuroblastoma cells is generally characterised by acquisition of an excitable membrane and high levels of specific neuronal enzymes, and indeed following dibutyryl cyclic AMP-induced differentiation, Nb2a neuroblastoma cells possess an increased resting membrane potential, increased GAP-43, tyrosine hydroxylase, choline acetyltransferase, acetyl cholinesterase, changes in glycoproteins, polyunsaturated fatty acids and a variety of other biochemical and biophysical properties that render these cells similar to neurones *in vivo* (Prasad, 1991; Shea *et al.*, 1991b). This makes differentiated Nb2a cells and the neurites they project suitable models for the assessment of neurotoxicity.

The neurotoxic compounds used in these assays, *viz.* colchicine and 2,5-hexanedione, were significantly more toxic to neurite outgrowth than cell proliferation because they affected proteins or characteristics that are peculiar to neuronal cells *i.e.* the axonal cytoskeleton. The non-specific toxic compounds did not affect any particularly neuronal quality of the cells and consequently were not especially toxic to neurite outgrowth. Colchicine has been demonstrated to inhibit extension of neurites from cultured cells (Smith, 1994a). It is a compound with neurotoxicity effects that binds to the αβ tubulin dimer. These colchicine-bound dimers bind to the polymerising end of the microtubule inhibiting further

polymerisation. As microtubules are in dynamic equilibrium, polymerising and depolymerising, this disturbs the equilibrium and the microtubule depolymerises (Darnell *et al.*, 1990). Microtubules are not, of course, a specifically neuronal protein. In proliferating cells depolymerisation of microtubules would cause a disturbance of the cytoskeleton, disorganisation of organelles and obstruction of cell division at metaphase and microtubule depolymerisation also has these detrimental effects on the cell bodies of neuronal cells. However, the neurotoxicity is a result of colchicine depolymerising the microtubules of the neurite cytoskeleton, disrupting axonal transport. 2,5-hexanedione is the neurotoxic metabolite of n-hexane that acts by binding to lysyl residues of neurofilaments, the intermediate filaments present in axons, causing their aggregation through covalently crosslinking them by cyclizing to a pyrrole (Genter St. Clair *et al.*, 1988; Lanning *et al.*, 1994) again disrupting axonal transport. Neuronal cells are especially vulnerable to compounds such as colchicine and 2,5-hexanedione that interact with microtubules and neurofilaments as a prominent feature of their cellular anatomy is the axonal cytoskeleton of which these are major components; vital metabolic and structural elements are of necessity transported along its length and any interference with this process can have a catastrophic effect on the functioning and survival of the axon in particular and the cell in general.

Sodium cyanide is a metabolic inhibitor; it blocks the oxidative metabolic pathway by inhibition of cytochrome-*c* oxidase leading to a depletion of cell ATP and disruption of metabolic processes and the ion gradients across membranes (Johnson *et al.*, 1994; Despande *et al.*, 1997). Ascorbate, the reduced form of

ascorbic acid, is an anti-oxidant compound but in the presence of transition metals such as iron or possibly copper it acts as a potent pro-oxidant (De Laurenzi *et al.*, 1995). It damages cells by reacting with reduced iron and hydrogen peroxide to produce the hydroxy radicals that induce DNA strand breaks. Neuroblastoma cells have been demonstrated to produce elevated levels of hydrogen peroxide exogenously. Moreover, they contain a large amount of iron-rich ferritin and ascorbate has been demonstrated to release this iron. Consequently, incubation with ascorbate alone is sufficient to produce the hydroxy radicals that damage cell DNA (Szatrowski & Nathan, 1991; Baader *et al.*, 1994). Ascorbate and sodium cyanide toxicity are not associated with neuronal targets and as a result these compounds might not be expected to possess greater toxicity toward differentiating Nb2a cells that express a phenotype closer to that of cells of the mature nervous system. This was confirmed by the results of section 3.3.2, where the IC_{50}s for the inhibition of neurite outgrowth by both compounds were not significantly greater than the IC_{50}s for inhibition of cell proliferation.

The sensitivity of the neurite outgrowth parameter to neurotoxic agents and dihydroartemisinin (and lack of sensitivity to non-specific toxins) suggests that inhibition of neurite outgrowth is a genuine measure of neurotoxicity rather than a reflection of the general well-being of the cell. This has also been confirmed by studies in this laboratory (Smith, unpublished) where the toxicity of artemisinin derivatives on the general metabolism of the differentiated cell (as measured by conversion of MTT) and their toxicity to neurite outgrowth were determined and the IC_{50} values for inhibition of general metabolism were found to be significantly

higher. Consequently, compounds that inhibit neurite outgrowth to a significantly greater degree than they inhibit proliferation probably exert neurotoxic effects on the cells. This confirms the hypothesis put forward in part one, *viz.* that dihydroartemisinin and the other artemisinin analogues are neurotoxic, as they are significantly more toxic to neurite outgrowth than proliferation and hence share the properties that are characteristic of neurotoxins rather than those of general toxins. This concurs with the findings of *in vivo* toxicity studies (Brewer *et al.*, 1994a, 1994b; Petras *et al.*, 1993a, 1993b) in which the drugs caused severe neuropathological lesions in dogs and rats but no significant damage to other tissues.

When the neurotoxicity of dihydroartemisinin, as measured as inhibition of neurite outgrowth, was compared with that of a range of established neurotoxicants it was found to be located in the centre of the range of IC_{50} values (table 3.5). The most toxic drugs, with IC_{50} values in the low nanomolar range, were colchicine and cytochalasin B. Interestingly, these compounds act in a very similar manner; colchicine as described above prevents polymerisation of microtubules, whilst cytochalasin B binds directly to the polymerising end of actin microfilaments preventing further polymerisation accordingly inducing depolymerisation by shifting the polymerisation / depolymerisation dynamic equilibrium in favour of depolymerisation. Paves and co-workers (1990) have also demonstrated that both of these compounds prevent neurite outgrowth in cultured neuronal cells. The least toxic compounds tested were 2,5-hexanedione and β,β`-iminodipropionitrile with IC_{50} values in the micromolar range. There are also similarities between the putative mechanisms of action of these compounds; 2,5-hexanedione as described above

causes aggregation of neurofilaments, β,β`-iminodipropionitrile reportedly interacts with neurofilament-associated proteins to increase binding interactions between microtubules and neurofilaments (Eyer *et al.*, 1989). It would appear that compounds that induce disassembly of protein components of the cytoskeleton are considerably more toxic than those which cause aggregation of neurofilaments and / or microtubules. This is analogous to the situation *in vivo,* where toxicology studies using rats and mice have demonstrated that colchicine and cytochalasin are at least an order of magnitude more potent than 2,5-hexanedione and β,β`-iminodipropionitrile (Sweet, 1987). The IC_{50} value for 2,5-hexanedione is suprisingly high. 2,5-hexanedione is a metabolite of n-hexane, and it is possible that *in vivo* n-hexane is metabolised to 2,5-hexanedione at its site of action and that the metabolite itself when incubated with cultured cells has difficulty gaining access to these sites. It may also be an illustration of a potential shortcoming of the *in vitro* study of neurotoxicity as it is possible that the length of the incubations - 24 h - may not be a long enough time period for 2,5-hexanedione neurotoxicity to manifest itself to its full extent. Unfortunately, due to the nature of Nb2a cells it is not possible for the neurites to survive if the cells are without serum for much longer than this period. Despite this, 2,5-hexanedione, like colchicine (and the artemisinin derivatives) still possessed significantly greater toxicity towards Nb2a neurite outgrowth than cell proliferation. The neurotoxicity of dihydroartemisinin falls between the two extremes of the "depolymerisers" and "aggregators" and is closest to the IC_{50} of calphostin C, a fungal agent, which binds irreversibly to protein kinase C.

One other purpose of using these neurotoxicants is that scanning and transmission electron microscopy demonstrated that the effects of the neurotoxic agents colchicine and 2,5-hexanedione did not resemble the effects of dihydroartemisinin on differentiated Nb2a cells. While this does not provide any hints as to the mechanism of action of the artemisinin derivatives, it does at least suggest that their toxicity is not mediated through colchicine- or 2,5-hexanedione-like effects on microtubules or neurofilaments and the results of the Western blotting and immunofluoresence of fixed cells to support this idea. The measurement of neurite outgrowth and the electron micrographs of drug-treated cells did, however, go some way toward validating the use of Nb2a cells for testing potential neurotoxicants. The effects of colchicine and 2,5-hexanedione on differentiating Nb2a cells appeared to be related to their mechanisms of action *in vivo* (Clarkson, 1986), *i.e.* in figure 3.22a colchicine caused the accumulation of vesicles at the base of neurites which may indicate damage to the axonal cytoskeleton and in figure 3.24a 2,5-hexanedione induced packing of the neurite with filaments, and this provides support for the use of the neurite outgrowth parameter and differentiated Nb2a cells as *in vitro* models for the study of neurotoxic agents.

It was found that proliferating Nb2a cells did not possess significantly greater sensitivity to dihydroartemisinin toxicity than non-neuronal cell lines. However, incubation of differentiating and proliferating Nb2a cells with neurotoxic agents and non-specific toxic compounds demonstrated that the neurotoxic agents had a greater effect on neurite outgrowth from differentiating cells than on proliferation of undifferentiated cells and that non-specific toxins did not.

Dihydroartemisinin shares these characteristics with the neurotoxins. Its effects on neurite outgrowth were compared with those of a range of neurotoxic agents; the IC_{50} of dihydroartemisinin for inhibition of neurite outgrowth was of a similar magnitude to those of the neurotoxins. These results suggested that differentiating Nb2a cells were a viable model for the study of the effects of chemical neurotoxicants, and provided evidence that the artemisinin derivatives are neurotoxic.

PART FOUR

4.4. CHARACTERISING DIHYDROARTEMISININ - PROTEIN BINDING

Most targets for toxic agents are proteins of some description, be they receptors, cytoskeletal components, ion channels or enzymes and the artemisinin derivatives, which are toxic *in vivo* and *in vitro*, bind extensively to proteins (Yang *et al.*, 1994). Part four of this thesis attempted to characterise drug-protein binding and determine the importance of haemin and the endoperoxide bridge in this process.

Before binding studies commenced it was necessary to determine that the artemisinin derivatives bound significantly to rat brain as, although this was known from *in vivo* studies to be the site of neuropathological lesions, the drug may generate toxic species at these sites rather than itself bind. These lesions are sharply confined to small groups of neurones rather than widely distributed (Brewer *et al.*, 1994a, 1994b; Petras *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997). However, there was no significant difference in the binding of ^{14}C-dihydroartemisinin to cells between the three gross brain areas either after a 2 h or 24 h incubation. Binding between the drug and the homogenised brain tissue was substantial, clearly involving more than the small number of neurones affected by the drugs *in vivo*, as total binding was substantial and similar in each area of the brain whereas the distribution of the lesions was not (Brewer *et al.*, 1994a, 1994b). This suggests that

structural features and the pharmacokinetics of the intact brain contribute towards the precision of the neuropathological lesions *in vivo*. Binding appeared to increase steadily over the 24 h period for both protein and non-protein components of the cell. Protein binding was significantly greater than non-protein binding after 24 h, and proportionally, protein binding increased more than non-protein binding between 2 h and 24 h indicating a greater binding capacity. This suggests that binding of ^{14}C-dihydroartemisinin to rat brain cells is mainly to protein and that it is likely to be of greater importance to toxicity than non-protein components such as lipids, *etc.* As there was no significant difference in drug binding between the three areas of the brain and as a great deal of material would be needed, the most plentiful of the three brain areas, the cortex, was used for all further incubations.

Rosenthal plots revealed that there were two discrete sets of binding sites. The curvature of the plotted data and the binding parameters calculated from them demonstrated that one set of binding sites had a very much lower affinity and higher capacity for ^{14}C- dihydroartemisinin than the other (figure 3.27), so much so that it is possible that this may represent non-specific binding rather than a defined population of binding sites. It was not possible to control for non-specific binding by the conventional method, *i.e.* using an excess of non-radioactive dihydroartemisinin, as the incubations involved living cells and the toxicity of dihydroartemisinin to these cells is sufficient to kill them at the concentrations that would be required to measure non-specific binding. By treating this putative non-specific binding as a discrete population of binding sites it was possible to separate it out from the high affinity / low capacity binding sites and examine the effects of haemin,

iodoacetamide and sodium cyanate on ^{14}C- dihydroartemisinin binding at lower dihydroartemisinin concentrations which are of greater relevance to dihydroartemisinin toxicity.

Pre-incubation of rat brain homogenate with iodoacetamide, which reacts with thiol groups, or with iodoacetamide and sodium cyanate, which react with thiol and amine groups (Means & Feeney, 1971), did not alter either k_D for ^{14}C-dihydroartemisinin binding to rat cortex homogenate, but reduced both B_{max} values, the maximum binding capacity of the preparation, of both the high and low capacity binding sites. This is indicative of non-competitive inhibition which one would expect to occur as iodoacetamide and sodium cyanate were pre-incubated with the homogenate and bind covalently to proteins (Means & Feeney, 1971). The blocking agents inhibited 60-70% of total binding illustrating that reactions with these groups constitute a large proportion of dihydroartemisinin -protein binding. However, sodium cyanate made little contribution to the total inhibition of ^{14}C-dihydroartemisinin binding demonstrating that the artemisinin derivatives did not react readily with the amine groups and the great majority of binding inhibition was caused by iodoacetamide protection of protein thiol groups. This may be significant as the thiol-containing molecule glutathione is an effective free radical scavenger *in vivo* and carbon-centred free radicals bind extensively to the thiol groups of cysteine residues (Soriani *et al.*, 1994). Furthermore, the incubation of isolated erythrocyte membranes with artemisinin and haemin causes extensive lipid peroxidation with approximately two thirds of membrane free thiols oxidised (Meshnick *et al.*, 1989, 1991) and damages erythrocyte membrane ATPases by

oxidation of thiol groups in the enzyme's active site (Wei & Sadrzadeh, 1994). Indeed many enzymes have thiol groups at their active sites (Ross, 1988). Therefore the toxic effects of the artemisinin derivatives may be mediated by binding to / alkylation of important thiol groups of target proteins.

The binding of ^{14}C-dihydroartemisinin to rat brain homogenate was significantly inhibited by co-incubation with arteether but not by co-incubation with desoxyartemisinin. This result demonstrates the importance of the endoperoxide bridge in protein binding, as without it none of the reactive products that bind can be generated. Arteether possesses an endoperoxide bridge and bound to rat brain proteins whereas desoxyartemisinin does not possess an endoperoxide moiety and did not bind to protein. The endoperoxide moiety is also a prerequisite for antimalarial activity and neurotoxicity, suggesting a relationship between protein binding and activity. Moreover, haemin increased protein binding and also increases the toxicity of the artemisinin derivatives to Nb2a cells (Smith *et al.*, 1997) providing further support for the postulated link between pharmacological activity, neurotoxicity and protein binding.

Arteether reduced ^{14}C-dihydroartemisinin binding implying that they compete for the same binding sites. Furthermore, as equal concentrations of arteether and ^{14}C-dihydroartemisinin reduced ^{14}C-dihydroartemisinin binding by ~50% it would appear that there are a finite number of specific binding sites at which the reactive products bound, and that the majority of drug-protein binding was to specific sites rather than non-specific interactions with rat brain proteins. If binding was largely non-specific reactions with protein then the number of binding sites

would be vast and there would be limited competition between the two compounds. The presence of arteether would not affect ^{14}C-dihydroartemisinin binding to a significant degree - the inhibition it would cause would be considerably less than the ~50% that actually occurred.

The effects of haemin on the binding parameters k_D and B_{max} of dihydroartemisinin binding sites of cells and cortex proteins varied. The effects on the low affinity / high capacity binding sites were different for each of the three preparations : the binding parameters which were affected in C6 cells or cortex proteins were unaffected in Nb2a cells and vice versa. However, low affinity / high capacity binding predominates at the higher, non-physiological, concentrations of dihydroartemisinin used and so may not have a major role in dihydroartemisinin neurotoxicity. Toxicity is more likely to be mediated through the high affinity / low capacity binding sites, yet the effects of haemin on those binding sites were also inconsistent. In each preparation B_{max} significantly increased, but in Nb2a cells, unlike C6 cells and brain cells, k_D also increased significantly

It is difficult to explain the differences in dihydroartemisinin binding in the three preparations and the different ways in which haemin affects their binding parameters as the proteins on which they act are not known, nor is the exact nature of the interactions between dihydroartemisinin, haemin and the binding sites. The differences may be due to the disparate characteristics of the preparations used. The properties of cells and rat cortex homogenate differ significantly. Use of intact, living cultured cells allowed study of binding in their normal environment, where the membrane, receptors and internal structures of the cell were not damaged and

any pH or ion gradients that normally exist were undisturbed. Dihydroartemisinin binding to cells was therefore modulated by these physiological factors. Furthermore, use of Nb2a and C6 cells meant that dihydroartemisinin binding properties were examined in the same system that was used to assess their *in vitro* toxicity (section 3.1). Rat cortex homogenate in contrast was largely a suspension of cells removed from their native environment but this was still a useful model as *in vivo* toxicity has been reported in rats on several occasions (Brewer *et al.*, 1994a; 1994b; Kamchonwongpaisan *et al.*, 1997). These factors may account for some of the differences observed between the cultured cells and homogenised rat brain.

The effects of haemin on the dihydroartemisinin binding parameters of rat cortex homogenate more closely resemble those on C6 cells than on Nb2a cells, and the effects of haemin on the high affinity / low capacity binding sites in the C6 cells and homogenate were identical. These similarities may occur because like the cortical tissue, the C6 cell line was originally derived from a rat whereas Nb2a cells originated in mouse. Furthermore, cortex homogenate contains both neuronal and glial cells, and as the volume of glial cells in many areas of the brain is greater than that of neuronal cells the effects of dihydroartemisinin and haemin on the glial material in the homogenate would make a large contribution to total brain protein binding (Holmes, 1993).

How might haemin bring about these changes ? Binding parameters describe the interactions between a ligand and a binding site and haemin could act on either dihydroartemisinin or its binding sites to alter B_{max} or k_D. An increase in B_{max} implies an increase in the number of sites to which the ligand can bind whilst a modification

to k_D means a change in the affinity of binding sites for the ligand. It is possible, although unlikely, that haemin interacts with the protein targets to uncover additional binding sites or that it acts directly on binding sites to alter their affinity for dihydroartemisinin. Haemin catalyses the conversion of artemisinin derivatives into potentially toxic compounds, *e.g.* organic free radicals, an electrophilic epoxide and at least one diketone (Meshnick *et al.*, 1991, 1993; Posner *et al.*, 1992; Zhang *et al.*,1992; Baker *et al.*,1993). Moreover, haemin can form covalent adducts with artemisinin derivatives (Meshnick *et al.*, 1991; Hong *et al.*, 1994). The presence of haemin increased the B_{max} of the high affinity binding sites and increased total binding to the preparation, so perhaps organic free radicals or dihydroartemisinin - haemin adducts are able to bind to sites that dihydroartemisinin alone cannot. Dihydroartemisinin-like free radicals or dihydroartemisinin-haemin adducts also perhaps act in some way on binding sites to alter their affinity for the drug. However, artemisinin-haemin adducts are not toxic when they are added exogenously to malaria parasites *in vitro.* Indeed pre-incubation of artemisinin with haemin reduces artemisinin efficacy against *P. falciparum* by ~75% (Meshnick *et al.*, 1991; Hong *et al.*, 1994), which suggests that artemisinin-haemin adducts do not bind to target proteins either in the parasite or neuronal cells / homogenate and are not themselves toxic. The most likely role of haemin in dihydroartemisinin-protein binding is that it catalyses the conversion of dihydroartemisinin into organic free radicals and other alkylating agents which then bind to their target proteins *i.e.* haemin greatly increases the amount of reactive products available for binding. Although an increase in the total amount of binding ligand should not alter binding

parameters this is probably not a classical ligand-receptor model; here the "ligand" probably binds covalently to, and damages, the proteins to which it binds. Alternatively, Yang *et al.* (1993) have suggested that alkylation of albumin by artemisinin derivatives can occur by multiple pathways, both iron-dependent and iron-independent, and perhaps this also happens with other proteins. If this is so then the changes observed in binding parameters in these assays may be the result of an increase in the iron-dependent pathway and an alteration in the relative contributions of the different binding mechanisms to total dihydroartemisinin-protein binding.

The effects of haemin on the dihydroartemisinin binding parameters of rat cortex homogenate, Nb2a cells and C6 cells are too diverse to draw any general conclusion about the exact nature of its influence on the binding of dihydroartemisinin to its target sites. What is clear, however, is that haemin dramatically increased the total binding of dihydroartemisinin in each case and has been shown previously to similarly increase dihydroartemisinin toxicity to the these cells (Smith *et al.*, 1997). The results of these assays are consistent with the idea that artemisinin derivatives exert their neurotoxic effects by specifically and covalently binding to as yet unidentified cell proteins. It is likely that haemin, by greatly increasing dihydroartemisinin conversion to the free radicals and other alkylating agents, increases the degree of binding taking place between the reactive agent and the proteins and in this way enhances dihydroartemisinin toxicity.

The results of these studies suggest that the requirements for binding of the artemisinin derivatives to proteins are a source of iron for the production of free radicals and other highly reactive species, and a protein with thiol groups available

for reaction with the drug. As artemisinin binding to protein is largely through interactions with specific, well-defined, binding sites this implies that the binding sites contain an amino acid with an available thiol moiety. Free radicals and electrophilic epoxides such as those that are produced by the reaction between artemisinin derivatives and haem are highly reactive, and in the case of free radicals short-lived, therefore if they are to react with the specific binding sites they are likely to be generated near their sites of action. This could either be with free iron or haem, and as the addition of exogenous haemin increases binding this clearly occurs, or the iron / haem could be attached to the target protein in some way. Many proteins, *e.g.* spectrin, possess binding sites for haem (Ma *et al.*, 1993) and others bind iron, *e.g.* transferrin (Morris *et al.*, 1992b), and a wide range of enzymes (Stadtman, 1990) utilise iron in their active sites. Indeed many electron-carrying complexes in mitochondria, an organelle which is a target in artemisinin antimalarial activity and toxicity (Ellis *et al.*, 1985; Zhao *et al.*, 1986; Maeno *et al.*, 1993; sections 3.3 & 4.3), contain haem moieties or iron-cysteine complexes in their active sites and these may be vulnerable to the toxicity of artemisinin derivatives *e.g.* the activity of the haem-containing cytochrome oxidase is inhibited by artesunate (Zhao *et al.*, 1986). Mitochondrial enzyme complexes such as succinate-coenzyme Q reductase possess an iron-cysteine complex in its active site and could be even more vulnerable, having iron and thiol groups in such close proximity. While inhibition of mitochondrial enzymes is one possible mechanism for the toxic effects (and antimalarial activity) of artemisinin, and is consistent with the results of this and other studies, it has not been demonstrated that artesunate inhibits cytochrome

oxidase activity through a reaction between the drug, iron / haem and thiol groups in the enzymes active site. Our limited knowledge of the requirements for the binding of the drug to its target site means that characterisation of the binding site and identification of the target proteins is not possible beyond speculating that the binding site may contain an available thiol moiety with a source of catalytic iron or haem, either bound or unbound to the protein, in close proximity.

4.5. GENERAL DISCUSSION

These studies have helped to elucidate some important aspects of the neurotoxicity of artemisinin derivatives. The mechanism of action of their neurotoxicity to differentiated Nb2a cells, the main model used in these assays, can be summarised as follows. The endoperoxide bridge of the artemisinin derivatives reacts with iron / haem either within the cell, and possibly the culture medium, to produce carbon-centred free radicals and other highly reactive products. These free radicals then react with and bind to various proteins within the cell and perhaps also cause lipid peroxidation of cell membranes. At least some of this binding to cell proteins appears to be specific rather than the general, indiscriminate, damage usually associated with free radical toxicity (Kamchonwongpaisan *et al.*, 1997). The free radicals appear to react with two discrete sets of binding sites, one of high affinity and low capacity for the drugs, the other low affinity and high capacity for the drugs. The free radicals react mainly with protein thiol moieties although some binding to protein amine groups also occurs. This damage manifests itself as the inhibition of cell proliferation and neurite outgrowth from differentiating cells. On an ultrastructural level, the mitochondria and endoplasmic reticulum and the microspike projections from the membrane surface of the cell neurites and soma all appear to be badly damaged by dihydroartemisinin.

Binding data in sections 3.4.1 - 3.4.3 strongly suggested that protein binding is important for drug toxicity, that the damage to Nb2a cells was induced by the binding of the free-radical products to cell proteins. The binding of artemisinin-

based free radicals to Nb2a cell proteins appears to be both specific and non-specific (Kamchonwongpaisan *et al.*, 1997). Additionally, indiscriminate binding occurs at concentrations of drug higher than those that produce specific binding (Kamchonwongpaisan *et al.*, 1997). This may provide an explanation for data in section 3.1 where the inhibition of the proliferation of undifferentiated Nb2a cells occurred at concentrations higher than those that caused inhibition of neurite outgrowth from differentiated cells. It may be that the non-specific binding is largely responsible for the inhibition of cell proliferation while one (or more) of the proteins that the drug selectively reacts with are the targets through which the artemisinin derivatives exert their neurotoxic effects, such as inhibition of neurite outgrowth.

As free radicals are relatively short-lived and are highly reactive it is likely that they are generated near their binding sites on the specific target proteins; those free radicals that are not are more likely to contribute to non-specific binding and toxicity. Proteins that actually bind iron are therefore potential targets for the drugs, especially as the iron / haem levels in neuronal cells are much lower than those in parasites and the probability of a toxic metabolite being randomly generated near a binding site is less. There are a wide range of proteins that can bind iron or haem or contain haem or iron moieties, *e.g.* catalase or the mitochondrial electron carriers, and a protein with similar characteristics may be the target for the drug (see section 4.4).

The selectivity of the specific drug-protein binding implies that the drug reacts with a well-defined binding site on the protein rather than undergoing the indiscriminate reactions with protein that are often associated with free radical

damage and which may be responsible for the non-specific binding. Stereoselectivity influences the *in vitro* neurotoxicity of the artemisinin derivatives (Wesche *et al.*, 1994) and may influence *in vitro* antimalarial activity (Shmuklarsky *et al.*, 1993), implying the involvement of a receptor, ion channel, enzyme, or some other protein that normally interacts with ligands. Indeed, it has been proposed that artemisinin neurotoxicity to neuroblastoma cells is mediated through opiate receptors (Wesche *et al.*, 1996) and demonstrated that artemisinin derivatives can inhibit cytochrome oxidase (Zhao *et al.*, 1986) and erythrocyte membrane ATPase (Wei & Sadrzadeh, 1994) activities.

Incubation of isolated erythrocyte membranes with artemisinin derivatives also results in non-specific labelling of the major protein components of the erythrocyte cytoskeleton *e.g.* spectrin, band 4.1, and actin (Asawamahasakda *et al.*, 1994a). Many neurotoxins act by binding to and disrupting the cytoskeleton components that are necessary for axonal maintenance. Dihydroartemisinin reactive products bind to specific proteins in Nb2a cells (Kamchonwongpaisan *et al.*, 1997) and a diketone is a product of the decomposition of arteether (Baker *et al.*, 1993). Diketones such as 2,5-hexanedione, the n-hexane metabolite, are potent neurotoxins and form pyrrole cross-links between cytoskeletal components resulting in protein aggregation (Genter St. Clair *et al.*, 1988). Consequently, it was initially postulated that the neurotoxicity of the artemisinin derivatives may be a result of aggregation of cytoskeletal proteins. However, while this was an attractive hypothesis, these studies have produced no evidence to verify it and, if binding to cytoskeleton is involved, there is no indication from any of the work that the cytoskeletal proteins are affected.

At present we can only speculate about the identity or likely properties of the target proteins. However as the drugs also selectively bind to proteins of malaria parasites (Asawamahasakda *et al.*, 1994b), they may not be intrinsically neuronal proteins, or if they are, they clearly share important structural features with the proteins in the malaria parasite. Indeed some of the proteins specifically labelled in Nb2a cells, rat brain and malaria parasites are of comparable molecular weights and it is possible that they represent the neural and parasitic forms of the same protein.

The necessity of the endoperoxide moiety, the importance of haem and / or iron, and the specific alkylation of proteins in both malaria parasites and Nb2a cells by free radical products of the drugs suggest that the mechanism of action of neurotoxicity and the mechanism of action of antimalarial activity of the drugs are in many ways analogous. Furthermore, damage to mitochondria and endoplasmic reticulum, prominent features of drug-induced damage to malaria parasites, has also been noted in both *in vivo* and *in vitro* artemisinin toxicity studies (Maeno *et al.*, 1993; Ellis *et al.*, 1985; Wang & Liu, 1983; section 3.2). However, if the mechanisms of action are analogous and are modulated by the same factors why are the drugs not also extremely toxic to man ?

The selective toxicity of the drugs towards the parasites is thought to be due to several factors. Malaria parasites actively take up the artemisinin derivatives (Gu *et al.*, 1984; Meshnick *et al.*, 1991). Furthermore, they then concentrate the drug with digested haemoglobin. The presence of very high concentrations of both the drug and iron / haem presumably generates large amounts of the toxic free radicals that alkylate various proteins, one or more of which may be the critical target for the

drug's antimalarial activity, leading ultimately to the death of the parasite. This combination of high concentrations of both iron / haem and drug, along with the presence of the target proteins, probably does not occur anywhere in the tissue of the host. However, target proteins or high levels of drug or iron / haem may be present in host tissues individually or, if in combination, at considerably lower concentrations than in parasites and this could account for the incidences of toxicity observed clinically and in recent animal toxicity studies.

The radiolabelled proteins of both Nb2a cells (Kamchonwongpaisan *et al.*, 1997) and rat brain (section 3.2.4) suggest that specific alkylation of proteins also occurs in neuronal cells, and *in vivo* neurotoxicity studies support this (Brewer *et al*, 1994a, 1994b; Petras *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997). Target proteins for the artemisinin derivatives may also exist in other tissues as electrocardiogram abnormalities have been recorded both clinically and in animal studies (Hien & White, 1993; Brewer *et al.*, 1994a, 1994b), and drug-induced ultrastructural damage to myocardium (Wang & Liu, 1983) has some parallels with that to malaria parasites and Nb2a cells. Reduced reticulocyte counts and degeneration of hepatocytes have also been frequently noted in animal studies (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982e), although it may be that the presence of the high concentrations of haem iron in these cells is the primary cause of this toxicity. It can be seen from these animal studies that toxicity has been observed in a number of tissues but only when very high doses of the artemisinin derivatives have been administered and therefore the clinical relevance is of most of

it is questionable. However, the neurotoxicity of the artemisinin derivatives differs in several important respects.

Unlike other organ toxicity observed *in vivo* the neurotoxic effects of arteether observed in rats and dogs (Brewer *et al.*, 1994a, 1994b) occurs at doses only fractionally higher than those used clinically. Furthermore, whereas the drug effects on the reticulocyte count, heart, liver, and other tissues, even at the extremely high concentrations used are reversible, damage to the CNS is usually permanent as it has little regenerative capacity. Finally, the damage to the brain is severe and very selective for certain neurones, causing necrosis of vestibular nuclei less than a millimetre away from completely unharmed Purkinje and granular cell neurones (Kamchonwongpaisan *et al.*, 1997).

There are several possible explanations for this highly selective pattern of damage to the brain. Selective uptake of a toxic agent by certain neurones has been demonstrated to be responsible for neurological damage, *e.g.* the Parkinsonism-like syndrome caused by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and neuronal cells possess many active transport systems for the uptake of neurotransmitters and other intercellular signals. Consequently, selective uptake and concentration of the drugs by neuronal cells is one explanation for the highly selective neuropathological damage caused by these drugs, and why the damage is so sharply confined. However, there is no evidence to suggest that this occurs; indeed Nb2a cells take up the drug far less readily than malaria parasites *in vitro* (Kamchonwongpaisan *et al.*, 1997).

Alternatively, specific target proteins for the drug may only exist in the particular cells that are affected or if the protein itself is more widespread the binding sites may only be available for binding in these cells, although if that were the case then drug-protein binding would not have been so substantial in the assays in sections 3.4.1-3.4.4. Moreover, it is unlikely that this unusual target protein would also be expressed in Nb2a cells.

The brain contains a huge number of highly specialised cells that are connected in a complex, integrated manner. Consequently, damage to small numbers of neurones can have a catastrophic effect on the animal. This is certainly true of the neurotoxicity of artemisinin derivatives in dogs and rats (Brewer *et al.*, 1994a, 1994b). Hence the complex structure of the brain, including localised variations in cerebral blood supply, may contribute to the selectivity of neuronal damage caused by the artemisinin derivatives. When rat brain tissue was homogenised, and the drug given unregulated access to individual brain cells (sections 3.4.1-3.4.3), the structural and pharmacokinetic factors that may influence local concentrations of the drug *in vivo* were abolished and protein binding at physiological concentrations of the drug was substantial, clearly involving more than a handful of cells. This suggest that the structure of the brain does contribute towards the selectivity of neurotoxicity observed *in vivo*.

The brain is notably sensitive to free radicals (Halliwell & Gutteridge, 1989) and several areas contain large amounts of iron, *e.g.* the red nuclei and the substantia nigra neurones. As has already been established, artemisinin toxicity is mediated by free radical damage and iron is crucial for their generation.

Consequently, these factors may contribute towards the sensitivity of the brain to these drugs. However, in rat brain the red nuclei were damaged but the substantia nigra was not. Other organs such as the liver, which is also the main site of metabolism, contain higher levels of iron but artemisinin-induced hepatoxicity at these concentrations has not been reported and only occurs transiently at much higher concentrations.

This illustrates the complicated role of iron in artemisinin toxicity. Total iron levels in a particular cell or tissue are an unreliable guide to its capacity for generating artemisinin-based free radicals. Iron is present in the body in many different forms from free iron, as part of a complex such as haem, or in a protein such as ferritin. Furthermore the oxidation state (iron (II) or iron (III) or both) in which it catalyses the formation of artemisinin-based free radicals is not certain. Additionally, artemisinin toxicity may not require high iron levels, in any form, if the target site is an iron-containing protein. To complicate matters still further, *in vivo*, neuronal cells are supported by a network of glial cells which are involved in protection of neurones but have recently also been implicated in neurotoxicity (Chao *et al.*, 1995). Glial cells also help regulate the iron levels in neurones by acting as a storage site; they may therefore influence the neurotoxicity of artemisinin in a number of ways. These complexities provide a good illustration of the advantages of studying the mechanism of action of neurotoxicants in an *in vitro* system rather than *in vivo*.

Any one of these factors, or the subtle combination of several, could be responsible for the neurotoxicity of the artemisinin derivatives, *in vivo* or *in vitro*,

and explain the selective patterns of damage within the brains of animals. Difficulties in determining which are involved and to what extent means that it is not possible to propose a comprehensive and cohesive mechanism of action for the neurotoxicity of the artemisinin derivatives from the studies of our group and those of others. Although some important aspects of the neurotoxicity of artemisinin derivatives have been elucidated in these studies the precise targets for this toxicity remain obscure. Discovering the targets of the drugs and understanding the mechanism of animal and cell culture neurotoxicity would help clarify the residual uncertainties about their clinical use, aid the development of better dosing regimens and the design and synthesis of new analogues.

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