# Investigation of the anti-*Toxoplasma* activity of arprinocid and the application of proteomics to the analysis of drug-resistance

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## Declaration

I declare that this thesis consists entirely of my own work, unless specifically indicated.

This thesis has not been accepted in any previous application for a degree.

12th June 2002

Adrian Cohen

## ABSTRACT

The *in vitro* efficacy and mechanism of action of two purine analogues, arprinocid and its *in vivo* metabolite arprinocid-1-N-oxide, were investigated against *T. gondii* tachyzoites using two contrasting approaches. Firstly, a global proteomics approach was undertaken for the analysis of proteins expressed in the tachyzoite stage of *T. gondii*, as a preliminary to analysing differences between arprinocid-1-N-oxide-resistant and -sensitive parasites. Secondly, a biochemical approach to investigate purine transporters of *T. gondii* as possible conduits or targets for arprinocid and arprinocid-1-N-oxide.

Initial work using $^{3}$H-uracil growth uptake to measure the growth of tachyzoites enabled the measurement of the basic anti-parasitic properties of the compounds. The $IC_{50}$ values for arprinocid and arprinocid-1-N-oxide were 22.4 ± 5.0 μM and 0.061 ± 0.028 μM, respectively. Both compounds were specific to *T. gondii* at therapeutic concentrations and acted irreversibly within a short period of time.

High-resolution two-dimensional electrophoresis (2-DE) using the pH ranges 4-7 and 6-11 reproducibly separated over 1,000 polypeptides, whilst further separations using narrow range gels suggested that at least 3,000-4,000 polypeptides should be resolvable by 2-DE using multiple single pH unit gels. Peptide mass fingerprint (PMF) data acquired by MALDI-time-of flight mass spectrometry, enabled unambiguous protein identifications to be made where full gene sequence information was available. However, interpretation of the *T. gondii* EST database using PMF data was less reliable. In contrast, peptide fragmentation data, acquired by MALDI-post-source decay mass spectrometry, proved a successful strategy for the putative identification of proteins using the *T. gondii* EST database. Moreover, peptide fragmentation data permitted the

identification of *T. gondii* proteins based on peptide homology to known proteins from other organisms. The data demonstrated that proteomic analyses are now viable for *T. gondii* and other protozoa for which there are good EST databases, even in the absence of complete genome sequence. Work presented in this thesis demonstrated the usefulness of proteomics for the investigation of strain variation, protein changes as a consequence of genetic manipulation, and protein expression differences between arprinocid-1-N-oxide-sensitive and -resistant *T. gondii* lines. Detailed analysis of these drug-sensitive and -resistant mutants indicated that they reproducibly differed in only one protein, although it is suspected that further analysis using narrow range IPG strips may yield more differences. Unfortunately, this differentially expressed protein remains unidentified probably because of the incompatibility of silver-staining with mass spectrometry analysis.

Although proteomics is a powerful collection of tools for the investigation of biological questions, currently it is in an early development phase. In contrast, classical biochemical approaches, such as the oil-stop technique for measurements of purine transport, are more standardised. Characterisation of purine transport in *T. gondii* resulted in the identification of a high affinity transporter for hypoxanthine (TgHT1; $K_m$ = 0.91 ± 0.19 μM) and low affinity transporters for inosine (TgIT1; 656 ± 259 μM) and adenosine (TgAT1; $K_m$ = 105 ± 22 μM). No saturable transport of [$^3$H]-adenine was observed. The discovery of a hypoxanthine transporter with a 100-fold higher affinity for substrate than the purine nucleoside transporters indicates that TgAT1 may not be the main carrier responsible for purine salvage in this organism. Both arprinocid and arprinocid-1-N-oxide inhibited TgAT1 with high affinity ($K_i$ = 3.3 ± 1.1 μM and $K_i$ = 10.4 ± 3.4 μM, respectively), suggesting that these drugs may be substrates for, or

inhibitors of, this transporter. Although their exact mechanisms of action remain to be elucidated, neither drug acts on *T. gondii* by interfering with the hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT)-mediated purine salvage.

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The continued support and encouragement from my family and Katrien throughout my post-graduate studies have been the source of the extra motivation to succeed, especially during occasions of heightened stress.

To my Parents and Grandparents.

## Table of Contents

Page

## List of Figures

## List of Tables

## Abbreviations

| | |
|---|---|
| 2-DE | Two-dimensional electrophoresis |
| A | amperes |
| AB | assay buffer |
| ADP | adenosine-5'-diphosphate |
| ATP | adenosine-5'-triphosphate |
| APS | ammonium persulphate |
| $cm^3$ | cubed centimetre |
| CHAPS | 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulphonate] |
| Ci | curie |
| CPM | counts per minute |
| $dddH_2O$ | double-distilled de-ionised water |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetra-acetic acid |
| EM | erythrocyte medium |
| FCS | foetal calf serum |
| g | gram |
| GDP | guanosine-5'-diphosphate |
| GTP | guanosine-5'-triphosphate |
| h | hour |
| HCCA | α-cyano-4-hydroxycinnamic acid |
| HEPES | N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] |

| | |
|---|---|
| Hz | hertz |
| IEF | isoelectric focussing |
| IMDM | Iscove's modified Dulbeccos medium |
| IMP | inosine-5'-diphosphate |
| IPG | immobilised pH gradient |
| l | litre |
| m | milli |
| M | Molar |
| MALDI-PSD | matrix-assisted-laser-desoption/ionisation post-source decay |
| MALDI-TOF | matrix-assisted-laser-desoption/ionisation time-of-flight |
| min | minute |
| MOPS | 3-[N-morpholino] propanesulphonic acid |
| MS | mass spectrometry |
| n | nano |
| NADH | nicotinamide-adenine dinucleotide (reduced form) |
| NADPH | nicotinamide-adenine dinucleotide phosphate (reduced form) |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| ppm | parts per million |
| RBC | red blood cell |
| rpm | revolutions per minute |
| s | second |
| SDS | sodium dodecyl sulphate |

| | |
|---|---|
| T25 | 25 $cm^3$ tissue culture flask |
| T75 | 75 $cm^3$ tissue culture flask |
| TBE | tris-borate-EDTA |
| TCA | trichloroacetic acid |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TFA | trifluoroacetic acid |
| *T. gondii* | *Toxoplasma gondii* |
| Tris | tris(hydroxymethyl)aminomethane |
| $\mu$ | micro |
| U | units of enzyme activity |
| U/ml | units per millilitre |
| V | volts |
| W | watts |
| w/v | weight by volume |
| XMP | xanthosine-5'-monophosphate |
| °C | degrees centigrade |

# CHAPTER 1

# Introduction

## 1.1 *Toxoplasma gondii* and toxoplasmosis

*Toxoplasma gondii*, an intracellular coccidian parasite belonging to the phylum Apicomplexa, is an important pathogen affecting both humans and animals. Over 500 million people are infected with *T. gondii* (Denkers and Gazzinelli, 1998). Although infection in humans is common, disease is rare and usually confined to risk groups, such as the immunocompromised for whom toxoplasmosis represents a life-threatening disease. Infection with *T. gondii* has been pinpointed as a common cause of central nervous system defects in AIDS patients, for example, toxoplasmic encephalitis. Toxoplasmosis also causes more than 3000 congenital infections per year in the USA alone (Joiner and Dubremetz, 1993). In addition to human infections, the economic impact to the farming industry is considerable and may be perceived on two levels. Firstly, the direct loss of livestock due to abortion storms (see Section 1.4.3), and secondly, meat products from *Toxoplasma*-infected livestock, such as sheep, goats and pigs, are a risk to human health.

The mainstay of treatment for human toxoplasmic encephalitis, a combination of pyrimethamine and sulfadiazine, has severe side effects often resulting in poor patient compliance. Furthermore, the lack of therapeutic agents to treat the tissue cyst stage of

*T. gondii*, which is responsible for the recrudescence of disease, is of great concern due to the persistence of the parasite in the host. In combination with a world-wide increase in the number of AIDS patients, the incidence of toxoplasmosis is certain to rise. Therefore, continued research into the biology and biochemistry of *T. gondii* is essential to improve our understanding of *Toxoplasma* and toxoplasmosis. This will provide information that is pivotal for the identification of novel therapeutic targets and ultimately for the development of new, improved anti-*Toxoplasma* drugs.

## 1.2 Life cycle

### 1.2.1 Historical perspective

*T. gondii* parasites were first described by Nicolle and Manceaux (1908) in the blood, spleen and liver of a North African rodent, *Ctenodactylus gondii.* At this time the biology of this newly described parasite, originally named *Leishmania gondii*, was poorly understood, resulting in confusion over its classification. However, this new species lacked a kinetoplast, in contrast to *Leishmania* species, and so in 1909 was renamed *Toxoplasma gondii*. *Toxoplasma* is derived from two Greek words, Toxon = bow or arc; and plasma = form, and is descriptive of the crescentic shape of the intracellular form of the parasites.

Soon after the discovery of *T. gondii*, researchers began to investigate its life cycle and the routes of transmission. Early work had demonstrated that *T. gondii* was an obligate intracellular parasite which invaded nucleated cells and produced fatal infections in mice, guinea pigs, rabbits and chickens (Sabin and Olitsky, 1937). Transmission of *T. gondii* using mice-to-mice contact experiments were negative until small numbers of

animals were allowed to feed on other mice recently deceased due to infection with *T. gondii* (Sabin and Olitsky, 1937). This led to the realisation that transmission *via* *Toxoplasma*-contaminated tissue was possible.

The first theory of *Toxoplasma* transmission suggested the cause of parasitaemia was a blood-sucking insect (Woke *et al.*, 1953). An insect responsible for transmission was not discovered. A few years later tissue cysts were identified in pork and subsequently linked with toxoplasmosis (Weinman & Chandler, 1956). These cysts were resilient to extremes of temperature and to strong acids (for example gastric juices), thereby supporting the idea that they are an important form of *Toxoplasma* and could be involved in transmission *via* ingestion (Jacobs *et al.*, 1960). However, this theory was insufficient to explain how vegetarians or herbivores became infected.

Speculation grew on the possible involvement of a nematode, *Toxocara cati*, in transmitting *T. gondii* from cat faeces. Initial work demonstrated that the faeces of a cat, infected with *Toxocara cati* two months prior to being infected with *T. gondii* tissue cysts (derived from murine infections), resulted in experimental transmission of toxoplasmosis to mice (Hutchison, 1965). Hutchison, (1967) proposed that *Toxoplasma* was contained within the ova of *T. cati* and was released when larval worms hatched in the intestine of a warm-blooded host. This hypothesis was further substantiated by the demonstration that filtration of *T. cati* ova destroyed the infectivity of the suspension (Hutchison, 1967). However, it was not realised that *Toxoplasma* oocysts tend to aggregate onto large particles, such as faecal debris, that are too big to pass through the filter (Frenkel *et al.*, 1969). Moreover, infection by *T. gondii* had previously been shown to be possible in the absence of *T. cati* (Dubey, 1968). Thus doubt was cast on the nematode theory.

Hutchison (1965) observed oocysts in the faeces of cats, but because they were similar to the genus *Isospora* they were not considered to be associated with *Toxoplasma*. The oocyst, originally described as a "new cystic form of *Toxoplasma*," was shown to be responsible for transmission of *T. gondii* from cat faeces without, as previously thought, the involvement of nematodes (Hutchison *et al.*, 1969; Work and Hutchison, 1969). Work and Hutchison (1969) showed that there was a correlation between the number of oocysts given to mice and the severity of infection. Freshly excreted faecal oocysts did not transmit toxoplasmosis to mice but if oocysts were left at room temperature for 2-3 days to allow sporulation, transmission of toxoplasmosis could occur. Cats excreted oocysts approximately 3 weeks (prepatent period) after being fed faecal oocysts from infected cats, 3 - 5 days after being fed tissue cysts from an infected mouse and 9 - 11 days after being fed tachyzoites (Frenkel *et al.*, 1970). Similar observations were reported by Dubey *et al.*, (1970). The discovery of the *Toxoplasma* oocyst led to the complete life cycle being fully understood and the realisation that infections could occur from tachyzoites, ingestion of tissue cysts or contamination from faecal oocysts. The complete life cycle was published by Hutchison *et al.*, (1971) (Figure 1.1). The life cycle comprises two distinct parts, (i) the sexual cycle, which only occurs in cats and felidae, the definitive hosts of *T. gondii*, and (ii) the asexual cycle, which occurs in any warm-blooded mammal or bird (Miller *et al.*, 1972). During each of these cycles the parasites undergoes distinct developmental changes.

### 1.2.2 Sexual cycle

*Toxoplasma gondii* infection of cats is by ingestion of one of the infective stages, i.e. tachyzoite, bradyzoite or sporozoite. Regardless of the route of infection, a common pathway exists in which parasites first undergo schizogeny and then gametogeny

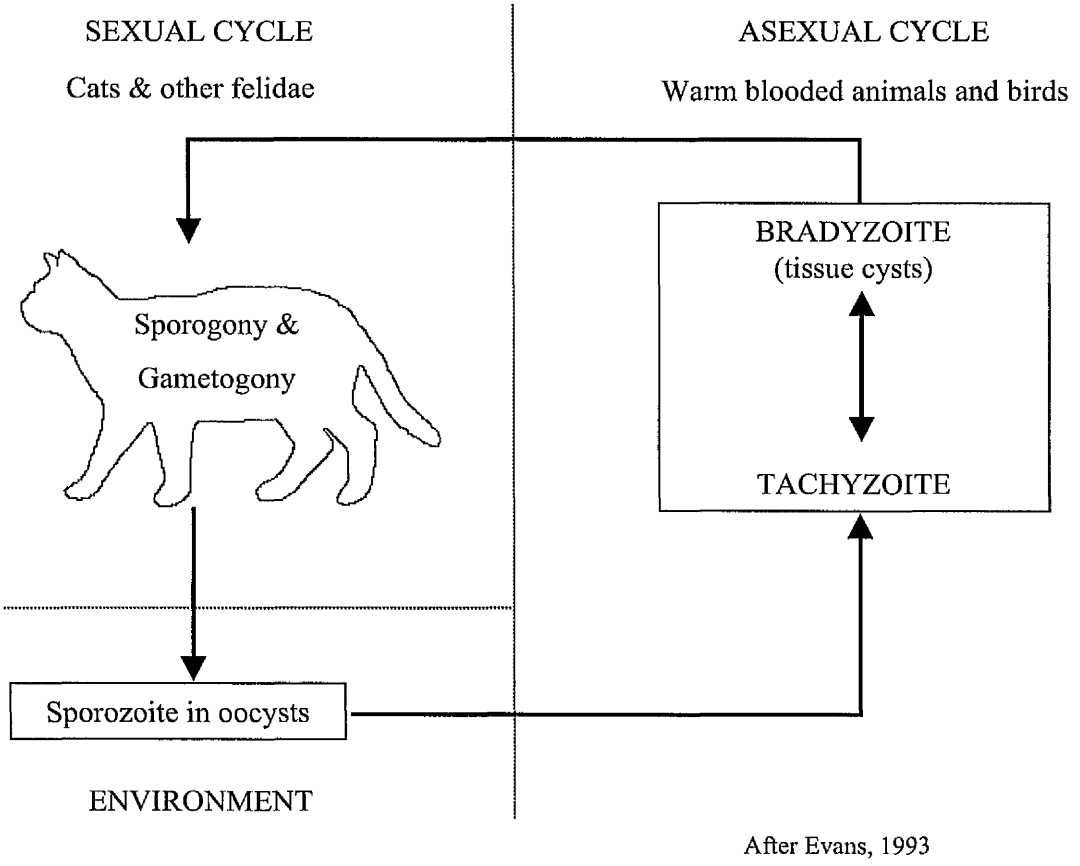



**Figure 1.1.** Simplified life cycle of *Toxoplasma gondii*. The life cycle may be considered in two parts (i) sexual cycle, which occurs in cats and other felidae resulting in excretion of oocysts, containing sporozoites, in the faeces (ii) asexual cycle, which occurs in warm-blooded animals and birds. Tachyzoites are the rapidly dividing form of *Toxoplasma* and are characteristic of acute infection. Bradyzoites are the slowly dividing form and are characteristic of latent chronic infections. In immunodeficient hosts, re-activation of bradyzoites into tachyzoites causes recrudescence of disease.

(Dubey and Frenkel, 1972; Hutchison *et al.*, 1971). However, the prepatent period (the time from infection to production of oocysts) varies depending on the source of infection (refer to Section 1.2.1; reviewed (Dubey, 1998).

### *1.2.2.1 Schizogony*

The transmission of *Toxoplasma* is enhanced by the resistance of the oocysts to environmental factors (Yilmaz and Hopkins, 1972) and by the shedding of millions of oocysts (Jackson and Hutchison, 1989). The production of such a large number of oocysts is achieved by the multiplication of *Toxoplasma* by a process known as schizogony. Schizogony occurs in the epithelial cells of the small and large intestines of the cat and is a process whereby the nucleus of the mother cell repeatedly divides resulting in multiple daughter cells.

### *1.2.2.2 Gametogony*

The development of the sexual stages of *Toxoplasma*, known as gametogony, occurs between 3 and 15 days post-infection. Male gametocytes, containing between 6 and 21 gametes, are few in number (2 - 4 %) compared with the total number of mature gametocytes. Each male gamete is elongated in appearance and contains two flagella, presumably used to help move towards female gametes. Fertilisation results in the production of a zygote, which is enclosed within a tough oocyst wall. Oocysts move through the small intestine and are excreted in faeces where they then undergo sporulation (Dubey and Frenkel, 1972).

*1.2.2.3 Sporulation*

Enclosed within the oocysts two sporoblasts form, which subsequently mature into two binucleate sporocysts. Inside each sporocyst, four sporozoites are produced resulting in a total of eight sporozoites inside a single oocyst. Sporulated oocysts may be formed within 24 h after being excreted in cats' faeces and may remain infective for up to one year outdoors in temperate climates (Yilmaz and Hopkins, 1972). Cats tend to be relatively clean animals and often bury their faeces, thus the warm moist texture of the soil may serve to increase viability of the oocysts and consequently increase the likelihood of transmission to a new host. Infection with oocysts may occur *via* a variety of routes including, contaminated vegetables, animal feed and grazing pastures or from accidental ingestion of oocysts during cleaning of cat litter trays.

**1.2.3 Asexual cycle**

The asexual cycle of *T. gondii* can be considered as two stages. Firstly, the production of tachyzoites which are characteristic of acute infection, and, secondly, the production of bradyzoites or tissue cysts present during latent chronic infection (Figure 1.1).

*1.2.3.1 Tachyzoites*

Infection of a new host with oocysts results in the release of sporozoites that may then enter the gastrointestinal tract and transform into tachyzoites. Subsequently, parasites disseminate *via* the bloodstream to organs and tissues within the body whereby they can invade any nucleated cell. Parasite replication takes place by a process of "internal budding,, called endodyogeny, derived from the Greek words: endon = within/inside;

dyo = two; and genesis = birth (Goldman *et al.*, 1957). Endodyogeny is a process by which a mother cell divides to produce two identical daughter cells, the parent being destroyed in the process (Goldman *et al.*, 1957).

*T. gondii* tachyzoites have highly developed organelles (rhoptries, micronemes and dense granules) which allow entry into the target cell by an active process rather than by phagocytosis. Invasion consists of many individual steps that occur individually or concurrently. The main processes involve attachment of the apical end of the parasite to the host cell surface, excretion of microneme and rhoptry proteins from specific organelles located at the apical end of the parasite, followed by the release of dense granules and vacuole formation (reviewed Dubremetz *et al.*, 1998; Joiner and Dubremetz, 1993). The complete process occurs in less than 15 - 40 s (Werk, 1985). It is hoped that understanding the precise and complex mechanisms involved in invasion will lead to the discovery of new chemotherapeutic targets.

*1.2.3.2 Microneme proteins*

Micronemes are organelles, located at the apical end of *T. gondii* tachyzoites, that release proteins that play a role in host-cell invasion (reviewed by Soldati *et al.*, 2001; Tomley and Soldati 2001). To date, 30 microneme proteins have been identified from *Cryptosporidium*, *Eimeria*, *Neospora*, *Plasmodium*, *Sarcocystis* and *Toxoplasma* (Tomley and Soldati, 2001), including the recently discovered *T. gondii* microneme protein, TgMIC10 (Hoff *et al.*, 2001). Considerable sequence homology in the adhesive domains of microneme proteins exists between these genera, suggesting that invasion processes may be conserved at the molecular level (Tomley and Soldati, 2001).

The identification of thrombospondin-related adhesive protein (TRAP) in *Plasmodium* sporozoites (Robson *et al.*, 1988) was central to the identification of homologues in *T. gondii* (Wan *et al.*, 1997). Like TRAP, TgMIC2 contains thrombospondin (TSP) repeats and similarly is considered to be important in host-cell attachment and invasion. During apical attachment to the host cell, TgMIC2 is distributed across the host cell membrane during invasion, but is not incorporated into the parasitophorous vacuole (Carruthers *et al.*, 1999; Carruthers and Sibley, 1999). TgMIC1 contains two (degenerate) TSP repeats with low homology to TRAP, suggesting that these proteins are also functionally similar (Fourmaux *et al.*, 1996). Other conserved functional structures, including I-domains, epidermal growth factor (EGF)-domains and apple domains, have been identified (reviewed Tomley and Soldati, 2001). TgMIC3 contains five EGF domains which are potentially involved in protein-protein interactions and thus may act as a link between parasite and host cell (Garcia-Réguet *et al.*, 2000). TgMIC4 consists of six conserved apple domains, each of which has four tandemly repeated structures that are stabilised by three internal disulphide bonds (Brecht *et al.*, 2001). The exact role of these apple domains remains to be elucidated, and thus far TgMIC4 is the only *T. gondii* microneme protein identified to have these domains. Recently another microneme protein, TgMIC10, has been identified. This lacks homology to any previously identified microneme protein, except one found in *Neospora caninum*, and may therefore indicate the presence of a new family of microneme proteins (Hoff *et al.*, 2001). Its role is yet to be determined.

The secretion of microneme proteins is regulated by cytoplasmic free $Ca^{2+}$ in the parasite (Carruthers and Sibley, 1999). Release of intracellular $Ca^{2+}$ by $NH_4Cl$ or thapsagargin treatment was shown to trigger microneme release from extracellular tachyzoites, suggesting that intracellular $Ca^{2+}$ stores are sufficient to initiate invasion

processes (Carruthers and Sibley, 1999). The recently defined acidocalcisome, found in bacteria, fungi, algae and protozoa contains millimolar concentrations of $Ca^{2+}$ and although the majority of this $Ca^{2+}$ is bound, it may be an essential source of free $Ca^{2+}$ required for release of micronemes (Docampo and Moreno, 2001). Since the acidocalcisome is not present in mammalian cells, it offers the exciting prospect of being a parasite-specific drug target. The acidocalcisome is membrane-bound, which will presumably cause difficulties in the design of drugs that can penetrate this barrier. However, this organelle, in addition to being a source of calcium, also has a range of ion pumps and proton exchangers, which also may be targeted (Docampo and Moreno, 2001). The exact nature and function of these pumps is not known but are thought to be involved in the regulation of pyrophosphates, polyphosphates and other cations (Docampo and Moreno, 2001). Whether these functions are essential to the parasite survival remains to be seen.

Some microneme proteins are proteolytically processed to an active form before being released from the parasite (Carruthers *et al* 2000). For example, prior to the secretion of TgMIC2 (115 kDa), the *C*-terminus and *N*-terminus domains are sequentially removed by two distinct proteases found on the parasite surface (termed MPP1 and MPP2).

The mechanism by which microneme proteins reach their designated organelle is not known. However, all micronemes are synthesised with an *N*-terminal signal sequence that is presumably involved in translocation of the protein (Tomley and Soldati, 2001). The extracellular adhesive domains of microneme proteins have been shown to interact with specific receptors on the surface of the host cell, and although the exact nature of these receptors is still unknown, lectins, laminins and glycans are thought to be important (Tomley and Soldati, 2001).

*1.2.3.3 Rhoptry proteins*

The initial contact between *T. gondii* tachyzoites and a host cell triggers the release of proteins from rhoptry organelles (reviewed Dubremetz *et al.*, 1998). Rhoptry proteins, in particular penetration enhancing factor (Lycke *et al.*, 1975), later renamed rhoptry protein ROP1, are found in the parasitophorous vacuole. Although their function is not certain, it is feasible that they are involved in the construction of this vacuole. One hypothesis is that rhoptry proteins may contribute to the formation of small pores in the vacuolar membrane which allow free exchange between the host cytoplasm and the vacuolar space (Schwab *et al.*, 1994). Current opinion on whether the parasite or the host cell contributes the material for the formation of the vacuole membrane has been extensively reviewed by Dubremetz and co-workers (1998), but the question remains largely unanswered. Capacitance of the host cell during invasion by *T. gondii* has been recorded by patch clamping and shown not to increase, and since capacitance is correlated with cell surface this would suggest that vacuoles consist primarily of invaginated host cell outer membrane (Suss-Toby *et al* 1996). During vacuole closure, capacitance is decreased which would indicate a drop in host cell surface area as the vacuole pinches off from the host cell membrane (Suss-Toby *et al* 1996). According to Suss-Toby and co-workers (1996), up to 18.5 % of material for the vacuole is parasite derived.

*1.2.3.4 Dense granule proteins*

Proteins released from dense granule organelles (GRA), during and subsequent to invasion of host cells, are thought to be involved in structural modifications of the parasitophorous vacuole (reviewed Cesbron-Delauw, 1994). These modifications are

unknown but are thought to be necessary for *T. gondii* survival. At least eight dense granules have been identified (Cesbron-Delauw, 1994). Several dense granule proteins, GRA1, GRA2, GRA4, GRA6 and GRA7 are all targeted to the vacuolar space, whilst, GRA5 is associated with the vacuole membrane and GRA3 is targeted to both (Fischer *et al.*, 1998; Labruyere *et al.*, 1999; Lecordier *et al.*, 1993; Mercier *et al.*, 1993). A mutant *T. gondii* line lacking GRA2 was shown to invade fibroblasts and macrophages *in vitro* but exhibited reduced virulence in mice, thus demonstrating that GRA2 (and perhaps other GRA proteins) may be important in *T. gondii* pathogenesis (Mercier *et al.*, 1998).

*1.2.3.5 Surface antigens*

The surface of *T. gondii* tachyzoites and bradyzoites is a complex entity being covered by many glycosyl-phosphatidylinositol (GPI)-anchored proteins (reviewed Lekutis *et al.*, 2001). Currently, there are 21 known surface antigens belonging to two main families, (i) surface antigen 1 (SAG1) and (ii) surface antigen 2 (SAG2). The SAG1 family consists of GPI-anchored proteins with 12 conserved cysteine residues of SAG1 and approximately 30 % (or more) overall homology (Manger *et al.*, 1998). The SAG2 family consists of those proteins related to the SAG2 antigen and have an incomplete set of cysteine residues and a low overall homology (approximately 20 %) (Lekutis *et al.*, 2000). In addition, SAG2 family proteins are smaller proteins, approximately 200 amino acids, compared with SAG1 family antigens which contain over 300 amino acids (Lekutis *et al.*, 2001).

The expression profile of many of the SAG genes has not been fully analysed, and although much work has been performed to determine which antigens are expressed in tachyzoites and/or bradyzoites, little is known about surface proteins found in the sexual

stages of *T. gondii*. To date eight surface antigens (SAG1, SRS1, SRS2, SRS3, SAG2A, SAG2B, SAG5B and SAG5C) have been found to be expressed only within tachyzoites and five SAG antigens (BSR4, SAG2C, SAG2D, SAG 4A and SAG5A) have been found to be expressed only within bradyzoites (Lekutis *et al.*, 2001). SAG3 has been identified in both tachyzoites and bradyzoites (Cesbron-Delauw *et al.*, 1994). The most likely reading frame of SRS5 contains four stop codons and a frame shift, indicating that it is a pseudogene; SRS5 is not expressed in either the tachyzoite or bradyzoite stage (Lekutis *et al.*, 2001).

The roles of SAG proteins are not completely understood but they are thought to be involved in host cell invasion, immune regulation, virulence and in the intracellular survival of parasites. One theory is that SAG proteins are used to adhere to host cells and that the vast array of antigens expressed by *T. gondii* may explain, to some extent, its ability to invade a wide range of nucleated cells (Boothroyd *et al.*, 1998). Analysis of surface proteins present in species with a more limited host range, such as *Neospora*, could give vital clues. Antibodies raised against SAG1 have been shown partially to block *T. gondii* invasion (Mineo *et al.*, 1993) and more recently a knockout mutant of SAG1 has been shown to have reduced virulence compared to parental wild-type control infections (Lekutis *et al.*, 2001). Similarly, a SAG3 null mutant was also less virulent than wild-type strains (Dzierszinski *et al.*, 2000). The creation of a double knockout of SAG1 and SAG3 has been attempted but failed, indicating that at least one of these proteins is required for invasion (Dzierszinski *et al.*, 2000). Further studies using single or double SAG gene knockout mutants are needed to elucidate which genes are essential and to obtain a more complete understanding of the host-parasite relationship. It seams feasible that differential expression of SAG genes between *T.*

*gondii* life stages will be important in tachyzoite-bradyzoite transition and therefore gene deletions in tissue-cyst forming strains could give valuable insights critical to drug or vaccine development strategies.

*1.2.3.6 Bradyzoites*

In immunocompetent hosts, tachyzoites are converted to bradyzoites which form cysts in a wide variety of tissues (especially the brain and skeletal muscle). They may persist for the lifetime of the host, thus acting as a reservoir of latent infection. The recrudescence of acute infection in immunodeficient hosts is of clinical significance, for example among AIDS patients over 95% of toxoplasmic encephalitis cases are due to the reactivation of *Toxoplasma* bradyzoites (Luft and Remington, 1992). Currently, there is no licensed drug proven to destroy or reduce the formation of tissue cysts. This is perhaps a consequence of the inability for sufficient concentrations of therapeutic agents to cross the blood-brain-barrier and/or to penetrate the cyst wall. Better understanding of the signalling pathways involved in tachyzoite-bradyzoite interconversion is critical and is likely to be important for the discovery of therapeutic targets against the bradyzoite stage.

The timing of tachyzoite-bradyzoite interconversion is dependent on the immune response (McCabe & Oster, 1989; Subauste and Remington 1993), although the extent of the involvement of the immune response in controlling interconversion is unknown. During this transition, it is likely that changes in metabolism and gene expression occur thus enabling *T. gondii* to adapt to a different environment. As discussed above, it is known that stage-specific antigens are expressed. Experimental evidence suggests that *in vitro* conversion of tachyzoites to bradyzoites occurs in response to external stress

conditions. For example, (i) incubation of tachyzoites with gamma interferon (Bohne *et al.*, 1993), (ii) incubation with mitochondrial inhibitors, such as oligomycin or antimycin A (Bohne *et al.*, 1993), (iii) culture in acidic (pH 6.8) or alkaline (pH 8.2) media (Bohne *et al.*, 1994; Soête *et al.*, 1994) and (iv) culture of tachyzoites at 43°C (Soête *et al.*, 1994).

Obtaining large quantities of bradyzoites is more difficult than for tachyzoites, and this is reflected in our current limited knowledge of this life-stage. However, the study of bradyzoites *in vitro* is now possible and should greatly contribute to our knowledge over the coming years. Recently, bradyzoite development has been linked to a family of heat shock proteins (hsp) and although the implications of this are unknown, future studies using gene knockouts should help elucidate the roles of hsps (Soête *et al.*, 1994). Interestingly, a bradyzoite-specific antigen, BAG1/hsp30 (BAG5), has *C*-terminal homology to hsps from plants that have been implicated in differentiation events, such as seed formation (Soête *et al.*, 1994). Despite BAG5 being specific for bradyzoites, it is not an essential gene for cyst formation since null mutants were able to form cysts *in vivo*, albeit at a reduced frequency (Zhang *et al.*, 1999). It would, therefore, appear that several genes are involved in cyst formation, and that alternative proteins are able to compensate for the lack of BAG5. The complexity of the process is indicated further by the asynchronous differentiation of bradyzoites and existence of intermediate stages that express both tachyzoite- and bradyzoite-specific antigens (Soête *et al.*, 1993). Recent evidence suggests that cyclic nucleotide signalling pathways are involved in stress-induced tachyzoite-bradyzoite conversion (an increase in cAMP induces bradyzoites formation) (Kirkman *et al.*, 2001).

## 1.3 Virulence

The severity of toxoplasmosis is related to both the virulence of the infecting strain and to host factors. In mice, some strains readily undergo conversion from the tachyzoite stage to the bradyzoite stage resulting in latent infections (such as the Beverly strain), whilst virulent strains remain in the tachyzoite form causing acute infections (for example RH *T. gondii*). Phylogenetic analysis, using restriction fragment length polymorphisms (RFLP), indicate that three clonal lineages exist. There are (i) Type I which are virulent in mice, (ii) Type II which are predominantly found in human infections and (iii) Type III which are predominantly found in animal infections (reviewed Sibley and Howe, 1996). However, recent evidence from sequencing loci of antigenic proteins suggests that only two clonal lineages exist and that the predominant genotypes arise from a genetic mixing of two distinct ancestral lines (Grigg *et al.,* 2001). The $F_1$ progeny of a cross between mice avirulent type II and mice avirulent type III *T. gondii* strains produced progeny that were three logs more virulent than the parental generation, thereby indicating virulence may occur by recombination of alleles at two or more loci (Grigg *et al.,* 2001). Strains that are virulent in one species may be avirulent in another, and *vice-versa*. These strain variations are important in disease manifestation and clinical treatment. Understanding, these differences should lead to better treatment strategies and development of vaccines. For example, the S48 *T. gondii* strain is not able to produce tissue cysts (Wilkins *et al.*, 1987) and has been successfully tested as a *Toxoplasma* vaccine, Toxovax™ (Buxton, 1993).

## 1.4 Incidence of disease

### 1.4.1 Immunocompromised host

The vast majority of acute infections in immunocompetent humans are asymptomatic and treatment is not warranted. However, onset of disease may be severe in immunocompromised hosts (AIDS, cancer chemotherapy and organ transplant patients) and consequently treatment is necessary. The predominant disease manifestation is toxoplasmic encephalitis (TE). In the USA one third of all seropositive AIDS patients will develop TE whilst in Europe it is estimated that 25-50% of seropositive AIDS patients will develop TE (Luft and Remington, 1992).

### 1.4.2 Congenital infection

*Toxoplasma* infection is only passed on to the foetus during pregnancy if the woman is infected after conception and the likelihood of foetal transmission is increased the later on in the pregnancy that infection is acquired (McCabe and Oster, 1989). Infections during pregnancy are usually asymptomatic and therefore the only method to detect a risk of foetal infection is by screening. However, political views on screening for toxoplasmosis in pregnancy vary. In France and Austria congenital toxoplasmosis is considered more of a public health hazard and both countries operate screening programmes, whilst in the UK and USA routine screening is not performed. In France, 87 % of pregnant women are seropositive to *Toxoplasma* whilst only 20 % of women in the UK are seropositive (Chatterton, 1992).

### 1.4.3 Domestic animals and cats

Many studies have been performed to assess the prevalence of *Toxoplasma* in domestic animals, but the results have shown high variability between different regions and different studies (reviewed Jackson and Hutchison, 1989; Evans 1992). Infections in horses and cattle generally result in few clinical symptoms, however, infections in ruminants can lead to abortions (Esteban-Redondo and Innes, 1997). In the past cases of toxoplasmosis in cattle were probably misdiagnosed, and instead may have been *Neospora caninum*, a closely related protozoan parasite (Dubey and Lindsay, 1993). Although infections in humans may occur from ingestion of tissue cysts from contaminated meat, the natural life cycle of infection is probably cat-rodent-cat transmission (Webster *et al.*, 1994).

The exact prevalence of infection in cats is unknown, although it appears that cats only excrete oocysts once in their lifetime (Dubey, 1970). In a survey of prevalence in cats in Scotland, 19 % were found to have significant antibody titres (>1:10) and exclusion of young cats from the data set (infection is more prevalent in older cats) indicated that stray cats are more susceptible to infection than domestic cats (Jackson *et al.,* 1987).

Congenital infection in sheep is a serious cause of loss of lambs due to abortion storms, stillbirth, mummification or neonatal death (Jackson and Hutchison, 1989). High losses (up to 80 % of sheep) have been recorded in Norway, with similar high rates of prevalence and abortion in Britain (up to 68 % prevalence), New Zealand and Australia (Jackson and Hutchison, 1989; Evans, 1992). Such severe losses are a significant economic drain to farmers and the farming industry.

Although cattle are susceptible to infection by *T. gondii*, disease rarely develops (Esteban-Redondo and Innes, 1997). As mentioned above, many cases of abortion in cattle were most likely to have been caused by *N. caninum*. Prevalence of *T. gondii* in cattle may be up to 25 % in some countries such as The Netherlands; in Scotland prevalence is reported to be less than 7 % (Jackson and Hutchison, 1989). Prevalence in pigs is generally lower than infection in sheep. Only three studies have been carried out on prevalence of toxoplasmosis in pigs in Britain, with a range of 4 - 12 %, although this may be higher in other countries (Evans, 1992). Studies of the prevalence of antibodies in goats estimated infection between 7 % to 20 % in California (Jackson and Hutchison, 1989).

## 1.5 Clinical and experimental treatment strategies

Chemotherapy of important human and veterinary coccidosis, including *Toxoplasmosis*, has been recently reviewed (Haberkorn, 1999; Coombs *et al.*, 1997). Currently there are only a few drugs that are licensed for the treatment of toxoplasmic encephalitis (TE). The first choice treatment for TE is usually a combination of pyrimethamine (a substituted phenylpyrimidine) and sulfadiazine (sulphonamide analogue) (Figure 1.2). However, the long-term therapy required and the adverse side effects mean that over 50 % of patients withdraw from medication (Haverkos, 1987). There is no proven licensed therapy against the tissue cyst stage of *T. gondii*, which is responsible for the recrudescence of disease. Many potential therapeutic drugs that have been shown to kill tachyzoites *in vitro* or *in vivo* do not affect tissue cysts. The reasons for the limited success of the treatment of bradyzoites remain unclear but may be related to the cyst wall acting as a barrier thereby preventing access of the drugs. Further research into the biology and biochemistry of coccidia is needed to identify new drug targets and to

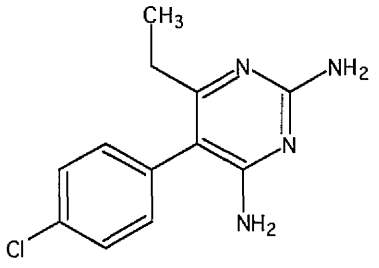
design novel inhibitors that have greater specificity for *T. gondii* and also destroy or reduce tissue cysts.

Treatment of congenital and ocular toxoplasmosis has been more successful. The most commonly used drugs are spiramycin used for the prevention of congenital infections and clindamycin used for the treatment of ocular toxoplasmosis (see below).

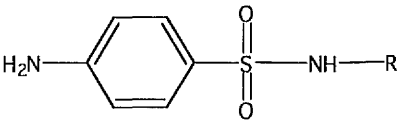
The mechanisms of action of many anti-*Toxoplasma* drugs are largely unknown but may be classified under four headings, (i) folic acid antagonists (ii) protein synthesis inhibitors (iii) purine analogues (iv) miscellaneous. The following is not an extensive list of compounds that have been tested against *T. gondii*, but is a discussion of previous and current work related to the development of anti-*Toxoplasma* drugs that is intended as an indication of areas of interest for future drug development. In particular, attention will be paid to the purine analogues, arprinocid and arprinocid-1-N-oxide, which are a main focus of this thesis.

### 1.5.1 Folic acid antagonists

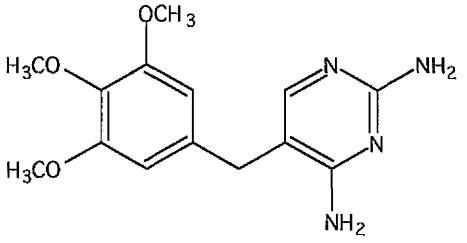
In contrast to mammalian cells, *T. gondii* has all the enzymes necessary for *de novo* folate synthesis thus providing possible targets for anti-*Toxoplasma* chemotherapy (Kovacs *et al.*, 1989). Pyrimethamine selectively binds to DHFR thereby inhibiting the conversion of dihydrofolate to tetrahydrofolate and ultimately reducing nucleic acid synthesis (Figure 1.3). Although this reaction occurs in mammalian and protozoan cells, the two enzymes are distinct (Kovacs *et al.*, 1990) and consequently pyrimethamine has 2000-fold greater specificity for DHFR of the parasite than for the equivalent human enzyme.



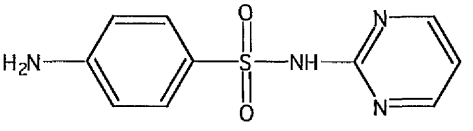
Pyrimethamine



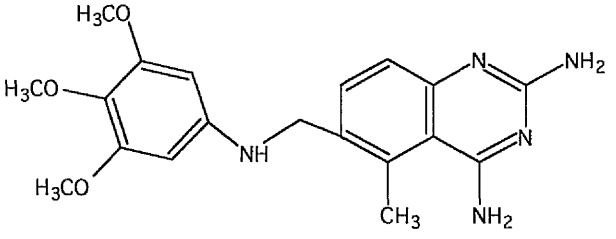
Sulfonamide



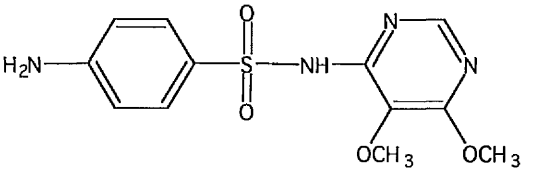
Trimethoprim



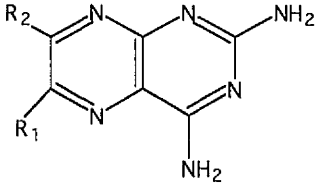
Sulfadiazine



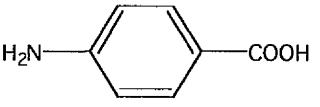
Trimetrexate



Sulfadoxine



6,7-Disubstituted 2,4-diaminopteridine



p-Aminobenzoic acid

**Figure 1.2.** Anti-folate chemotherapeutic drugs. Pyrimethamine, which inhibits dihydrofolate reductase (DHFR), in combination with a sulfonamide analogue is the mainstay of treatment of toxoplasmic encephalitis. Sulfadiazine is the most commonly used sulfonamide, although recently sulfadoxine has been successfully tested. p-Aminobezoic acid (pABA) is a precursor of for the synthesis of dihydropteroate (see Figure 1.3). Trimetrexate and analogues of 6,7-disubstituted 2,4-diaminopteridine are novel compounds which selectively inhibit DHFR.

*De novo* folate synthesis in *Toxoplasma gondii*

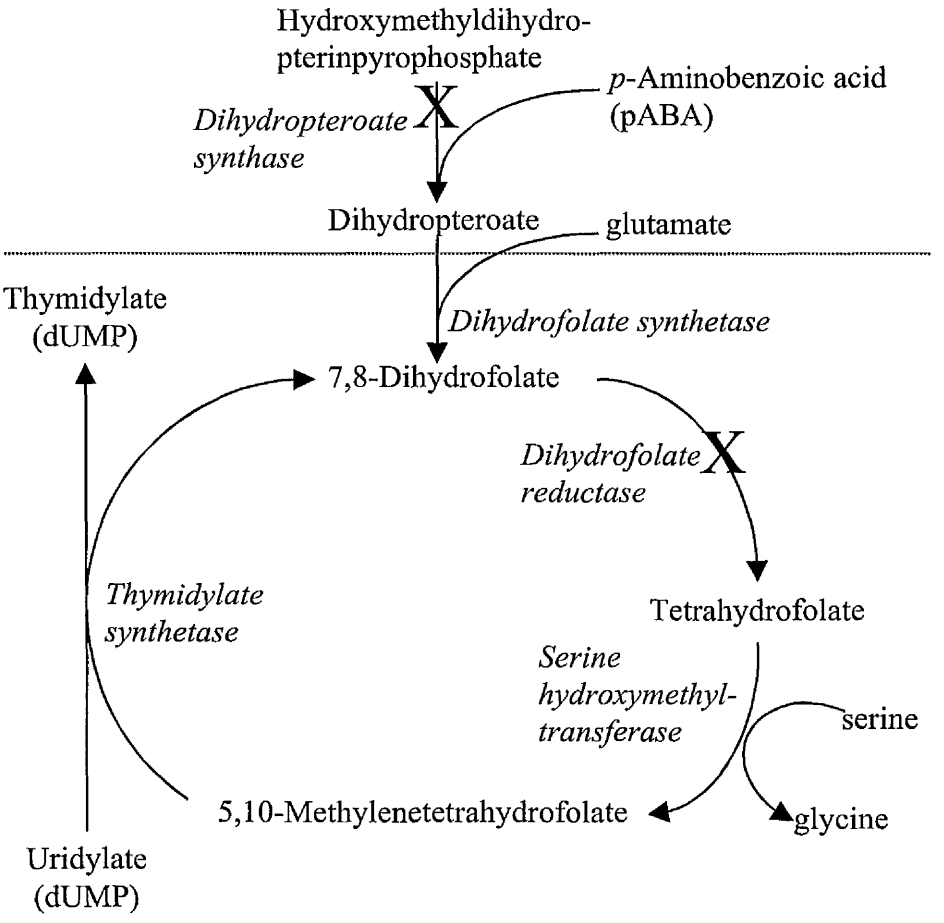



**Figure 1.3.** *De novo* folate synthesis in *T. gondii*. Folate synthesis pathway is shown above the centre dotted line. The precursor for synthesis is GTP which is metabolised in a four-step reaction to hydroxymethyldihydropterinpyrophosphate (omitted from figure). The product of folate synthesis, dihydropteroate, is subsequently converted to dihydrofolate, reduced and methylated. The cycle is completed by the formation of thymidylate. Key enzymes are written in *italics*. Dihydrofolate reductase and thymidylate synthetase are part of a single bifunctional enzyme. The two enzymes, each marked by a cross (X), are the chemotherapeutic targets of the pyrimethamine and sulfonamides (refer to text).

In clinical therapies, the activity of pyrimethamine is usually enhanced by the co-administration of a sulphonamide drug. Sulphonamides are synthetic analogues of para-aminobenzoic acid (pABA), a precursor for the synthesis of dihydropteroate, and competitively inhibit dihydropteroate synthase (Figure 1.3). Although sulphonamides do not kill *Toxoplasma* they act synergistically with pyrimethamine, presumably because both drugs act against a common metabolic pathway but it may also be because some of the pterin substrate binds to sulfonamides forming complexes that inhibit dihydrofolate synthetase (Barrett *et al.*, 1998). There are many sulfonamide compounds with proven efficacies including sulfanilamide, sulfapyridine and sulfathiazole (Sabin and Warren, 1942). However, sulfadiazine (Figure 1.2) is usually the sulfonamide of choice for the treatment of toxoplasmosis (Sheffield and Melton, 1975). Other, newer sulfonamides have been synthesised such as sulfadoxine (Figure 1.2) and sulfametopyrazine which have longer half-lives and thus need only be administered once a week. The combination of sulfadoxine and pyrimethamine, Fansidar, is commonly used for treatment of chloroquine-resistant malaria.

As mentioned above, the side effects of pyrimethamine-sulfadiazine long-term treatment are often so severe that withdrawal from therapy is common. When the two compounds are administered together they result in depression of bone marrow production causing leukopenia (abnormally low white blood cell count), megaloblastic anaemia (abnormally large red blood cells) and thrombocytopenia (abnormally low platelet count). However, co-administration of folinic acid may reverse some of these effects (folic acid antagonises the toxic effects of pyrimethamine). Pyrimethamine is teratogenic and therefore cannot be used during the first trimester of pregnancy. Sulphonamides tend to cause a wide range of side effects primarily due to excessive and

unwanted protein binding which invokes an immune response causing hypersensitivity and fever. Other adverse reactions to sulfonamides include bone marrow suppression (discussed above), crystalluria, nephrolithiasis (kidney stones) and renal failure (McCabe and Oster, 1989); Joss, 1992).

Apart from the severe side effects of pyrimethamine-sulfadiazine treatment, resistance of some protozoan parasites to therapy is an increasing problem. Point mutations in enzymes have been shown to decrease the ability of drugs to bind to their target enzymes. For example, mutations in *Plasmodium falciparum* dihydropteroate synthase and dihydrofolate reductase result in resistance to sulfadoxine and pyrimethamine, respectively (Wang *et al.*, 1997). Similar mutations conferring resistance to antifolates in *T. gondii* have been observed under laboratory conditions (Reynolds and Roos, 1998). However, pyrimethamine resistance in *T. gondii* is rare, probably because drug-resistance mutations are unlikely to pass from one human to another. Should there be an increase in resistance to antifolate therapy in toxoplasmosis, the mainstay of treatment, pyrimethamine and sulfadiazine, would be rendered inadequate (Reynolds and Roos, 1998).

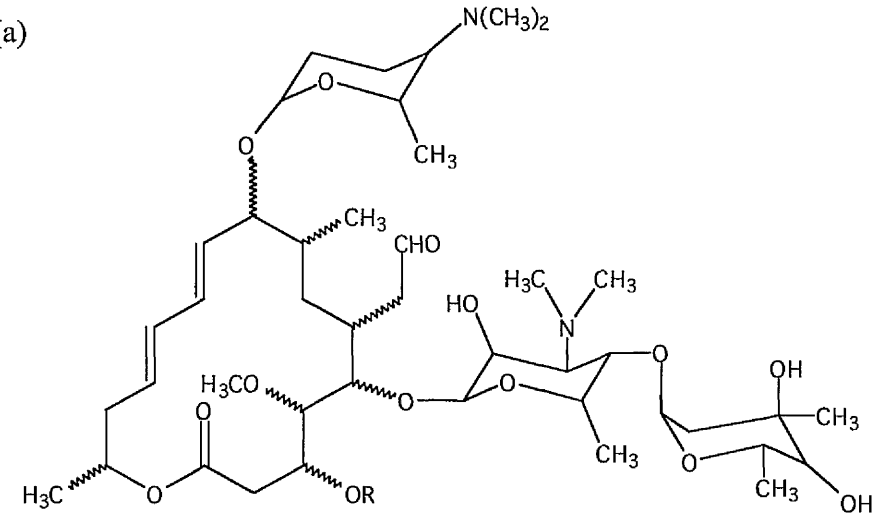
Other anti-folate compounds have been shown to be efficacious by inhibiting DHFR and thus are promising chemotherapeutic agents. Trimethoprim-sulfamethoxazole (co-trimoxazole) (Figure 1.2) has been shown to be effective during clinical trials (Norrby *et al.*, 1975) and more recently the combination trimethoprim-piritrexim has been demonstrated to inhibit *in vitro* growth of *T. gondii* tachyzoites (Derouin and Chastang, 1989). Epiroprim, an analogue of trimethoprim, has been shown to be effective alone or in combination with dapsone in reducing brain cysts (Brun-Pascaud *et al.*, 1996; Chang *et al.*, 1994). Trimetrexate ($IC_{50}$ = 1.4 nM), an analogue of piritrexim, was 600-fold

more active against DHFR enzyme compared with pyrimethamine and has been shown to be efficacious *in vitro* and *in vivo* (Allegra *et al.*, 1987). Other newer compounds that have been shown to inhibit *T. gondii* DHFR, such as analogues of 6,7-disubstituted 2,4-diamino-pteridines (Jackson *et al.*, 1996) (Figure 1.2), 2,4,-diamino-5-methyl-5-deazapteridines (Piper *et al.*, 1996) and di- and tri-cyclic 2,4-diaminopyrimidines (Lau *et al.*, 2001), may prove superior to trimetrexate and piritrexim.

### 1.5.2 Protein synthesis inhibitors

Spiramycin (Figure 1.4) is a macrolide antibiotic, i.e. it contains a macrocyclic lactone ring structure. Spiramycin is unsuitable for the treatment of cerebral toxoplasmosis because it is unable to penetrate into the cerebrospinal fluid (CSF), but because it does accumulate within tissues, particularly the placenta, spiramycin may be used for the prevention of congenital toxoplasmosis. Spiramycin is available in Europe for the treatment of pregnant women with acute *Toxoplasma* infection, although its efficacy has never been proved (Derouin *et al.*, 2000). However, the success of treatment is a contentious issue; most studies use incomparable control groups and thus the potential benefits of treatment versus the side-effects of treatment are difficult to conclude (Wallon *et al.*, 1999). Clindamycin (Figure 1.4), structurally very different to spiramycin, is a highly lipophilic compound that is used mainly to treat ocular toxoplasmosis (McCabe and Oster, 1989). There are few adverse reactions to treatment except when prescribed in large doses (McCabe and Oster, 1989). Clindamycin has been demonstrated to reduce parasite invasion, possibly by reducing protein synthesis in extracellular parasites (Blais *et al.*, 1993b).

(a)




(b)

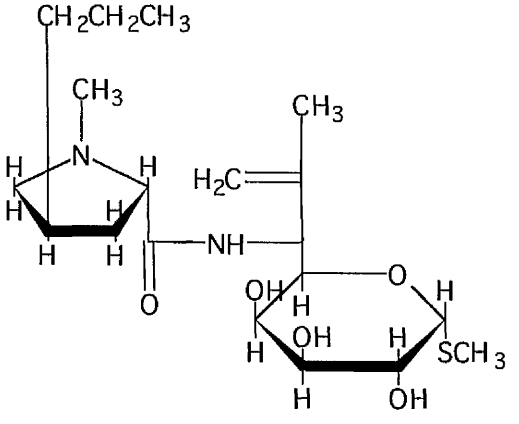



**Figure 1.4.** Structures of (a) spiramycin and (b) clindamycin. Spiramycin is used to prevent congenital toxoplasmosis whilst clindamycin is used against ocular toxoplasmosis. Spiramycin is actually composed of 3 compounds, spiramycin I (R = H), spiramycin II (R = $COCH_3$) and spiramycin III (R = $COCH_2CH_3$).

Of the newer macrolides, based on semisynthetic derivatives of 14- and 15-membered macrolides, azithromycin, clarithromycin and roxithromycin have been shown to have increased bioavailability and therefore more persistent levels of drug in serum and tissues may be achieved (Kirst and Sides, 1989). Moreover, these newer macrolides penetrate polymorphonuclear leukocytes, macrophages and lymphocytes, which is particularly important in the treatment of intracellular pathogens, such as *T. gondii*. Azithromycin has been shown to be concentrated inside mouse macrophages at up to 2.7 mM, well above therapeutic levels ($IC_{50}$ = 140 μM; $IC_{90}$ = 334 μM) and well above concentrations needed to inhibit protein synthesis; azithromycin (53 μM) inhibited [$^{35}$S]-methionine incorporation by 67 % compared with controls (Blais *et al.*, 1993). Azithromycin has been shown to be effective both *in vitro* against tachyzoites and tissue cysts (Chang and Pechere, 1988; Huskinson-Mark *et al.*, 1991) and in treating murine toxoplasmosis (Chang *et al.*, 1989). Clarithromycin has been shown to be effective both *in vitro* (Chang and Pechere, 1988) and in murine models of toxoplasmosis (Chang *et al.*, 1988). The exact mode of action of macrolide antibiotics is not completely known, although recent evidence suggests that targeting to the plastid organelle results in inhibition of protein synthesis (Beckers *et al.*, 1995). Recently, a combined treatment of azithromycin and pyrimethamine for the treatment of toxoplasmic encephalitis in AIDS patients has undergone phase I and II clinical trials (Jacobson *et al.*, 2001). Results demonstrated that whilst the combination was safe, there was a significant relapse rate and severe side effects in patients receiving higher doses of drugs.

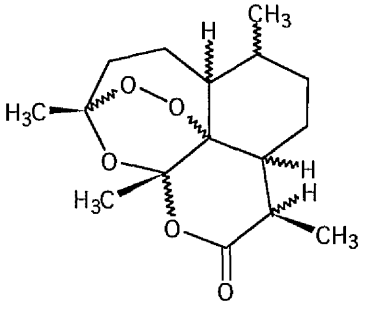
### 1.5.3 Purine analogues

*Toxoplasma gondii* is incapable of *de novo* purine synthesis (Perrotto *et al.*, 1971) and as such relies on purine transporters and salvage enzymes (the details of these pathways will be discussed in Chapter 6). Consequently, these uptake mechanisms provide potential chemotherapeutic targets for the design of novel anti-*Toxoplasma* compounds. Since several of the enzymes involved are specific to *T. gondii*, a suitable level of parasite specificity may be obtained, resulting in the ability to administer low doses with fewer side effects. The purine analogues, arprinocid and arprinocid-1-N-oxide, have both been shown to be effective against *T. gondii* tachyzoites when tested *in vitro* and *in vivo* (Luft, 1986; Pfefferkorn *et al.*, 1988), and against *Neospora caninum* (Lindsay *et al.*, 1994) and *Cryptosporidium parvum* (Rehg and Hancock, 1990). These compounds are discussed in more detail in Sections 1.7.1 - 1.7.5. Another purine analogue, 2',3'-dideoxyinosine, has been shown to reduce the numbers of *T. gondii* cysts within cerebral tissue and re-infection experiments with these tissue cysts showed that they had lost their viability (Sarciron *et al.*, 1997). Although, the mechanism of action is not known, it is feasible that interference with essential purine metabolic pathways results in parasite death. A recent study of purine metabolism in the brains of mice infected with a tissue-cyst forming strain of *T. gondii* tissue cysts showed that treatment with 2',3'-dideoxyinosine resulted in an increase in activity of purine enzymes in cerebral tissue, in particular adenosine deaminase (Gherardi *et al.*, 1999). The implications of this are not known although it suggests that 2',3'-dideoxyinosine targets purine metabolic enzymes.

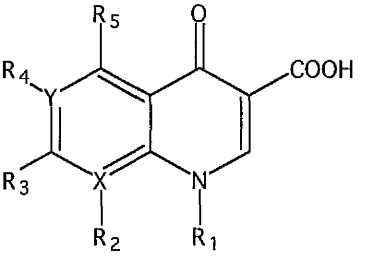
### 1.5.4 Miscellaneous compounds

Among the first compounds to be tested against *T. gondii* were lasalocid and monensin, both polyether antibiotics. They have both been proven to be potent inhibitors of *T. gondii* tachyzoites *in vitro* (Melton and Sheffield, 1975) and monensin was also effective in reducing faecal oocyst production in cats (Frenkel and Smith, 1982). Treatment of domestic cats using monensin may reduce human infections and congenital transmission but this strategy would have no impact on transmission from stray cats. Monensin and lasalocid have been shown to be effective in reducing the viability of *Eimeria tenella* sporozoites (Long and Jeffers, 1982) and have activity against *Cryptosporidium parvum* (Armson *et al.*, 1999).

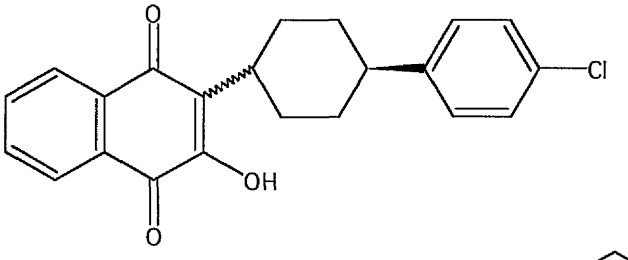
During the early 1970's the active antimalarial ingredient, artemisinin (alternative name: qinghaosu), of the chinese herb qinghao, was isolated (Figure 1.5). Artemisinin belongs to a class of compounds called trioxanes that have been found to be rapid acting, highly effective antimalarials with low host toxicity (reviewed van Agtmael *et al.*, 1999; Dhingra *et al.*, 2000; Olliaro, 2001; Olliaro *et al.*, 2001; Posner and Meshnick, 2001). Interestingly, the use of qinghao for malaria fever dates back to 340 A.D (cited Ke *et al.*, 1990). Artemisinin acts on the early trophozoite and schizont stages of malaria, and also against gametocyte development, resulting in reduced transmission. Moreover, more than two million patients have been treated with artemisinin (Olliaro *et al.*, 2001) and no incidence of resistance has been reported (Olliaro, 2001). Holfels *et al.*, (1994) postulated that artemisinin may act *via* free radicals, although their exact mechanisms of action are still a mystery and of great scientific debate. Malaria-infected erythrocytes digest haemoglobin thus releasing haem in the process, a toxic substance for the parasite that is neutralised by polymerisation to haemazoin. Haem, or more



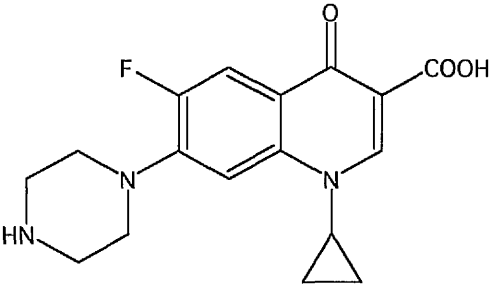
Artemisinin



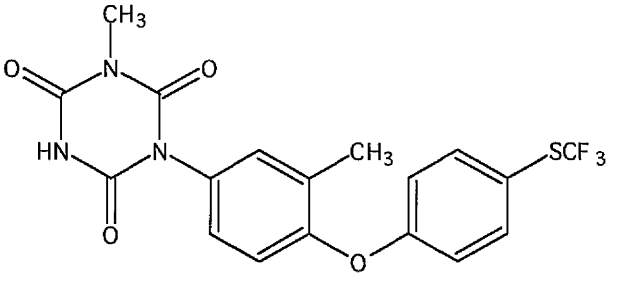
General quinolone structure



Atovaquone



Ciprofloxacin



Toltrazuril

**Figure 1.5.** Anti-*Toxoplasma* compounds. Basic quinolone structure; X and Y can be a carbon or nitrogen atoms, and $R_1$ - $R_5$ can be wide variety of structures. Ciprofloxacin is a quinolone analogue.

specifically $Fe^{2+}$, catalyses the breakdown of the peroxide bridge of artemisinin (Figure 1.5), leading to the formation of free radicals. These free radicals are toxic to malaria parasites (van Agtmael *et al.*, 1999). The precise mechanism whereby free radicals are formed and how they exert their action remains unclear. Several semi-synthetic analogues of artemisinin, for example dihydroartemisinin, artemether, arteether and artesunate have been demonstrated to have greater efficacy and better drug solubilities than artemisinin (van Agtmael *et al.*, 1999). Several of these derivatives and also second-generation completely synthetic 1,2,4-trioxanes display *in vitro* anti-*Toxoplasma* activity, however further work is necessary to determine their usefulness as drugs (Chang *et al.*, 1989; Ke *et al.*, 1990). Cloned *T. gondii* mutants resistant to artemisinin have been demonstrated to be cross-resistant to dihydroartemisinin and artemether, but not deoxyartemisinin (derivative of artemisinin containing a reduced endoperoxide bond) and pyrimethamine, suggesting resistance is specific to artemisinin and its endoperoxide-bond-containing derivatives (Berens *et al.*, 1998). One resistant clone was found to be avirulent for mice and offered protection against lethal challenge from a wild-type strain, whilst another clone resulted in the formation of unusually large vacuoles. Together, these clones will be useful for investigation of the mechanism of action of artemisinin and for studies of parasitophorous vacuole formation and virulence factors (Berens *et al.*, 1998).

Hydroxynaphthoquinones, in particular atovaquone (Figure 1.5), have been shown to be effective against tachyzoites *in vitro* and significantly reduce the numbers of brain tissue cysts in murine models of toxoplasmosis (Araujo *et al.*, 1991; Khan *et al.*, 1998). There was no synergistic effect when atovaquone was combined with pyrimethamine, sulfadiazine, clarithromycin or minocycine (Romand *et al.*, 1993) although another

publication presented evidence that the combination of atovaquone and pyrimethamine was synergistic (Araujo *et al.*, 1993). Atovaquone combined with proguanil has recently been licensed, as a drug called Malarone, for the treatment of malaria, however resistance to this combination has already been reported. Mutations in the parasite cytochrome b, where the drug is thought to bind and inhibit mitochondrial electron transport, have been found to confer resistance resulting in a 9000-fold reduction in sensitivity to atovaquone (Korsinczky *et al.*, 2000).

Another class of compounds, the triazines, including diclazuril and toltrazuril (Figure 1.5) have been shown to be effective against *T. gondii* both *in vitro* and in murine models (Lindsay and Blagburn, 1994). The mechanism of action of triazines is not completely known, however, one report has suggested that toltrazuril primarily affects the respiratory chain (Harder and Haberkorn, 1989), although it was reported that one target of triazines is the chlorophyll a-D1 complex located within the apicoplast (Hackstein *et al.*, 1995).

## 1.6 Novel drug targets

### 1.6.1 Apicoplast

*T. gondii* parasites contain three distinct genomes, (i) nuclear genome, (ii) mitochondrial genome and (iii) 35 kb extrachromosomal DNA. The latter, identified by *in situ* hybridisation and electron microscopy, is localised to a discrete organelle surrounded by four bilayer membranes, termed the apicoplast, and has a structure similar to chloroplast genomes (Kohler *et al.*, 1997). The function of this organelle, thought to be acquired by endosymbiosis of green alga, is largely unknown although it

is certain that the parasite replicates the 35 kb DNA during endodyogeny. Therefore, the apicoplast is likely to be a source of novel chemotherapeutic targets (discussed below) (Kohler *et al.*, 1997; McFadden and Roos, 1999).

*T. gondii* bradyzoites as well as tachyzoites have been shown to contain a morphologically similar plastid organelle (Roberts *et al.*, 1998) allowing the possibility of identifying chemotherapeutic targets in both life stages, to which a single drug may act. However, inevitably, designing therapeutic agents that readily cross the tissue cyst wall remains a challenge.

Ciprofloxacin, a quinolone analogue (Figure 1.5), has been shown to inhibit replication of the *T. gondii* tachyzoite apicoplast genome, clearly validating this organelle as a potential chemotherapeutic target (Fichera and Roos, 1997). Furthermore, the same characteristic kinetics of delayed parasite death (described below) that have been demonstrated for some protein synthesis inhibitors, including clindamycin, also occur with ciprofloxacin indicating that these compounds all act upon the apicoplast (Beckers *et al.*, 1995; Fichera *et al.*, 1995). Parasites treated with ciprofloxacin or clindamycin replicate normally at the beginning of treatment but in the subsequent invasion round replication is severely disrupted, giving rise to the phenomenon of delayed parasite death. Concentrations of clindamycin over 1000 times greater than its $IC_{50}$ against tachyzoites do not inhibit parasite growth in the first round of replication, however, different treatment do affect the speed of replication upon entry into a second host cell (Fichera *et al.*, 1995). The reasons for continued parasite survival in the presence of what would appear to be a malfunctioning apicoplast is a mystery but perhaps the damage to the apicoplast only manifests itself during some function required for correct invasion of a new cell, like, for instance, parasitophorous vacuole formation.

A fusion protein consisting of the nuclear-encoded apicoplast acyl-carrier protein (ACP) and the green fluorescent protein (GFP) results in the targeting of GFP to the apicoplast. Transient expression of this construct containing a fragment of ROP1, named ACP-GFP-mROP1, inhibits apicoplast segregation (He, *et al.*, 2001). It was noted that a parasite within a vacuole replicates normally but the apicoplast is only segregated into one of the two daughter cells. This process is repeated in the next generation, i.e. only one granddaughter cell acquires the apicoplast. Parasites with and without apicoplasts grow normally, although the apicoplasts in some cells grow abnormally large, indicating a loss of cell cycle control. Parasites lacking an apicoplast were sorted by FACS and used successfully to re-infect cells. Although vacuoles were formed, replication ceased. This work further substantiates the 'delayed death' effect seen with ciprofloxacin or clindamycin and illustrates the importance of the apicoplast in establishing a functional parasitophorous vacuole (He *et al.*, 2001). The ability to generate apicoplast deficient isolates will allow further characterisation of this organelle and should also provide a convenient system for testing whether or not potential chemotherapeutic agents are targeted to the apicoplast.

In contrast to the work of Fichera *et al.* (1995), one study found that ciprofloxacin was toxic to host cells and that a related compound, trovafloxacin, had increased specificity both *in vitro* and *in vivo* with no host cell toxicity (Khan *et al.*, 1996). An assessment of the structure-activity relationships of quinolones and fluoroquinolones confirmed the potency of trovafloxacin against *T. gondii* (Gozalbes *et al.*, 2000) and a later report suggested that this compound may be important for treatment of malaria (Hamzah *et al.*, 2000).

The exact function of the apicoplast is not known, although its genome has been sequenced for *P. falciparum* and for *T. gondii* (Wilson *et al.*, 1997; http://www.sas.upenn.edu/~jkissing/toxomap.html). The apicoplast does not encode all of the genes required for protein synthesis and no proteins are apparently involved in the functioning of the apicoplast, but rather nuclear-encoded proteins possess peptide leader sequences that target proteins to the apicoplast (reviewed (Gleeson, 2000); this makes it more difficult to determine the exact functions of the apicoplast. The discovery that herbicides were effective against some Apicomplexa led to the identification of the apicomplexan shikimate pathway, a source of amino acid synthesis. As such, the shikimate pathway is of potential significance in the discovery of novel therapeutic targets. Other pathways such as isoprenoid biosynthesis (Section 1.6.3) and fatty acid synthesis (Section 1.6.4) have been located to the apicoplast and demonstrated to be attractive chemotherapeutic targets.

### 1.6.2 Shikimate pathway

Roberts *et al.* (1998) published evidence for the presence of a shikimate pathway in Apicomplexan parasites. The shikimate pathway is conserved in plants, algae, bacteria and fungi, and is responsible for the synthesis of aromatic compounds including the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, in addition to the synthesis of ubiquinone and pABA. All of these compounds are formed from the 'branch' product of the shikimate pathway, chorismate, which is formed from the condensation of phosphoenolpyruvate and erythrose-4-phosphate (Figure 1.6). In total seven enzymes are utilised, initially forming 3-deoxy-*d*-arabinoheptulosonate-7-phosphate, which is sequentially metabolised to 3-dehydroquinate, 3-dehydroshikimate,

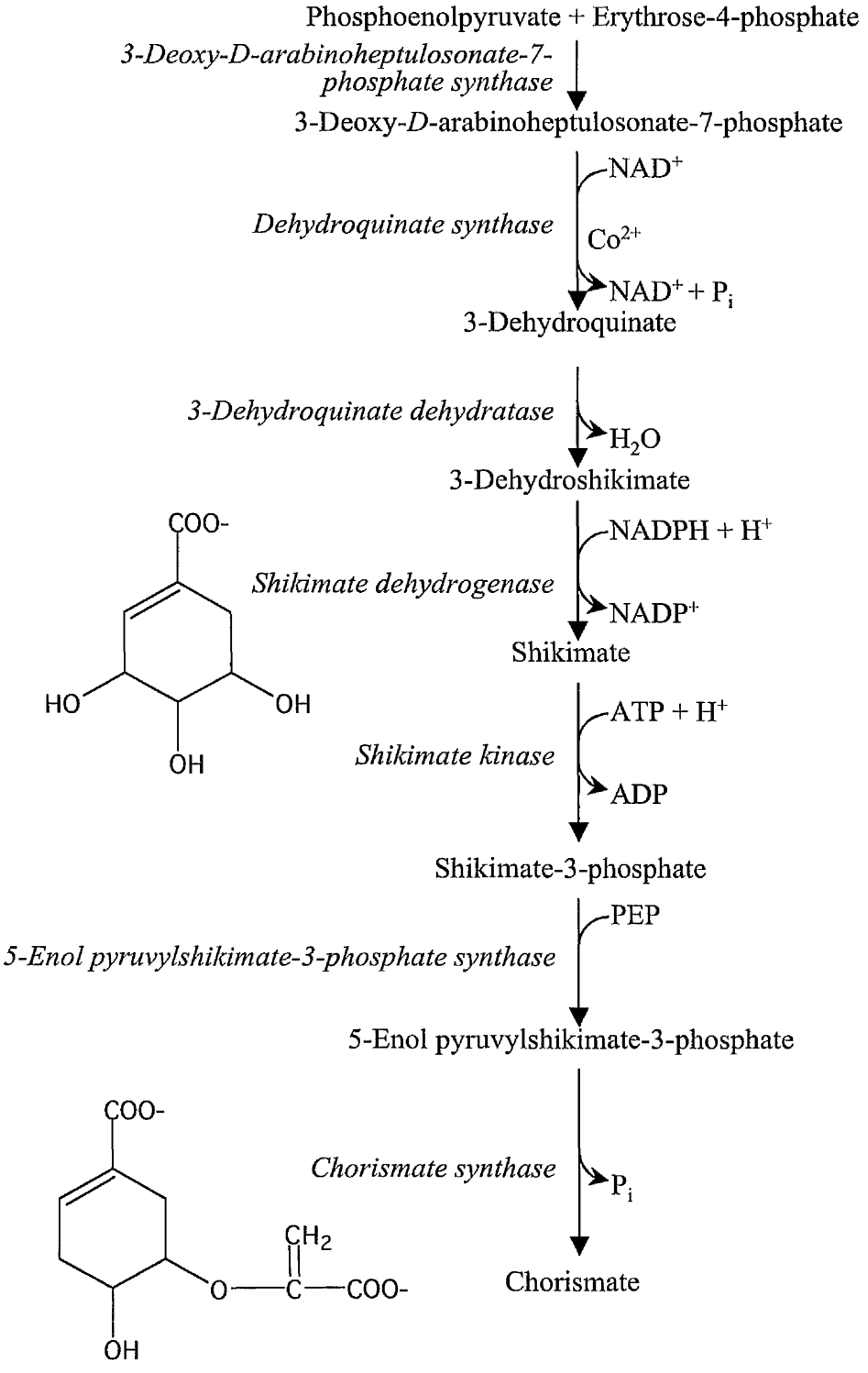



**Figure 1.6.** Shikimate pathway present in Apicomplexa. Chorismate, formed in a seven-step process from phosphoenolpyruvate and erythrose-4-phosphate, is then used in the formation of aromatic amino acids, ubiquinone and pABA.

shikimate, shikimate-3-phosphate, 5-enol pyruvylshikimate-3-phosphate and finally chorismate (Figure 1.6).

Glyphosate (*N*-(phosphonomethyl)-glycine), also known as RoundUp® or Tumbleweed®, is a selective inhibitor of 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase, was found to inhibit the growth of *T. gondii*, *P. falciparum* and *C. parvum* thereby indicating the presence of a functional shikimate pathway (Roberts *et al.*, 1998). Furthermore, addition of pABA to the medium reversed the effects of glyphosate against *T. gondii*, *P. falciparum* but not *C. parvum*. The reasons for the non-antagonistic effect of pABA against glyphosate treatment of *C. parvum* are unknown, but may be because of poor transport or the involvement of the shikimate pathway in other obligatory metabolic pathways (Roberts *et al.*, 1998).

Enzymes involved in the synthesis of chorismate are potential chemotherapeutic targets. Furthermore, mammalian cells do not have a shikimate pathway and therefore drugs should be specific to the Apicomplexa. Fluorinated analogues of shikimate, an intermediate in the shikimate pathway, have been found to inhibit the *in vitro* growth of *P. falciparum* (McConkey, 1999). Chorismate synthase, the final enzyme in the shikimate pathway, has considerable homology with chorismate synthases from several species, including *P. falciparum* (49.7 %) and *Saccharomyces cerevisiae* (49.6 %), although chorismate synthases from *T. gondii* and *P. falciparum* have unusually long insertions (Roberts *et al.*, 1998). The function of these insertions is not known, but clearly the distinct homology between the enzymes of some Apicomplexa may allow the development of common treatments against malaria, toxoplasmosis and coccidiosis. Moreover, many patients with immune deficiencies are susceptible to simultaneous infection with opportunistic pathogens, such as *Pneumocystis carnii* and

*Mycobacterium tuberculosis*, which also have shikimate pathways, thus permitting the attractive prospect of developing drugs with broad-spectrum activities.

In plants the shikimate pathway is localised in the chloroplast whilst in fungi and bacteria the enzymes are localised in the cytoplasm. Recent evidence suggests that the apicomplexan shikimate pathway is present within the cytoplasm because the amino terminal leader sequence that target nuclear-encoded proteins to the plastid, present in plants, are absent from apicomplexan chorismate synthases (Keeling *et al.*, 1999). Recently, *P. falciparum* chorismate synthase was shown to be dispersed within the parasite cytoplasm, and not located discretely within the apicoplast (Fitzpatrick *et al.*, 2001).

### 1.6.3 Isoprenoid biosynthesis

Another important function of the plastid is isoprenoid biosynthesis (Jomaa *et al.*, 1999; Vial, 2000). Isoprenoid synthesis is an important pathway in protein prenylation, ubiquinone-related electron transport systems and *N*-glycosylation of proteins. It was long assumed that Apicomplexa were unable to synthesise isopentenyl diphosphate (IPP), since they lack enzymes of the acetyl-CoA/mevalonate (MVA) pathway present in mammals (Figure 1.7). In bacteria, plants and algae, IPP synthesis is MVA-independent and occurs *via* 1-deoxy-*D*-xylulose 5-phosphate (DOXP). Condensation of pyruvate and glyceraldehyde-3-phosphate by DOXP synthase and subsequent conversion to 2-C-methyl-*D*-erythritol 4-phosphate by DOXP reductoisomerase results in the formation of IPP (Figure 1.7). Database mining of sequences on chromosome 14 of *P. falciparum* from the Malaria Genome Project, suggested the existence of DOXP

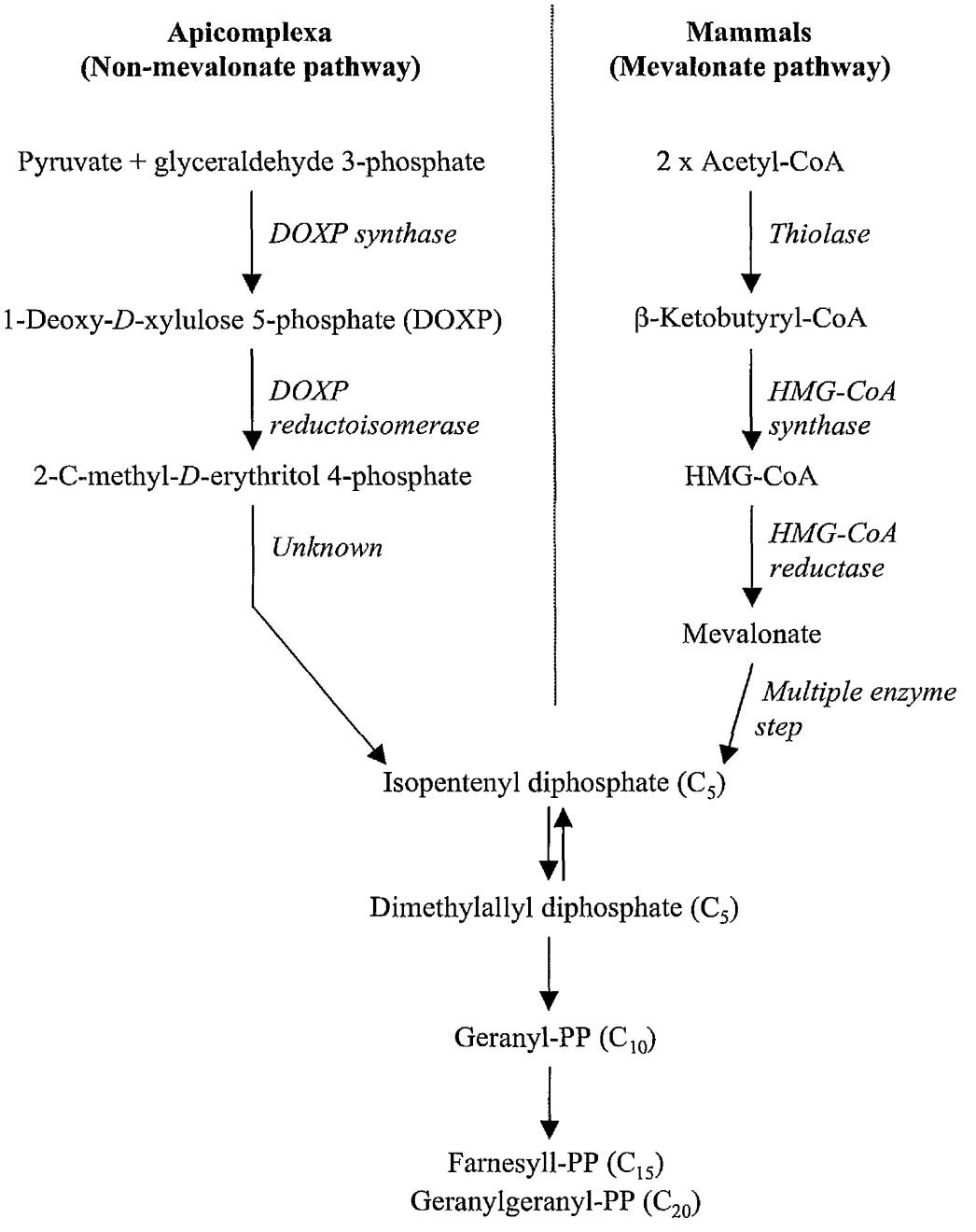



**Figure 1.7.** Apicomplexan and mammalian isoprenoid biosynthesis. In both apicomplexan and mammalian systems, isoprenoid biosynthesis occurs from isopentenyl diphosphate (IPP) and its isoform dimethylallyl diphosphate. In mammals, the condensation of two molecules of acetyl-CoA results in the formation of mevalonate, which is then converted to IPP in a three enzyme step (Vance, 1993). In contrast, apicomplexan IPP is synthesised from pyruvate and glyceraldehyde 3-phosphate (Vial, 2000). Not all of the enzymes in this pathway have been identified. Abbreviation: HMG-CoA, β-hydroxy-β-methylglutaryl-CoA; PP, pyrophosphate.

synthase and DOXP reductoisomerase homologous genes. Moreover, analysis of these sequences identified plastidial targeting sequences, and transient expression in *T. gondii* of a construct encoding the leader peptide of DOXP-reductoisomerase fused with GFP co-localised with extranuclear plastid DNA, strongly indicating the presence of a functional IPP pathway in the apicoplast (Jomaa *et al.*, 1999). In addition, *in vitro* and *in vivo P. falciparum* growth was inhibited by fosmidomycin and FR-900098 (a derivative of fosmidomycin), compounds which are known to inhibit DOXP-reductoisomerase. The presence of isoprenoid biosynthesis in *P. falciparum* suggests that apicomplexan IPP synthesis is a potential chemotherapeutic target, and since parasite and mammalian IPP synthesis pathways are divergent, these drug targets are likely to be parasite specific. It would be interesting to elucidate whether inhibitors of isoprenoid biosynthesis exhibit the same 'delayed-death' phenotype observed with other inhibitors of apicoplast function, for example ciprofloxacin.

### 1.6.4 Fatty acid biosynthesis

Fatty acid biosynthesis is important in cell growth, and since the apicomplexan fatty acid synthesis pathway (Type II) is distinct from the mammalian pathway (Type I), it is therefore an attractive parasite-specific drug target (reviewed Coombs and Müller, in press). One major difference in these pathways is that enzymes of the Type I fatty acid synthesis are present as a complex, whereas, Type II enzymes exist as mono functional proteins. Triclosan, commonly found in toothpaste and other household goods, inhibits *T. gondii* and *P. falciparum* growth by inhibiting the Fab I (enoyl-acyl carrier protein (ACP) reductase) step of fatty acid biosynthesis (Heath *et al.*, 1999; McLeod, 2001). As further evidence of the presence of Type II fatty acid biosynthesis in Apicomplexa, thiolactomycin, an inhibitor of FabB, FabF (β-ketoacyl-ACP synthase II and FabH (β-

ketoacyl-ACP synthase III) in *E. coli*, also inhibits the *in vitro* growth of *P. falciparum* (Waller *et al.*, 1998), and in addition aryloxyphenoxyproprionate and cyclohexadione herbicides inhibit *T. gondii* growth by interacting with acetyl-CoA carboxylase (ACC) (Zuther *et al.*, 1999). It would be interesting to elucidate whether or not lipids manufactured by the fatty acid synthesis pathway contribute to parasitophorous vacuole formation, and perhaps consequently whether this would be related to the 'delayed-death' phenotype described above.

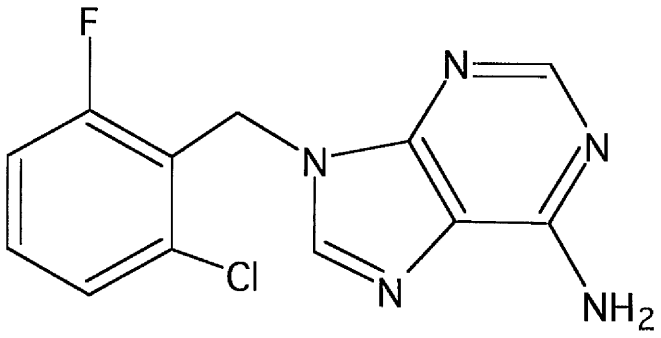
## 1.7 Arprinocid

### 1.7.1 Activity against chicken coccidiosis

A new class of chemical compounds were synthesised in the early 1970's, 6-amino-9-(substituted benzyl)-purines (Lire *et al.*, 1974). Unpublished observations identified one compound in particular, 6-amino-9-(2-chloro-6-fluorobenzyl)-purine (MK-302) later known as arprinocid (Figure 1.8a), as having anticoccidial activity (cited Miller *et al.*, 1977).

The efficacy of arprinocid was proven against *Eimeria* species infections in broiler chickens and the most effective dose determined to be 75 parts per million (ppm) (Kilgore *et al.*, 1976). The final weights of drug-treated infected chickens were significantly greater than untreated-infected control birds. Moreover, none of the medicated chickens showed any evidence of coccidiosis (Kilgore *et al.*, 1976). A similar, but larger scale, study of battery chickens infected with *Eimeria tenella* also

(a)




(b)

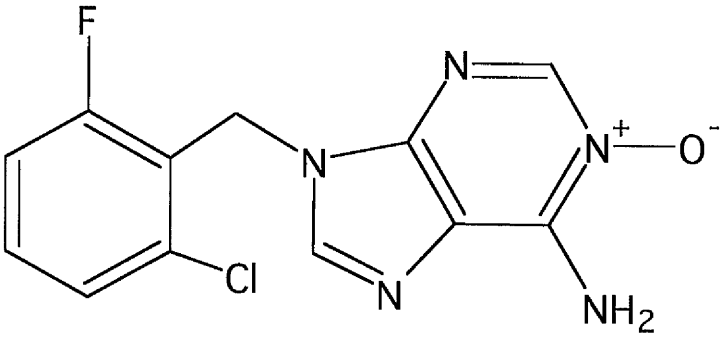



**Figure 1.8.** Structures of (a) arprinocid and (b) arprinocid-1-N-oxide.

concluded that arprinocid could exert complete control of coccidiosis (McManus *et al.*, 1976).

A structural analogue of arprinocid, 6-amino-9-(2, 6-dichlorobenzyl)-purine (L-628,914), was shown to have a similar efficacy, compared to arprinocid, against *E. tenella* infections in broiler chickens (Miller *et al.*, 1977). Both compounds reduced mortality, lesions and oocyst production resulting in an increased growth rate (Miller *et al.*, 1977). As well as decreased oocyst production, both arprinocid and L-628,914 reduced the sporulation of oocysts (Tamas *et al.*, 1978). Treatment of *E. tenella*-infected chickens with 70 ppm arprinocid completely inhibited oocyst production, and although oocyst production occurred to a limited extent with lower doses of arprinocid, sporulation was less than 5 % of controls (Tamas *et al.*, 1978). Similar observations were made with L-628,914 (Tamas *et al.*, 1978). Despite higher concentrations of arprinocid, compared to L-628,914 (45 ppm), being needed to control infections, the maximum tolerated concentration for arprinocid was 1.5 times greater than for L-628,914 (Miller *et al.*, 1977). The increased tolerance of chickens to arprinocid presumably resulted in the lack of interest of L-628,914, although other factors, such as cost of synthesis, may have been a consideration.

Results from a subsequent much larger study confirmed the potential of arprinocid as an anticoccidial drug (Olson *et al.*, 1978). Arprinocid was shown to prevent mortality, reduce lesions, inhibit oocyst production and improve weight gain, comparable to uninfected controls, in battery chickens infected with 20 field isolates of *E. tenella*, *E. acervulina*, *E. necatrix*, *E. maxima* and *E. brunetti* (Olson *et al.*, 1978). Furthermore, arprinocid showed no cross-resistance with other drug-tolerant isolates (Olson *et al.*, 1978). Seven marketed anticoccidials (amprolium and ethopabate, robenidine, clopidol,

zoalene, monensin, nicarbazine and lasalocid) were tested for comparison and all isolates, with the exception of one *E. tenella* isolate, were insensitive to one or more of the drugs tested (Olson *et al.*, 1978).

In order to confirm the potential of arprinocid as an anticoccidial drug, a large scale study was conducted to evaluate the activity of arprinocid under floor-pen conditions, thus allowing the collection of data in situations that would simulate commercial use (Kilgore *et al.*, 1978). Eight such trials of arprinocid against *Eimeria* coccidiosis in broiler chickens were performed at various locations within the United States and, in agreement with previous small-scale studies, arprinocid-treated birds (70 ppm) had significantly reduced lesions, increased feed efficiency and improved weight gain compared with control birds (Kilgore *et al.*, 1978). The same conclusions about the efficacy of arprinocid were also made subsequent to similar large-scale trials of arprinocid, conducted in France, Germany and England, against artificially induced and natural coccidial infections in broiler chickens ((Schindler *et al.*, 1979). Arprinocid controlled coccidiosis (as judged by prevention of mortality, reduced lesions and increased weight gain) and was shown to have equal, or greater efficacy, than four commercially available products; halofuginone, monensin, nicarbazine and Pancoxin® (amprolium, ethopabate and sulfaquinoxaline). In addition, unpublished results from a study with 99 isolates of *Eimeria* from 9 European countries (cited (Schindler *et al.*, 1979) and data from three trials in South Africa (Schroder *et al.*, 1980) confirmed the efficacy of arprinocid.

Heat stress in poultry is an occasional but severe problem, especially in hot climates, resulting in mortality that is often enhanced by anticoccidial drugs such as nicarbazin. Several floor-pen experiments, coinciding with hot summer temperatures, were

conducted to study the heat tolerance of a number of drugs, including arprinocid. Nicarbazin treatment of birds resulted in a heat related mortality of 59 % compared to 12 % for arprinocid and 13 % for unmedicated controls, further indicating the potential of arprinocid as a safe and effective anticoccidial drug (Keshavarz and McDouglad, 1981).

In addition to the remarkable efficacy of arprinocid in controlling chicken coccidiosis, arprinocid was found to have little toxicity (Keshavarz and McDougald, 1982). Five separate battery trials were conducted in which test drugs were used at up to three times the recommended levels. The general health of chickens was only impaired at concentrations of arprinocid 2.5 to 3 times normal dosages, although the increased mortality seen was not significantly different from control chickens (Keshavarz and McDougald, 1982). Inconsistencies in the preparation of chicken feeds may result in varying doses causing drug toxicity problems, in addition to coccidiosis. The finding that arprinocid has little toxicity at high doses is advantageous since many other compounds, such as nicarbazine, cause severe growth depression above therapeutic concentrations.

### 1.7.2 Morphological effects of arprinocid on *Eimeria*

Arprinocid was reported to have 3 distinct effects on oocysts: (i) reduction in the number of excreted oocysts; (ii) decreased sporulation; and (iii) oocysts that did sporulate were less viable (Ruff *et al.*, 1978). The ability of arprinocid to reduce sporulation appeared not to be a direct effect on oocysts themselves, since oocysts from untreated infected chickens sporulated as normal when incubated in drug-treated sporulation medium (Tamas *et al.*, 1978). Consequently, the drugs were thought to

interfere with sporulation by acting against either the micro- or macrogametes or zygotes.

Arprinocid (70 ppm) inhibited the development of first- and second-generation schizonts and altered the structure of the wall-forming bodies of macrogametes (McManus *et al.*, 1980). More detailed investigations of macrogamete structure using electron microscopy, however, concluded that there was little morphological change in type I wall forming bodies, although type II wall forming bodies became granular in appearance and ceased to develop further (Pittillo *et al.*, 1981). Normal development of second-generation schizonts results in complete merogony (Ball *et al.*, 1985). In chicks treated with arprinocid (70 ppm) two days after infection with *E. tenella*, large masses of the residual cytoplasm were found to be vacuolated and surrounded by unusually small schizonts. These effects could account for the reduction in oocyst numbers after arprinocid treatment (Ball *et al.*, 1985).

### 1.7.3 Mechanism of action of arprinocid and arprinocid-1-N-oxide

Urine taken from chickens dosed with arprinocid was analysed and a compound, 16 atomic mass units heavier than the parent drug, was identified as 6-amino-9-(2-chloro-6-fluorobenzyl) purine-1-N-oxide (arprinocid-1-N-oxide) (Jacob *et al.*, 1978) (Figure 1.8b). This urinary metabolite of arprinocid was later demonstrated, using an arprinocid-1-N-oxide resistant strain, to be the active *in vivo* metabolite of arprinocid (Pfefferkorn *et al.*, 1988). Other active metabolites have never been identified. Chicken and dog liver microsomes have been shown to convert arprinocid to arprinocid-1-N-oxide whilst mouse liver microsomes incubated with arprinocid predominantly gave 2-chloro-6-fluorobenzyl alcohol (Wolf *et al.*, 1978). Unpublished observations have

shown that 2-chloro-6-fluorobenzyl alcohol is inactive against chicken coccidiosis (cited Wang *et al.*, 1979a) (discussed further in Section 1.7.5).

Multiplication of *E. tenella* tachyzoites (trophozoites) in chickens was inhibited by arprinocid (60 ppm), even when treatment was delayed by 72 h post-infection. No change in the activity of arprinocid was observed after the addition of up to 1000 ppm adenine or adenosine to the diet, suggesting that there was no competition between drug and these substrates (Wang *et al.*, 1979a).

Arprinocid (67 μM) had no detectable effect on cow milk xanthine oxidase, chicken liver hypoxanthine-guanine phosphoribosyl transferase (HGPRT), chicken liver adenosine deaminase, pig nucleoside phosphorylase and *E. tenella* cAMP phosphodiesterase (Wang *et al.*, 1979a). Arprinocid also had no inhibitory effects on other NADH/NADPH-dependent dehydrogenases found in *E. tenella* unsporulated oocysts, for example glutamate dehydrogenase and isocitrate dehydrogenase (Wang *et al.*, 1979a). However, arprinocid did exert a potent inhibition of *E. tenella* dihydrofolate reductase ($K_i$ = 3 μM) by competing with NADPH, and inhibited glucose-6-phosphate dehydrogenase ($K_i$ = 90 μM) in *E. tenella* by competing with NADP (Wang *et al.*, 1979a).

Based on the assumption that arprinocid may inhibit *E. tenella* dihydrofolate synthesis, experiments were conducted to measure DNA synthesis in the presence of drug (Wang *et al.*, 1979a). Uptake of [$^3$H]-adenine and [$^{14}$C]-uracil by *E. tenella* schizonts was not affected by arprinocid. However, in contrast arprinocid (67 μM) resulted in a reduction in the incorporation of [$^3$H]-hypoxanthine into DNA of the host cell and parasite indicating that perhaps the mechanism of action of arprinocid was by inhibition of

hypoxanthine transport (Wang *et al.*, 1979a). Although, the activity of arprinocid and arprinocid-1-N-oxide was not affected by the presence of hypoxanthine (Pfefferkorn *et al.*, 1988).

In *E. tenella*, nucleic acid metabolism only occurs intracellularly - which poses difficulties for direct experimental analysis of isolated parasites. Therefore, further studies on the activity of arprinocid were conducted mainly using uninfected host cells. The mechanism of action of arprinocid was thought to be blockade of the hypoxanthine-guanine salvage pathway since 67 μM arprinocid inhibited hypoxanthine, guanine and inosine incorporation by 64 %, 58 % and 43 %, respectively (Wang *et al.*, 1979a). High voltage paper electrophoresis of purine nucleotides labelled with [$^3$H]-hypoxanthine demonstrated that arprinocid had no affect on any specific nucleotide (XMP, IMP, ADP, ATP, GDP or GTP) but reduced the incorporation of [$^3$H]-hypoxanthine into all nucleotides in HeLa cells (Wang *et al.*, 1979a). It is not known whether this effect is significant to the anti-coccidial activity of arprinocid. The inhibition of inosine incorporation could either be as a result of the conversion of inosine to hypoxanthine by inosine phosphorylase or perhaps there is a common receptor site (or transporter) for which hypoxanthine, inosine and guanine compete that is blocked by arprinocid. There was no change in the incorporation of radiolabelled uridine, thymidine, and adenine.

Subsequent work provided further evidence for arprinocid inhibition of hypoxanthine transport (Wang *et al.*, 1979b). Although, arprinocid (0.5 mM) had no noticeable effect on hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in HeLa cells, arprinocid acted as a potent competitive inhibitor of hypoxanthine transport ($K_i$ = 33 μM) and guanine transport ($K_i$ = 79 μM) (Wang *et al.*, 1979b). Arprinocid showed no effect against adenine transport ($K_i$ = 4.4 mM) (Wang *et al.*, 1979b) which confirmed a

previous report that adenine does not alter the efficacy of arprinocid (Wang *et al.*, 1979a). However, these effects do not necessarily correlate with activity against the parasite.

Wang and co-workers (1979 a and b) concluded that arprinocid was the active compound against coccidia based on two distinct observations. Firstly, since metabolic activity in the allantoic cavity of embryonic eggs is minimal, metabolism of arprinocid to arprinocid-1-N-oxide would be unlikely. The higher activity of arprinocid compared to arprinocid-1-N-oxide in embryonic eggs therefore suggested that arprinocid was the active form (Wang *et al.*, 1979a). Secondly, inhibition of hypoxanthine-guanine transport by arprinocid-1-N-oxide was only half that of arprinocid (Wang *et al.*, 1979b). However, Latter and Wilson (1979) postulated that arprinocid-1-N-oxide was the active compound against coccidia based on the observation that arprinocid was active in chick liver cultures but not in chick kidney cells (Latter and Wilson 1979). As mentioned above, chicken liver microsomes can metabolise arprinocid to arprinocid-1-N-oxide (Wolf *et al.*, 1978), a compound which was active in both chick kidney and liver cells (Latter and Wilson, 1979). Moreover, tissue residue levels of chicks fed arprinocid-1-N-oxide correlated with the $IC_{50}$ of arprinocid-1-N-oxide against *E. tenella* (Wang and Simashkevich, 1980). Specifically, chickens medicated with [$^{14}$C]methylene-arprinocid (70 ppm) for 22 h were found to have (i) 0.64 ppm (2.1 μM) arprinocid and 0.33 ppm (1.1 μM) arprinocid-1-N-oxide in their livers, and (ii) 0.42 ppm (1.4 μM) arprinocid and 0.3 ppm (1 μM) arprinocid-1-N-oxide in the intestines (Wang and Simashkevich, 1980). The $IC_{50}$ of arprinocid against *E. tenella in vitro* was determined as 67 μM and the $K_i$ for inhibition of hypoxanthine transport is 33 μM (see above), both values far higher than the levels of drug found with tissues of medicated chickens. In contrast, the $IC_{50}$ of

arprinocid-1-N-oxide against *E. tenella in vitro* was determined as 1 μM, a level much closer to the chicken tissue residue levels (Wang and Simashkevich, 1980). Hence, it was evident that arprinocid-1-N-oxide was in fact the active compound *in vivo*.

The activity of arprinocid or arprinocid-1-N-oxide against HeLa cells may not translate into a similar effect on the parasite and therefore HeLa cells are not a good model for studying anticoccidial activity. For example, uptake of arprinocid by HeLa cells is not altered by the presence of hypoxanthine suggesting that hypoxanthine transport is not involved in its activity (Wang & Simashkevich, 1980). In contrast, second generation schizonts of *E. tenella* grown in chick kidney epithelial cells and incubated in 67 mM arprinocid resulted in a decreased incorporation of hypoxanthine (53 %) indicating that arprinocid interferes with hypoxanthine transport (Wang & Simashkevich, 1980).

Arprinocid-1-N-oxide (67 μM) only decreased hypoxanthine incorporation in HeLa cells by 19 % which could not explain its potent inhibition of the growth of *E. tenella* (Wang & Simashkevich, 1980), suggesting that arprinocid-1-N-oxide may have an alternative mechanism of action. Wang *et al.,* (1981) presented evidence that the activity of arprinocid-1-N-oxide may be related to interference with microsomal metabolism. HeLa cells incubated with high doses of arprinocid-1-N-oxide (67 μM) resulted in the dilation of the rough endoplasmic reticulum (ER) and vacuole formation which were prevented by SKF-525A, an inhibitor of microsomal metabolism (Wang *et al.*, 1981). Furthermore, arprinocid-1-N-oxide, but not arprinocid, has been shown to bind to cytochrome P450 (Wang *et al.*, 1981). From the evidence published by Wang and co-workers (1981) it appears that arprinocid-1-N-oxide undergoes microsomal drug metabolism. However, no further metabolites of arprinocid-1-N-oxide have been

discovered and more importantly it is not known whether *T. gondii* has cytochrome P450 enzyme.

### 1.7.4 Development of resistance to arprinocid

The first published report on the development of resistance to arprinocid came two years after it was first used commercially in controlling coccidiosis in broiler chickens. In 1981, in England, after an outbreak of coccidiosis occurred, *E. tenella* was isolated and tested for sensitivity to arprinocid. There was no significant difference in weight gain, mortality, and oocysts sporulation, between arprinocid treated (60 ppm) infected chickens and unmedicated infected chickens (Chapman, 1982). Previously arprinocid had already been shown to be highly effective at 60 ppm and 70 ppm against *E. tenella* (Olson *et al.*, 1978; Schindler *et al.*, 1979). A similar experiment was repeated using a range of concentrations of arprinocid and it was concluded that this particular isolate was arprinocid resistant (Chapman, 1982). Shortly after the publication of this work a letter was published stating results that did not support the theory of a rapid onset of coccidial resistance to arprinocid, and that this drug could be highly beneficial if used in rotation with other drugs (Voeten, 1982). A more detailed study was needed.

The efficacy of arprinocid against *Eimeria* isolates obtained from areas where arprinocid had previously been used was compared to the efficacy from sites where arprinocid had not been used, and shown to be effective against *Eimeria* species only from areas where the drug had never been used. Isolates from areas where arprinocid had been used for 5-7 successive flocks were resistant (Chapman, 1983).

Chapman (1982, 1983) showed that the development of resistance to arprinocid in *Eimeria* isolates was a major problem and around this time it was withdrawn from use. However, it was not known if the resistance was stable. If this was not the case, then strategies for increasing the "life„ of a drug could be implemented, possibly by using the drug in rotation with 1 or more compounds (Chapman, 1986; Voeten, 1982). Unpublished observations indicated that it was possible to induce resistance by continuous passage in chickens medicated with increasing concentrations of drug (Chapman, 1986). Resistance to 150 ppm arprinocid was found to be stable whereas resistance to arprinocid 60 ppm (recommended level) was found to be unstable (Chapman, 1986). The question must be raised though, if resistance is unstable in the field then maybe arprinocid could be re-introduced under a new drug regime? This question has never been addressed because arprinocid was taken off the market.

### 1.7.5 Activity of arprinocid and arprinocid-1-N-oxide against *T. gondii*

Arprinocid was shown to be effective in mice infected with *T. gondii* tachyzoites (Luft, 1986) although in the light of evidence published by Latter and Wilson (1979) and Wang and Simashkevich (1980) (refer Section 1.7.3) the effects were most likely to be due to metabolism to arprinocid-1-N-oxide. Whilst mice infected with $2 \times 10^3$ RH *T. gondii* tachyzoites and treated 2 h later with 50 μg/day arprinocid survived for a significantly longer period of time compared with untreated infected mice, overall mortality was unchanged (Luft, 1986). In contrast, mice infected with $2 \times 10^6$ RH tachyzoites and treated with 1 mg/day arprinocid were completely protected, although *Toxoplasma* was found in the brain of 58% of those mice treated with 100 μg/day arprinocid (Luft, 1986). Unfortunately, Luft did not make clear what was meant by

*Toxoplasma* in the brain and whether this referred to tissue cysts. Nonetheless, arprinocid was effective against murine models of *Toxoplasma* and was demonstrated to have better efficacy than pyrimethamine (Luft, 1986).

Pfefferkorn *et al* (1988) published further evidence that arprinocid-1-N-oxide was the active metabolite of arprinocid using a drug-resistant mutant. A mutant isolate, R-ANO$^{R}$-1, resistant to arprinocid-1-N-oxide, was obtained by chemical mutagenesis using ethylnitrosourea and subsequent passage in the presence of drug (Pfefferkorn *et al.,* 1988). This resistant line was 16- to 20-fold less sensitive to arprinocid-1-N-oxide than the parental RH line, but just as sensitive to arprinocid *in vitro*. However, whilst arprinocid was effective in treating mice infected with RH *T. gondii* tachyzoites, it was not effective against toxoplasmosis in mice infected with R-ANO$^{R}$-1, suggesting that *in vivo* metabolism to arprinocid-1-N-oxide occurs and that this 1-N-oxide is the active form (Pfefferkorn *et al* 1988). Where this metabolism takes place is unknown. The most obvious place would be in the liver of the host animal, however, mouse liver microsomes incubated with arprinocid predominantly gave 2-chloro-6-fluorobenzyl alcohol, which was found to be inactive against chicken coccidiosis (Wolf *et al.*, 1978). One possibility is that the parasite, itself, may have drug-metabolising enzymes.

An *in vitro* method was developed for evaluating the effect of drugs on *T. gondii* tissue cysts and bradyzoites using a fluorescent dye that changes colour depending on the viability of the bradyzoites (discussed further in Chapter 3). Treatment of intact tissue cysts and released bradyzoites with 10 μg/ml arprinocid-1-N-oxide for 6 days resulted in deformed bradyzoites (Huskinson-Mark *et al.*, 1991). The authors did not describe this deformity. Whilst this evidence suggests that arprinocid-1-N-oxide may eradicate

the cyst stage of *Toxoplasma*, further work in animal models is essential to draw conclusions.

## 1.8 Identification of novel drug targets: a global approach

Chemotherapy is the mainstay of treatment of many animal and human diseases, including toxoplasmosis. The search for new drugs relies on the identification of novel targets, preferably essential biochemical pathways present in parasites and absent in their mammalian hosts. One recent example, is the identification of the shikimate pathway in *Plasmodium* species and *Toxoplasma gondii* as discussed above (Section 1.6.2).

The process of identification of novel biochemical pathways is rapidly changing. Biochemical techniques, such as kinetic assays of recombinant enzymes or HPLC analysis of enzyme activity, are directed at characterising parasite enzymes or proteins that differ between parasites and mammalian cells. In addition to these techniques, recent advances in bioinformatics and reverse genetics (Soête *et al.*, 1999), together with high-throughput differential gene analysis (Humphery-Smith and Blackstock, 1999), have made possible a rational approach to the identification of drug targets.

### 1.8.1 Gene sequencing

The expansion of genome databases, such as the Malaria Sequencing Project (Anonymous, 2001), and expansion of expressed sequence tag (EST) databases, particularly for *T. gondii* tachyzoite and bradyzoites genes (Ajioka, 1998a; Manger *et al.*, 1998), have enhanced our ability to discover previously unidentified genes. In

particular, the identification of genes that are conserved between Apicomplexa not only suggests the presence of common pathways, but allows the prospect of designing broad-range chemotherapeutic agents (Ajioka *et al.*, 1998b). At present there are over 16, 000 *T. gondii* ESTs available (http://www.ebi.ac.uk/parasites/paratable.html), from both tachyzoite and bradyzoite stages, therefore providing valuable information on genes expressed during stage transition. For example, searching the *T. gondii* EST database allowed the discovery of some microneme proteins based on homology to *P. falciparum* TRAP (reviewed Tomley and Soldati, 2001). However, whilst mining EST databases may prove fruitful, it has the disadvantage that many essential genes may be under-represented.

### 1.8.2 Reverse genetics

The high frequency of stable transformation coupled with the haploid genome of *T. gondii* tachyzoites makes this parasite a suitable model for investigation of gene function by reverse genetics (Roos *et al.*, 1994). The feasibility of creating mutant *T. gondii* lines with specific gene deletions, modified genes or expressing additional genes, permits the exploration of many important biological questions. For example, questions related to protein trafficking, host cell invasion and drug resistance. The *T. gondii* hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT) and uracil phosphoribosyl transferase genes were cloned (Donald *et al.*, 1996; Donald and Roos, 1995, respectively). Both enzymes are parasite-specific and were therefore thought to be good candidates for drug targeting. However, null mutants were found to be viable (Donald *et al.*, 1996; Donald and Roos, 1995). The use of HXGPRT vectors for targeting specific genes whose function is unknown has also been demonstrated, by using mycophenolic acid or 6-thioxanthine as positive or negative selection,

respectively (Donald and Roos, 1998). Other *T. gondii* genes have been deleted, including GRA2 and SAG3 which resulted in decreased virulence in mice (Mercier *et al.*, 1998; Dzierszinski *et al.*, 2000, respectively), and the gene encoding for the BAG1 bradyzoite antigen which resulted in decreased *in vivo* cyst formation (Zhang *et al.*, 1999).

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, may be expressed as a fusion protein in *T. gondii*, thereby allowing the visualisation of protein trafficking. For example, secretion of surface antigens, and real time studies of protein movement have been investigated (Striepen *et al.*, 1998, 2001; Kim *et al.*, 2001). Extracellular tachyzoites or infected host cells expressing GFP fusion proteins can be readily be sorted by FACS, facilitating a convenient selection strategy (Striepen *et al.*, 1998). Moreover, targeting studies in deletion mutants allows the elucidation of those genes essential for parasite survival (Striepen *et al.*, 2001).

Although the list of available reverse genetics techniques for *T. gondii* is growing, currently there is no system that allows inducible control of gene expression, thus limiting the study of essential genes (Soldati, 1996). An inducible expression system has been developed for the protozoan parasite, *Trypanosoma brucei*, utilising the tetracycline repressor system of *Escherichia coli* (Wirtz and Christine, 1995), but this system in not available for *T. gondii* yet. The hormonally regulated Cre-lox system, which has been used successfully in mammalian cells for inducible gene targeting, has been stable expressed in *T. gondii* tachyzoites (Brecht *et al.*, 1999). However, the fusion between Cre recombinase and a mutated progesterone receptor binding domain (CrePR1) was active even in the absence of hormone, thus preventing this as a system for gene regulation in *T. gondii*.

### 1.8.3 Differential gene expression

Whilst molecular biology and reverse genetics techniques can be successful in identifying and validating gene targets, they usually allow for only one gene at a time to be analysed. The wealth of information that has become available recently due to sequencing projects, such as the *P. falciparum* sequencing project, renders these techniques insufficient to mine this wealth of information optimally.

Methodologies for the quantification of mRNA using hybridisation techniques such as Northern Blotting or by reverse transcription polymerase chain reaction (RT-PCR) are also limited to just a few genes per assay. However, developments in PCR technology have led to quantification of mRNA on a larger scale. Differential display-PCR is a relatively simple and inexpensive technique for identifying differentially expressed genes and potential drug targets. However, the large number of false positives/negatives that are obtained can reduce its efficiency. Serial analysis of gene expression (SAGE) tends to be less error prone but requires large amounts of high quality starting material. This technique has been proven effective in studies of gene expression in normal versus cancer cells (Zhang *et al.*, 1997).

In the last decade, the development of high-throughput methods based on gene-chip technologies has allowed many thousands of genes to be analysed simultaneously. DNA array hybridisation allows the simultaneous analysis of tens of thousands of genes. Individual cDNA (or oligonucleotide) sequences are imprinted as spots onto a solid support, typically a nylon membrane or glass slide. After hybridisation of labelled mRNA to the array, the intensity of the label associated with each spot directly

correlates with the level of expression of that mRNA. Double labelling experiments permit the identification of differentially expressed mRNA sequences.

Genetic techniques, such as insertional mutagenesis and gene deletion, are not however redundant, neither are the classical biochemical analyses. Instead, these approaches are likely to be more directed to genes/proteins of interest that were identified by chip-based technologies. The cost of array-based technology has been beyond the finance of many research groups but hopefully this will change. The technology is exciting and, when more readily available, will provide rapid analysis of parasite gene expression.

Analysis of the protein expression by two-dimensional electrophoresis (2-DE) will complement these other approaches. Recent advances enabling reproducible separation of proteins (Chapter 4) allows not only global analysis of gene products expressed by an organism (or tissue), but also gives detailed information about co- and post-translational modifications, such as phosphorylation and glycosylation. These changes, not detectable at the mRNA level, are of tremendous importance in understanding biochemical processes within a cell. Furthermore, proteins of interest may be identified by using mass spectrometry data to search protein or genome databases (Chapter 5). This, therefore, allows the validation of an increased/decreased expression of a gene at the protein level. Moreover, comparison of protein expression changes in healthy versus diseased cells can give vital information about the onset of disease. The applications of these techniques are wide and have particular benefits for investigation of the mechanism of action of drugs and drug-resistance.

There is no doubt that classical biochemical and genetic techniques have added a tremendous wealth of knowledge to the study of Apicomplexa. Developing global

techniques aimed at analysing many hundreds or thousands of genes/proteins simultaneously are also powerful techniques, but their full potential is yet to be realised. The question is not which techniques will take over, but, rather, how will we be able to integrate the techniques in a more rational approach to the more rapid identification and validation of drug targets.

## 1.9 Aims of project

The overall focus of this study was to provide information on the action against *Toxoplasma gondii* of arprinocid and arprinocid-1-N-oxide. The availability of drug-sensitive and -resistant lines facilitated the investigation and was a central theme to the thesis. The following were the main approaches and objectives of this thesis.

1) Efficacy of arprinocid and arprinocid-1-N-oxide

   a) To examine the sensitivity of *T. gondii* tachyzoites to arprinocid and arprinocid-1-N-oxide and investigate the sensitivity of two mutant *T. gondii* lines resistant to arprinocid-1-N-oxide.

   b) To investigate whether the effects of arprinocid and arprinocid-1-N-oxide against *T. gondii* were directed specifically towards the tachyzoites or dependant on disruption of normal host cell metabolism.

   c) To investigate whether or not arprinocid and arprinocid-1-N-oxide interfere with the HXGPRT-mediated salvage of purines.

   d) To analyse the relative efficacy of arprinocid and arprinocid-1-N-oxide against *T. gondii* using different dose regimens.

2) Two-dimensional electrophoresis (2-DE) analysis and mass spectrometric identification of *T. gondii* tachyzoite proteins.

a) To develop the tools necessary for the reproducible, high-resolution separation of *T. gondii* tachyzoite proteins by 2-DE, and apply these techniques to investigate acquisition of drug-resistance.

b) To create a preliminary 2-D gel map of *T. gondii* tachyzoite proteins using MALDI mass spectrometry in combination with protein and EST database searching.

c) To use mass spectrometry techniques to identify a protein differentially expressed between the drug-sensitive and -resistant parasites.

3) Purine transport in *T. gondii* tachyzoites

a) To characterise nucleoside/nucleobase transporters in *T. gondii* as likely conduits for the transport or targets of arprinocid and arprinocid-1-N-oxide.

# CHAPTER 2

## General Materials and Methods

The purpose of this Chapter is to describe general materials and methods including parasite maintenance, protein estimation, preparation of protein and DNA, and statistical analysis. Detailed methods for *in vitro* drug assays, two-dimensional electrophoresis of *T. gondii* proteins, mass spectrometric identification of *T. gondii* proteins, and membrane transport, are described in Chapters 3, 4, 5 and 6, respectively.

## 2.1 Materials

### 2.1.1 *Toxoplasma gondii* tachyzoites

The following *Toxoplasma gondii* isolates were kindly supplied by E.R. Pfefferkorn (Dartmouth College, New Hampshire, USA): (i) a cloned parental line "pRH", (ii) an arprinocid-1-N-oxide-resistant mutant "$ARP^R$ 5-86" derived from pRH (Pfefferkorn *et al.*, 1988) and (iii) an arprinocid-1-N-oxide-resistant mutant "$ANO^R$", also derived from pRH (Rickets Pfefferkorn, 1993). For convenience $ARP^R$ 5-86 *T. gondii* will be referred to as $ARP^R$. The following *T. gondii* strains were also used: (iv) *T. gondii* strain, RH (Sabin, 1941), (v) incomplete vaccine line, S48 (Wilkins *et al.*, 1987) and (vi) a cloned line of RH lacking the hypoxanthine-xanthine-guanine-phosphoribosyl-

transferase (HXGPRT) gene (Donald *et al.*, 1996). A summary of the above *T. gondii* lines is given in Table 2.1.1.

**Table 2.1.1.** Description of *Toxoplasma gondii* lines available for analysis.

| *T. gondii* strain | Description | Cloned | Reference |
|---|---|---|---|
| pRH | Parental RH line of $ARP^R$ and $ANO^R$ | Yes | Pfefferkorn *et al.*, 1988 |
| $ARP^R$ | Arpinocid-1-N-oxide-resistant mutant | Yes | Pfefferkorn *et al.*, 1988 |
| $ANO^R$ | Arpinocid-1-N-oxide-resistant mutant | No | Ricketts and Pfefferkorn, 1993 |
| S48 | Vaccine strain | No | Wilkins *et al.*, 1987 |
| RH | Virulent strain | No | Sabin, 1941 |
| $RH\text{-}HX^-$ | HXGPRT knockout mutant derived from RH | Yes | Donald *et al.*, 1996 |

### 2.1.2 Culture medium and solutions

(i) Iscove's Modified Dulbecco's Medium (Life Technologies) used for cell culture was supplemented with 5 % (v/v) foetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. Culture medium was filter sterilised using a 0.2 μm membrane pore-size Sartolab® V500 filter (Sartorius, Surrey, UK) connected to a diaphragm-based vacuum pump. Unless stated otherwise, the abbreviation IMDM refers to filter sterilised supplemented medium containing FCS and antibiotics.

(ii) HEPES buffered balanced salt solution for washing cell monolayers prior to trypsinisation was made as follows: 140 mM sodium chloride, 5 mM potassium chloride, 5 mM *D*-glucose, 10 mM HEPES and 0.001 % phenol red. The buffer was adjusted to pH 7.5 using 5 M sodium hydroxide and filter sterilised as described above.

(iii) Suspension medium for the cryopreservation of *T. gondii* tachyzoites and host cells consisted of Iscove's Modified Dulbecco's Medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate and 12.5 % (v/v) DMSO. Suspension medium was filter sterilised using syringe filters.

All solutions were stored at 4°C for a maximum of one month.

### 2.1.3 Drugs and chemicals

Arprinocid (6-amino-9-(2-chloro-6-fluorobenzyl)-purine), arprinocid-1-N-oxide (6-amino-9-(2-chloro-6-fluorobenzyl) purine-1-N-oxide), and adenosine-N-oxide were kindly donated by Pfizer Ltd, Kent, UK. They were supplied in powder form and dissolved in 100 % dimethysulphoxide (DMSO). Aliquots at various concentrations were stored at -20°C. For *in vitro* experiments using compounds dissolved in DMSO, drugs were diluted to a final concentration of 1 % DMSO, which had no noticeable effect on Vero cells or *T. gondii* tachyzoites as judged by visual analysis of control cultures incubated in 1 % DMSO.

Laboratory chemicals used were the best available. Chemicals and their suppliers are listed in Table 2.1.2. All buffers and reagents were made using double-distilled-deionised water (ddd$H_2O$), except those used for two-dimensional electrophoresis which were made using 18 Megohm (18 MΩ) ultrapure water (Millipore, UK).

**Table 2.1.2** List of chemicals.

| | |
|---|---|
| Acetonitrile | Sigma, Dorset, UK |
| Acetic acid (glacial) | BDH, Poole, UK |
| Acrylamide PAGE solution, 40 % (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Adenine | Sigma, Dorset, UK |
| [2, 8-$^{3}$H]-Adenine (32.2. Ci/mmol) | NEN, Hounslow, UK |
| Adenosine | Sigma, Dorset, UK |
| [2-$^{3}$H]-Adenosine (25 Ci/mmol) | Amersham Pharmacia Biotech, Bucks, UK |
| Adenosine-N-oxide | Pfizer Ltd, Kent, UK |
| Agarose, Seakem LE | BMA, ME, USA |
| Ammonium bicarbonate | BDH, Poole, UK |
| Ammonium persulphate (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Angiotensin I | Sigma, Dorset, UK |
| Arprinocid (6-amino-9-(2-chloro-6-fluorobenzyl)-purine) | Pfizer Ltd, Kent, UK |
| Arprinocid-1-N-oxide (6-amino-9-(2-chloro-6-fluorobenzyl) purine-1-N-oxide) | Pfizer Ltd, Kent, UK |
| n-Butanol | BDH, Poole, UK |
| di-n-butylphthalate | BDH, Poole, UK |
| Calcium chloride | BDH, Poole, UK |
| CHAPS (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Coomassie Brilliant Blue R250 | Sigma, Dorset, UK |
| α-Cyano-4-hydroxycinnamic acid | Aldrich, Dorset, UK |
| Dilazep | Sigma, Dorset, UK |
| Dimethylsulphoxide | Sigma, Dorset, UK |
| Dipyridamole | Sigma, Dorset, UK |
| Dithiothreitol (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Ethanol (Anal$^{R}$) | Bamford Laboratories, Rochdale, UK |
| Ethylenediaminetetraacetic acid (EDTA) | Fisher Scientific, Leics, UK |
| Foetal Calf Serum | Labtech, Sussex, UK |
| Formaldehyde | BDH, Poole, UK |
| *D*-Glucose | Fisher Scientific, Leics, UK |
| Glutaraldehyde | BDH, Poole, UK |
| Glycerol, 87 % (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Glycine (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |

**Table 2.1.2. (continued)**

| | |
|---|---|
| HEPES | Fisher Scientific, Leics, UK |
| [8-$^{3}$H]-Hypoxanthine (32.0 Ci/mmol) | Amersham Pharmacia Biotech, Bucks, UK |
| Iscove's modified Dulbeccos medium | Life Technologies, Paisley, UK |
| Immobiline drystrip cover fluid (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Immobiline pH gradient buffers (3-10NL, 4-7L, 6-11L and 5.5-6.7L) | Amersham Pharmacia Biotech, Bucks, UK |
| Inosine | Sigma, Dorset, UK |
| [2, 8-$^{3}$H]-inosine (33.4 Ci/mmol) | Moravek Biochemicals, Ca, USA |
| ∝-Iodoacetamide | Calbiochem, Germany |
| Magnesium chloride | Riedel de Haën, Germany |
| Magnesium sulphate | Sigma, Dorset, UK |
| Methanol (Anal$^{R}$) | BDH, Poole, UK |
| N-N'-Methylene-bisacrylamide solution, 2 % (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Mineral oil (light) | Sigma, Dorset, UK |
| Monensin | Sigma, Dorset, UK |
| MOPS (USB) | Amersham Pharmacia Biotech, Bucks, UK |
| Papaverine | Sigma, Dorset, UK |
| Penicillin G sodium / streptomycin sulphate (100 U/ml / 100 μg/ml) | Life Technologies, Paisley, UK |
| Phenol red | BDH, Poole, UK |
| Phosphate Buffered Saline (tablets) | Sigma, Dorset, UK |
| Potassium carbonate | BDH, Poole, UK |
| Potassium chloride | BDH, Poole, UK |
| Potassium tetrathionate | Sigma, Dorset, UK |
| Scintillation fluid (Ecoscint A) | National Diagnostics, Hull, UK |
| Silver nitrate | Sigma, Dorset, UK |
| Silver stain kit, protein (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Silver stain kit, protein | Genomic Solutions |
| Sodium acetate | Sigma, Dorset, UK |
| Sodium chloride | Riedel de Haën, Germany |
| Sodium dihydrogen phosphate | BDH, Poole, UK |
| Sodium dodecyl sulphate (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Sodium hydrogen carbonate | Riedel de Haën, Germany |
| Sodium hydroxide | Riedel de Haën, Germany |
| Sodium thiosulphate | Sigma, Dorset, UK |

**Table 2.1.2. (continued)**

| | |
|---|---|
| [methyl-$^3$H]-Thymidine (6.7 Ci/mmol) | NEN, Hounslow, UK |
| TEMED (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Tris base (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Triton X-100 | Sigma, Dorset, UK |
| Trypsin-EDTA (1x) | Life Technologies, Paisley, UK |
| Trichloracetic acid | BDH, Poole, UK |
| Trifluoracetic acid | Sigma, Dorset, UK |
| Trypsin, sequence grade (modified) | Promega, Southampton, UK |
| Uracil | Sigma, Dorset, UK |
| [5, 6-$^3$H]-Uracil (45.0 Ci/mmol) | Amersham Pharmacia Biotech, Bucks, UK |
| Urea (PlusOne™) | Amersham Pharmacia Biotech, Bucks, UK |
| Versine | Media Services, University of Glasgow, UK |

## 2.2 Methods

### 2.2.1 Culture of Vero cells and *Toxoplasma gondii*

*T. gondii* tachyzoites were maintained by twice-weekly serial passage using African Green Monkey kidney fibroblasts (Vero cells) as host cells. Cells and parasites were grown in filter sterilised IMDM in 25 $cm^3$ (T25) or 75 $cm^3$ (T75) vented tissue culture flasks (Greiner, Germany) at 37°C in a 5 % $CO_2$ humidified incubator.

Confluent uninfected host cells were detached from the surface of culture flasks by washing the cell monolayer twice in HEPES saline for 1 min (5 ml for a T25, 10 ml for a T75) and incubating the cells in versine : trypsin (4 : 1) for approximately 5 min at 37°C. Gentle tapping of the flask aided the removal of cells from the flask surface. Cells were centrifuged at 1500 x g for 5 min at 4°C and resuspended at a density of 2 x $10^6$ cells/ml IMDM. Cells were re-seeded at 1 x $10^5$ (total) in 5 ml IMDM for a T25 flask and 4 x $10^5$ (total) in 15 ml medium for a T75 flask. Vero cells were infected 24 h later at a parasite-to-cell ratio of 3:1 and 4:1 for a T25 and T75, respectively. Tachyzoites were harvested 3 or 4 days later using a sterile cell scraper (Greiner). Cells and parasites were counted using an improved Neubauer hemacytometer (Weber Scientific Ltd, UK).

### 2.2.2 Preparation of *T. gondii* tachyzoites for protein or DNA extraction

Tachyzoites were separated from host cells by filtration through 47 mm diameter 3 μm pore-size Nuclepore® polycarbonate filters (Whatman, UK). Filtered tachyzoites,

typically containing less than 0.1 % host cell contamination as judged by microscopic observation, were washed twice in PBS (pH 7.4) by centrifugation at 1500 × g for 20 min at 4°C. Tachyzoites were given a final wash in PBS (pH 7.4) and pelleted by centrifugation at 16,000 x g for 3 min at 4°C.

### 2.2.3 Protein estimation (BCA assay)

Protein content estimations were performed using a BCA Protein Assay kit (Pierce Ltd, UK). Working Reagent was prepared by combining 50 parts Reagent A with 1 part Reagent B. Bovine Serum Albumin protein standards (10 µl) were made by serial dilutions from a stock of 2 mg/ml. Each standard, blank or unknown sample was prepared in triplicate and pipetted into the appropriate microtitre plate wells. Working Reagent (200 µl) was added to each of the wells and the samples mixed on a shaker for 1 min. Samples were incubated at 37°C for 1 h and absorbances read at 560 nm in a Titertek Multiskan® MCC/340 microtitre plate reader (Labsystems & Flow Laboratories, Finland). A standard curve of blank-corrected absorbances of known standards versus protein concentration was plotted and used to calculate unknown protein concentrations.

### 2.2.4 Preparation of genomic DNA from *T. gondii* tachyzoites

DNA from *T. gondii* parasites was extracted from a minimum of 2 x $10^7$ filter-purified and washed tachyzoites (as described in paragraph 2.2.2) using the GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech, UK). Parasites were resuspended in a final volume of 40 µl PBS (pH 7.4) and gently mixed with 600 µl Cell

Lysis Buffer. RNAse A (3 µl) was added and mixed by inverting 25 times. Samples were incubated at 37°C for 1 h, cooled to room temperature and vortexed vigorously for 20 s in the presence of 200 µl Protein Precipitation Solution. Following centrifugation at 16,000 x g for 3 min, the supernatant (containing the DNA) was decanted into a clean 1.5 ml capped tube. Isopropanol (600 µl) was added and mixed by inverting 50 times before being centrifuged at 16000 x g for 1 min. The sedimented DNA was washed in 70 % ethanol, air dried and dissolved in 100 µl water.

### 2.2.5 Cloning and sequencing of gene fragments

DNA was amplified using the polymerase chain reaction (PCR) on a Dyad™ DNA Engine (MJ Research Inc, USA). DNA samples were separated by electrophoresis in a 1 % agarose gel using 1x TBE buffer containing 0.5 µl/ml ethidium bromide and visualised on a UV transilluminator. These techniques were performed by Ms Janice Brock. Sequencing was performed directly on the PCR fragment at the Molecular Biology Service Unit at Glasgow University.

### 2.2.6 Cryopreservation of *T. gondii* tachyzoites and Vero cells

*T. gondii* tachyzoites and Vero cells were stored in liquid nitrogen using dimethysulphoxide (DMSO) as a cryoprotectant. Freshly harvested tachyzoites were washed twice in IMDM containing 10 % (v/v) FCS by centrifugation at 1500 x g for 10 min at 4°C and resuspended in suspension medium at a density of 1 x $10^8$/ml. Detached Vero cells were washed as above and resuspended in suspension medium at a density of 1 x $10^6$/ml. Aliquots (1 ml) were pipetted into cryotubes, which were placed in a

polystyrene box insulated with cotton wool and cooled slowly to -80°C. Vials were catalogued and immersed in liquid nitrogen for long-term storage.

Cryopreserved tachyzoites were removed from liquid nitrogen and thawed rapidly in a clenched fist. One vial of tachyzoites was used to infect two T25 flasks of Vero cells. Parasite growth was monitored daily and cells passaged as required until the normal 3-4 day cycle of invasion of host cells, replication and lysis occurred.

### 2.2.7 Statistical analysis.

Equations and methods for statistical analysis were obtained from Fowler and Cohen (1992).

#### *2.2.7.1 Measurement of variance*

Statistical variance ($s^2$) was calculated according to the formula below

$$s^2 = \frac{\sum (x - \bar{x})^2}{n - 1}$$

where $\sum$ = the sum of, $x$ = data value, $\bar{x}$ = mean of $x$, and $n$ = number of samples. Standard deviation (s) was calculated by taking the square root of variance.

#### *2.2.7.2 F-test analysis*

Prior to performing a Student's *t*-test for significance in difference between means of two samples, data were analysed to test for equal or unequal variance. A two-tailed F-

test was performed using the following equation, where the degrees of freedom are ($n_1$ - 1) and ($n_2$ - 1) for samples 1 and 2, respectively.

$$F = \frac{\text{greater variance}}{\text{lesser variance}}$$

*2.2.7.3 Student's t-Test for comparing the means of samples*

Comparison of the means of two small samples (under 30 observations) was performed using a two-tailed *t*-test (unequal or equal variance depending on result from F-test) in which the mean difference between two samples was divided by the standard error of the difference. Calculated *t* values were compared with a standard table of distribution of *t* under the appropriate degrees of freedom. The equation below, where degrees of freedom = ($n_1$ + $n_2$)- 2, was used to calculate *t* manually. *T* values were checked using Microsoft Excel 97. P values of ≤ 0.05 were considered significant.

$$t = \frac{(\bar{x_1} - \bar{x_2})}{\sqrt{\left(\frac{(n_1 - 1)\cdot s_1^{\,2} + (n_2 - 1)\cdot s_2^{\,2}}{(n_1 + n_2 - 2)}\right)\cdot\left(\frac{n_1 + n_2}{n_1 \cdot n_2}\right)}}$$

*2.2.7.4 Student's t-Test for comparing the means of paired samples*

Analysis of the means of matched pairs was performed using a paired two-tailed *t*-test. The difference between matched pairs was determined as '*d*.' The equation is given below, where n is the number of matched pairs and (n - 1) is the degrees of freedom.

$$t = \frac{\sum d}{\sqrt{\left[\left(n \cdot \sum d^2 - (\sum d)^2\right)/(n-1)\right]}}$$

# CHAPTER 3

## Evaluation of the *In Vitro* Effects of Arprinocid and Arprinocid-1-N-oxide on *T. gondii* Growth and Survival

### 3.1 Introduction

Understanding the mode of action of drugs and the mechanism of acquisition of drug-resistance is crucial to the development of new anti-coccidials with increased potency and specificity (Luft and Remington, 1992). *In vitro* investigations into the efficacy of novel therapeutic compounds is an important step in designing improved drugs and combination therapies. In addition, the testing of chemical analogues allows the identification of chemical structures that have increased affinity and greater specificity for parasites, thus enabling rational drug design. *In vitro* investigations are invaluable studies and are comparatively inexpensive for high-throughput screening of large numbers of compounds.

For some coccidia, including *Eimeria spp* and *Cryptosporidium*, the comparative difficulty in growing life stages *in vitro* makes the assessment of anti-coccidial agents problematic. By contrast, measurement of the incorporation of $^{3}$H-uracil, which specifically labels intracellular *T. gondii* parasites, has been successfully used to analyse quantitatively the *in vitro* growth of tachyzoites (Mack and McLeod, 1984; Pfefferkorn and Pfefferkorn, 1977a). Although this technique may also be used to measure the

growth of *Eimeria tenella* first generation schizonts, $^{3}$H-uracil is not incorporated into mature schizonts and merozoites, possibly due to reduced RNA synthesis in these later stages (Schmatz *et al.*, 1986). Furthermore, the asynchronous growth of *Eimeria* parasites makes interpretation of the results difficult. The *in vitro* assessment of drugs against *Cryptosporidium parvum* has been performed semi-quantitatively either by manually counting parasite life stages (Armson *et al.*, 1999; Theodos *et al.*, 1998) or by using monoclonal antibodies specific to intracellular stages of *C. parvum* and immunofluorescence microscopy (Perkins *et al.*, 1998). However, manual counting of parasites is tedious and not suitable for high-throughput analysis, although it should be mentioned that microscopic analysis is invaluable in interpreting data from quantitative assays. In contrast, *T. gondii* tachyzoites can be grown in tissue culture allowing quantitative *in vitro* drug-screen assays to be performed with relative ease and therefore is an excellent model for testing potential anticoccidial agents *in vitro*. Likewise, analysis of compounds against *T. gondii* tissue cysts *in vitro* is possible. Tissue cysts may be obtained by (i) extraction from brains of infected mice (Huskinson-Mark *et al.*, 1991); (ii) infection of murine brain cells/human fibroblasts with bradyzoites obtained from brain cysts (Ricard *et al.*, 1999a, b; Sahm *et al.*, 1997); or (iii) decoquinate-induction of tachyzoite infected fibroblasts (Lindsay *et al.*, 1998). The measurement of drug-activity against tissue cysts may be performed by using a fluorescent stain (acridine orange-ethidium bromide) which changes colour depending on the viability of the cyst (Huskinson-Mark *et al.*, 1991). Results may be confirmed by inoculating mice with the drug-treated tissue cysts and monitoring time to death (Araujo *et al.*, 1991).

Several methods of quantitatively measuring the efficacy of anti-*Toxoplasma* drugs have been developed. In addition to the $^{3}$H-uracil assay, enzyme-linked-immuno-

sorbent-assays (ELISA) have been performed to test for inhibitors of *T. gondii* and *Neospora caninum* growth (Derouin and Chastang, 1988; Lindsay *et al.*, 1994). Recently, a microtitre colorimetric assay has been developed using a strain of *T. gondii* expressing bacterial β-galactosidase providing a non-radioactive alternative to the $^{3}$H-uracil incorporation assay (McFadden *et al.*, 1997). An alternative and more sophisticated method, allows the measurement of the percentage of infective cells and number of intracellular tachyzoites by flow cytometry using a monoclonal antibody against cell surface antigen, for example *T. gondii* SAG1 (Gay-Andrieu *et al.*, 1999). Recently a flow cytometry approach has been applied to *C. parvum* sporozoite viability (Giacometti *et al.*, 2000). Whilst these techniques avoid the use of radiolabelled precursors and can be readily automated, ELISA-based screens require additional experiments to determine the effect of compounds on host cells and flow cytometric quantification methods require the use of expensive FACScan equipment which is not available to many researchers.

An understanding of the biological and biochemical differences between parasites and their hosts is not only of great importance in the development of new antiparasitic drugs, but can also be exploited in the design of drug screen assays. For example, as mentioned above, the measurement of the incorporation of $^{3}$H-uracil that specifically labels intracellular *T. gondii* and not extracellular tachyzoites or host cells. This assay was discovered accidentally during a series of investigations into resistance to 5-fluorodeoxyuridine (Pfefferkorn, 1978; Pfefferkorn and Pfefferkorn, 1977b). In the late 1970's, subsequent to the study of an enzyme defect in a mutant *T. gondii* strain resistant to 5-fluorodeoxyuridine, it was proposed that deoxyuridine was incorporated by *T. gondii* (Pfefferkorn and Pfefferkorn, 1977b). In a subsequent study, it was discovered

that the mutant strain was unable to incorporate radiolabelled uracil, deoxyuridine, or uridine and consequently it was thought that uridine kinase, an enzyme responsible for the conversion of uridine to UMP, was absent. However, uridine kinase activity could not be detected in wild-type parasites. Thus, the previously published salvage pathway was modified (Figure 3.1) (Pfefferkorn, 1978). Uptake of $^{3}$H-uracil by intracellular *T. gondii* can only occur after permeating the host outer membrane and therefore uracil must be available to the host cell. Since uracil is not utilised by the host cell, a parasite-specific salvage enzyme was thought to be responsible. Further work concluded that a 40-fold higher activity of uridine phosphorylase in infected cells compared to uninfected cells was responsible for the conversion of uridine to uracil. Autoradiographic analysis of the uptake of $^{3}$H-uracil indicated that nearly all the radiolabel was associated with intracellular tachyzoites and that there was no reverse flow into the host cell. Once the intracellular parasite has incorporated uracil it is converted to UMP by uracil phosphoribosyl transferase, an enzyme absent in mammalian cells. $^{3}$H-uracil is specifically incorporated into *T. gondii* nucleic acids in proportion to the number of parasites and therefore can be used as a measure of tachyzoite growth (Pfefferkorn and Pfefferkorn, 1977a). The $^{3}$H-uracil assay has been used in many studies to assess the efficacy of anti-*Toxoplasma* compounds. For example, rifapentine (Araujo *et al.*, 1996), naphthoquinones analogues (Khan *et al.*, 1998) and synercid (Khan *et al.*, 1999a). It is used in this chapter as the principle tool to evaluate the *in vitro* effects of arprinocid and, its *in vivo* metabolite, arprinocid-1-N-oxide on *T. gondii* tachyzoites.

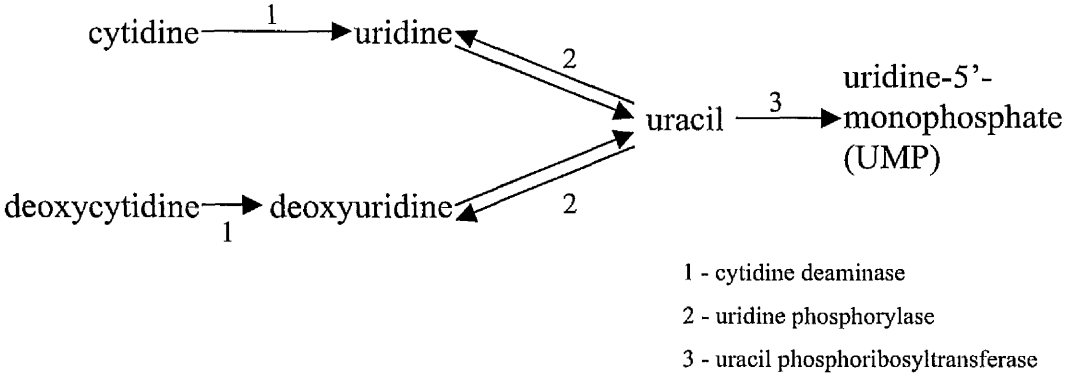



**Figure 3.1.** Pyrimidine salvage pathways present in *T. gondii* tachyzoites. The end product, uridine-5'-monophosphates (UMP), is used directly in the formation of nucleic acids. Uridine phosphorylase catalyses the reversible phosphorlysis of uridine and deoxyuridine to form uracil which is salvaged by *T. gondii* and converted to UMP by the enzyme, uracil phosphoribosyltransferase. Neither of these enzymes are present in mammalian cells and consequently these cells are unable to utilise uracil for RNA and DNA synthesis.

## 3.2 Aims

Arprinocid has been shown to be effective against both chicken coccidiosis (Miller *et al.*, 1977) and murine toxoplasmosis (Luft, 1986; Pfefferkorn *et al.*, 1988). Previous work suggested that the activity of arprinocid was related to inhibition of hypoxanthine transport (Wang *et al.*, 1979a; Wang *et al.*, 1979b). The production of an arprinocid-1-N-oxide-resistant *T. gondii* strain allowed further investigation of the mechanism of action of arprinocid (Pfefferkorn *et al.*, 1988) and led to the conclusion that arprinocid-1-N-oxide was the active *in vivo* metabolite of arprinocid. Furthermore, hypoxanthine was shown not to alter the efficacy of either compound. However, the mechanism of action of arprinocid and arprinocid-1-N-oxide remains to be elucidated.

The main objectives of work presented in this chapter were to

(i) Examine the sensitivity of pRH *T. gondii* tachyzoites to arprinocid and arprinocid-1-N-oxide, and investigate the decreased sensitivity of two mutant *T. gondii* strains ($ARP^R$ and $ANO^R$) to arprinocid-1-N-oxide.

(ii) Compare the $IC_{50}$ values of arprinocid and arprinocid-1-N-oxide with those for adenosine-N-oxide and monensin.

(iii) Investigate whether the effects of arprinocid, arprinocid-1-N-oxide, adenosine-N-oxide or monensin against *T. gondii* were specific to tachyzoites as a consequence of the disruption of normal host cell metabolism.

(iv) Analyse the efficacy of arprinocid and arprinocid-1-N-oxide against a mutant *T. gondii* line lacking the gene encoding for HXGPRT (RH-HX$^-$) to investigate whether these compounds interfere with the HXGPRT-mediated salvage of purines.

(v) Analyse the efficacy of arprinocid and arprinocid-1-N-oxide when *T. gondii* tachyzoites were treated using different dose regimes.

## 3.3 Methods

### 3.3.1 *In vitro* activity of drugs on *T. gondii* tachyzoites.

Vero cells (2 x $10^4$ cells/well) were incubated in 24-well plates overnight to form 60 % confluent monolayers and were infected with freshly harvested tachyzoites using a tachyzoite-to-cell ratio of 3:1. In order to measure the effects of both invasion and replication of *T. gondii*, compounds were added to cell cultures 1 h before infection, unless otherwise stated. Cultures were incubated for 24 h, 48 h, or 72 h, at 37°C in a 5 % $CO_2$ humidified incubator. Subsequently, the cultures were radiolabelled by the addition of 5 μCi [5, 6-$^3$H]-uracil per well and then further incubated for 4 h at 37°C. The 24-well plates were then chilled for 3 min at -20°C and the cell monolayers fixed to the bottom of the wells by the addition of 1 ml of ice-cold 0.6 N trichloroacetic acid (TCA) per well. After incubating on ice for 1 h, the TCA solution was removed and excess soluble radiation washed away by immersing the plates in a cold water bath overnight. Plates were air-dried for 5-10 min and the TCA precipitate dissolved by the addition of 0.5 ml of 0.1 N sodium hydroxide per well. After incubation of the 24-well plates at 37°C for 1 h, 0.25 ml of the liquid phase was removed from each well and added to 3 ml scintillation fluid that was acidified to neutralise the sodium hydroxide (3 μl glacial acetic acid per 3 ml scintillant). Experiments were conducted in triplicate. Counts per minute (CPM) were recorded from both infected and uninfected Vero cells (background) using a 1219 RockBeta Spectral scintillation counter (LKB Wallac, Finland).

### 3.3.2 *In vitro* activity of drugs on host Vero cells.

The sensitivity of Vero cells to the drugs was assayed by measuring $^3$H-thymidine incorporation, a similar technique to the measurement of $^3$H-uracil incorporation into tachyzoites (Pfefferkorn *et al.*, 1988). Uninfected Vero cells ($2 \times 10^4$) were incubated in 24-well plates in the presence of drug for 48 h. The cultures were radiolabelled by the addition of 5 μCi [$^3$H]-thymidine per well and incubated for 6 h at 37°C. $^3$H-thymidine incorporation was quantified as described above.

### 3.3.3 Graphical analysis.

Dose-effect curves were plotted as percentage of control $^3$H-uracil incorporation against drug concentration (μM) using FigP version 2.98 (Biosoft, UK) and $IC_{50}$ values calculated by FigP using the sigmoidal curve-fit equation below:

$$y = \frac{Min + (Max - Min)}{1 + ((x / x_{50})^{\wedge} - P)}$$

Bar-charts were drawn using Microsoft® Excel 97. Other graphs were drawn using GraFit version 4.0.14 (Erithacus Software Ltd, UK).

## 3.4 Results

### 3.4.1 Reproducibility of the $^{3}$H-uracil assay

The reproducibility of the $^{3}$H-uracil assay was measured using triplicate 24-well plates and measuring the incorporation of $^{3}$H-uracil into *T. gondii* tachyzoites during a 4 h period 48 h after infection of Vero cells. The mean counts per minute (CPM) ± s.d. (n = 24) from each 24-well plate were (i) plate 1 - 4289 ± 287 (ii) plate 2 - 4435 ± 315 (iii) plate 3 - 4514 ± 299. Mean CPM values ± s.d. for corresponding wells (n = 3) from each plate are displayed in Figure 3.2a. A frequency histogram showed that 75 % of observations fell within 1 s.d. of the mean and that 95 % of observations fell within 2 s.d of the mean (Figure 3.2b). On this basis it was concluded that the data were normally distributed. It was assumed that the residual observations (< 5 %) were equally divided at either end of the distribution and therefore Student's *t*-Test statistics were calculated as two-tailed.

The calculated values of *F* (P = 0.05) for data between plates 1 and 2, between plates 2 and 3 and between plates 1 & 3 were 1.21, 1.11 and 1.09, respectively. Since these values were below the critical value of *F* (P = 0.05) the samples were assumed to be from populations of similar variance. Student's *t*-Test analysis (assuming equal variance) showed that there was no significant difference (P > 0.05) in the means of the groups of data. A similar more sensitive test for matched pairs, whereby the well positions from one plate were fixed against the well positions from a second plate (wells were assumed to be numbered 1 to 24, from top left to bottom right) was performed. The calculated values of *t* between plates 1 and 2, between plates 2 and 3 and between

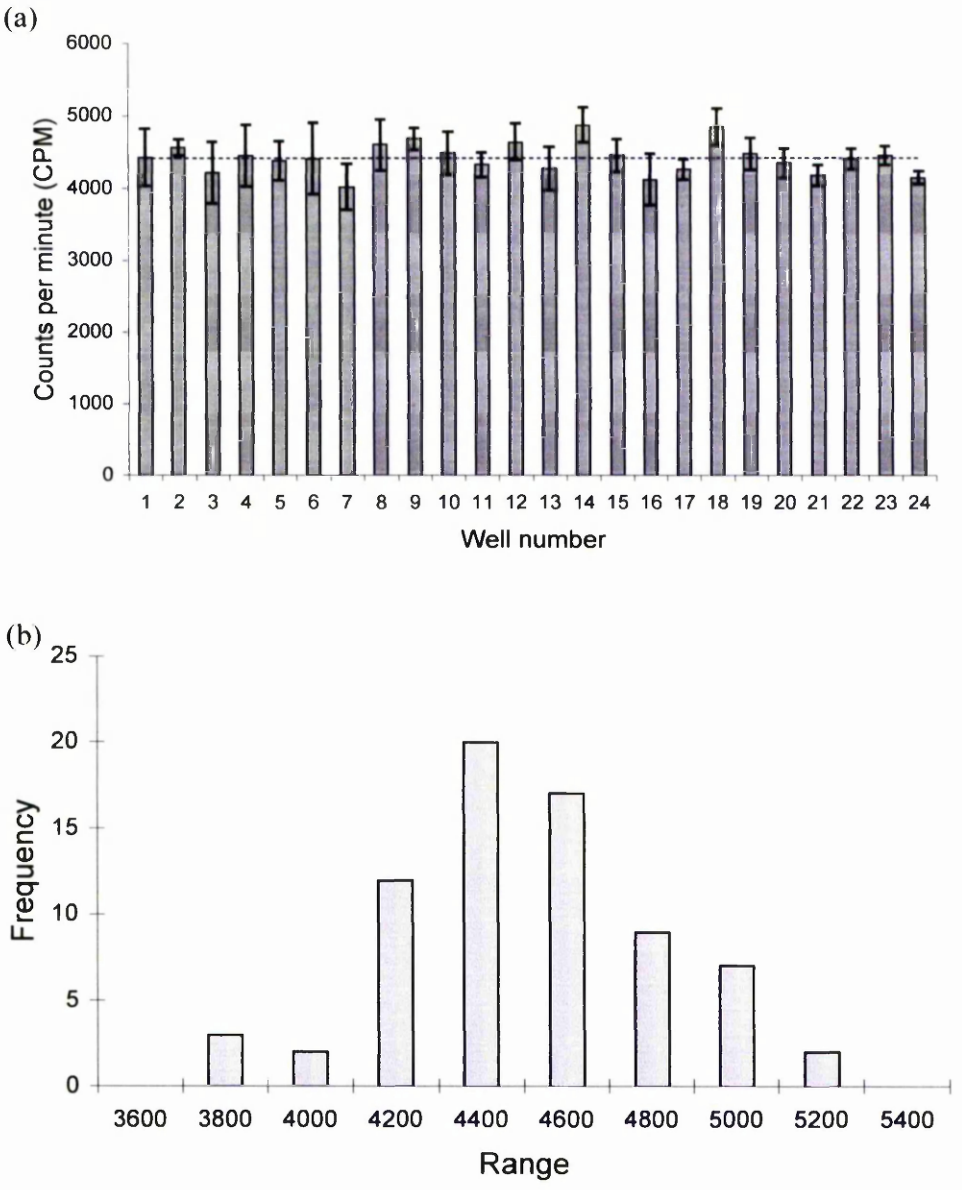



**Figure 3.2.** Reproducibility of $^{3}$H-uracil assay used to measure growth of *T. gondii* tachyzoites (a) Parasites and host cells were grown in replicate 24-well plates and $^{3}$H-uracil incorporation measured during a 4 h period 48 h after infection of Vero cells. Data are the mean ± s.d. (n = 3) of uracil uptake in each well (numbered consecutively from top left to bottom right). Dotted line is mean of 72 wells, 4412 ± 310 (b) Frequency histogram of the distribution of the data from all observations (n = 72).

plates 1 and 3 indicated there was no significant difference in the means ($P > 0.05$). As a final test, row and column reproducibility within each plate was measured. There was no significant difference between rows of plates ($P > 0.05$) or between columns of plates ($P > 0.05$).

### 3.4.2 Growth rate of 5 different strains of *T. gondii*

Growth curves of five strains of *T. gondii*, pRH, $ARP^R$, $ANO^R$, RH-$HX^-$ and S48, were quantified by measuring $^3$H-uracil incorporation during a 4 h period at 0 h, 4 h, 24 h, 48 h and 72 h relative to the time of infection of Vero cells (Figure 3.3a). All five *T. gondii* strains exhibited similar growth rates up to 24 h. However, the continued growth of parasites varied between strains. At 48 h post-infection, compared with 24 h post-infection, $^3$H-uracil uptake by pRH and $ARP^R$ *T. gondii* tachyzoites increased by approximately 30 % whilst, for $ANO^R$, $^3$H-uracil uptake decreased. For the same time period $^3$H-uracil incorporation by RH-$HX^-$ and S48 *T. gondii* increased by 188 % and 176 %, respectively.

A concurrent experiment to the $^3$H-uracil growth assay, using 25 $cm^3$ tissue culture flasks and exactly the same parasite:cell infection ratio, was performed to analyse the relationship between parasite growth and $^3$H-uracil uptake. At several time intervals after the infection of Vero cells: (i) extracellular tachyzoites were removed from the flask and counted; (ii) the cell monolayer was disrupted and the numbers of intracellular tachyzoites counted. Data collected for each strain indicated that there was a good correlation between $^3$H-uracil incorporation (CPM) and numbers of intracellular tachyzoites. Data for pRH tachyzoites are shown in Figure 3.4. Therefore, in all subsequent experiments an increase or decrease in CPM was assumed to be a reflection

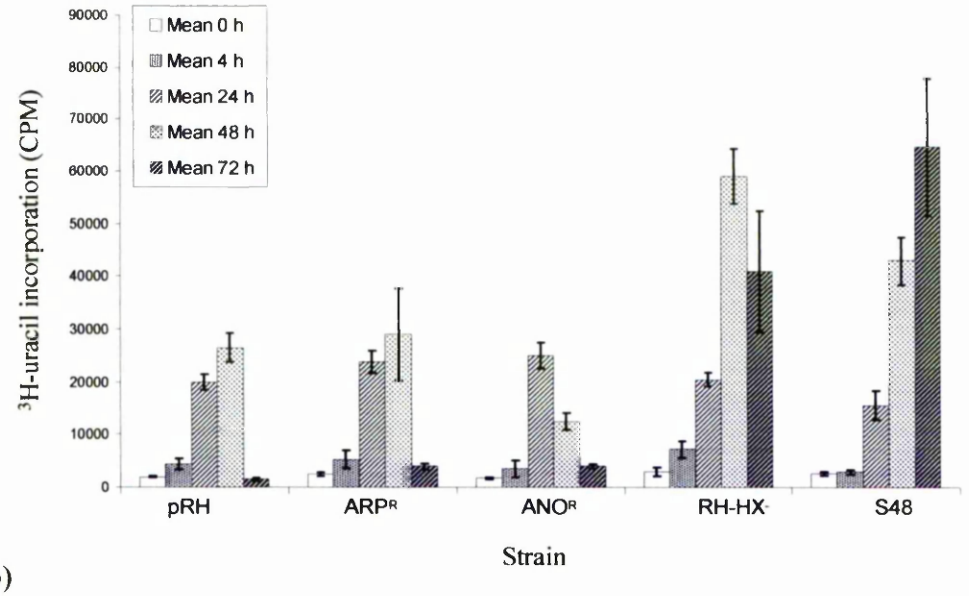
of the growth or inhibition of growth of *T. gondii* tachyzoites, respectively. The total numbers of tachyzoites for each strain are shown in Figure 3.3b. It was noticeable that RH-HX$^-$ and S48 tachyzoites grew at a faster rate than pRH, ARP$^R$ or ANO$^R$. For instance, at 144 h there were 25-fold more RH-HX$^-$ tachyzoites and 17-fold more S48 tachyzoites than pRH, ARP$^R$ or ANO$^R$.

### 3.4.3 Efficacy of arprinocid, arprinocid-1-N-oxide, adenosine-N-oxide and monensin on growth of *T. gondii*

Having investigated the reproducibility of the $^3$H-uracil incorporation assay and established the correlation between CPM and parasite growth, the assay was used to quantify the efficacy of arprinocid, arprinocid-1-N-oxide, adenosine-N-oxide and monensin against *T. gondii* tachyzoites.

$^3$H-Uracil uptake by pRH *T. gondii* tachyzoites grown within Vero cells was diminished significantly ($P < 0.005$) by treatment with arprinocid (Figure 3.5). Specifically, when drug was added to the culture 1 h before infection and $^3$H-uracil uptake measured during a 4 h period 48 h later, arprinocid (50 μM) inhibited the growth of *T. gondii* by 82.8 ± 7.3 % (n = 3). Arprinocid (0 - 50 μM) exhibited similar toxicity against pRH, ARP$^R$ ($P < 0.005$) and ANO$^R$ ($P < 0.001$), with $IC_{50}$ values of 22.4 ± 5.0 μM (n = 3), 18.7 ± 2.9 μM (n = 3) and 17.7 ± 2.2 μM (n = 3), respectively. There was no significant change ($P < 0.05$) in the efficacy of arprinocid when $^3$H-uracil incorporation was measured during a 4 h period 24 h post infection (data not shown).

(a)




(b)

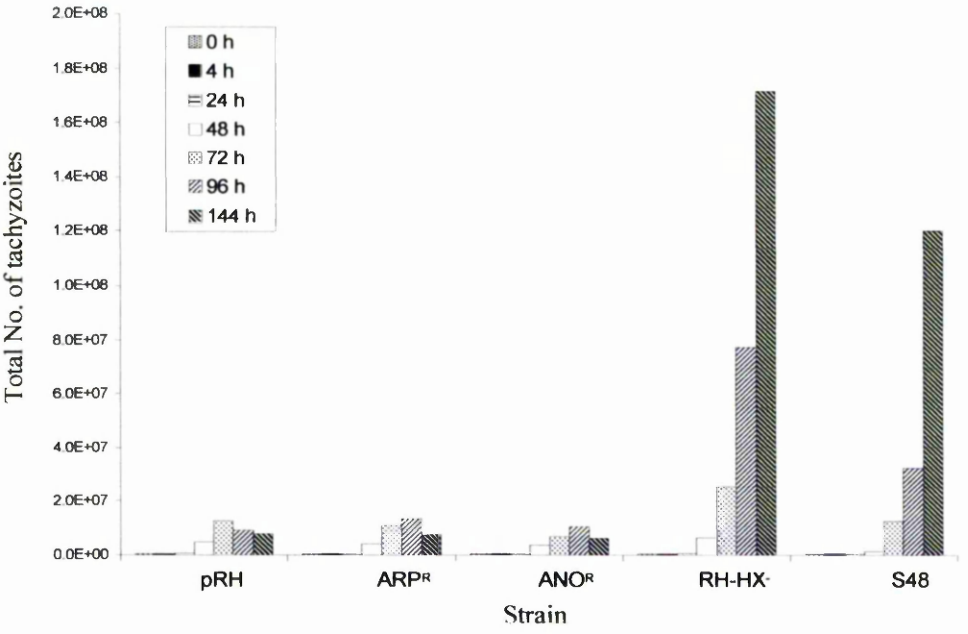



**Figure 3.3.** Measurement of the growth of pRH, ARP$^R$, ANO$^R$, RH-HX$^-$ and S48 *T. gondii* strains (a) $^3$H-uracil incorporation was measured during a 4 h period 0 h, 4 h, 24 h, 48 h and 72 h post-infection of Vero cells. Data points are the mean ± s.d. of 3 experiments (b) Measurement of the growth characteristics of pRH, ARP$^R$, ANO$^R$, RH-HX$^-$ and S48 *T. gondii* strains using microscopic analysis. Parallel cultures to those in the $^3$H-uracil assay (Figure 3.3a) were monitored at 0, 4, 24, 48, 72, 96 and 144 h post-infection of Vero cells and the total number of parasites counted.

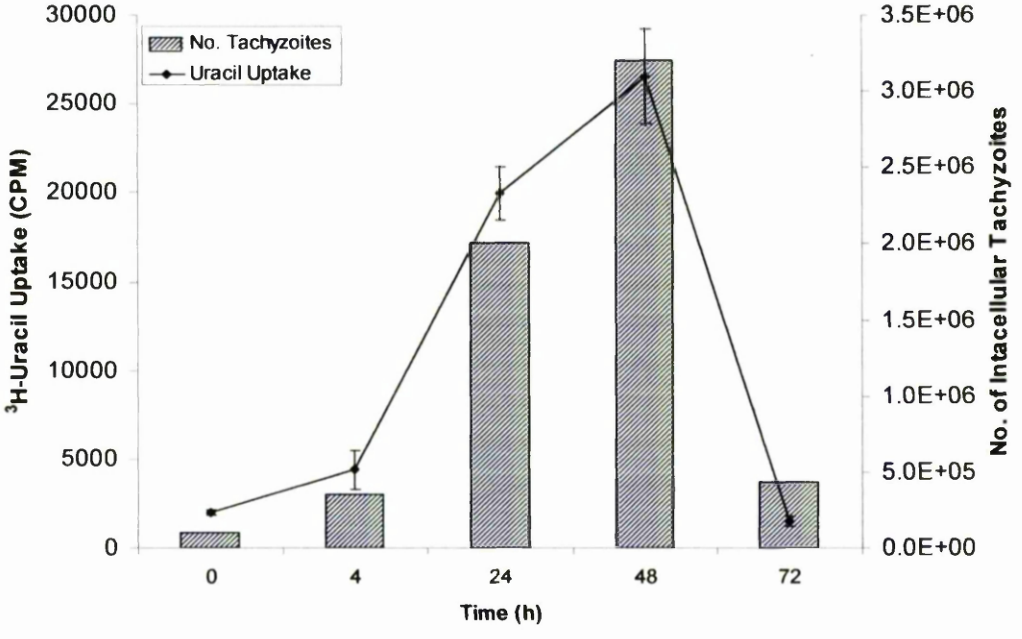
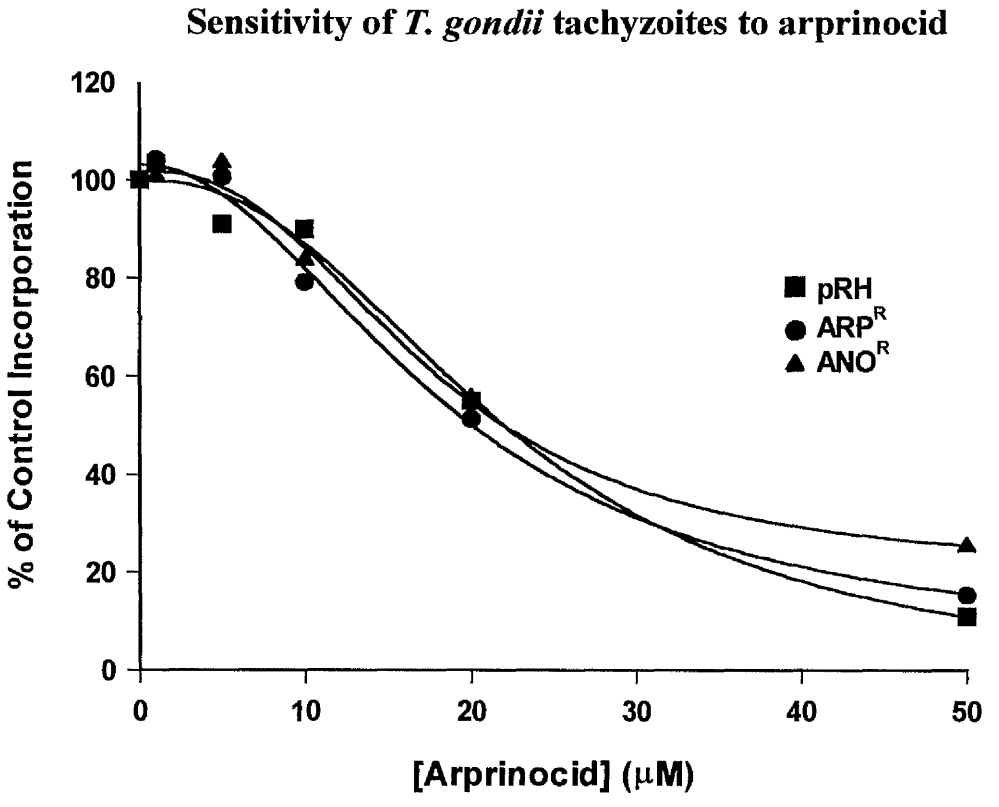



**Figure 3.4.** Correlation between $^3$H-uracil incorporation and growth of intracellular tachyzoites (pRH). Parallel cultures to those used in the $^3$H-uracil assay (Figure 3.3 a) were monitored at each time interval and number of intracellular parasites counted. Data points are the mean ± s.d. of 3 experiments. A direct correlation between $^3$H-uracil incorporation and parasite growth was observed for each strain.




| *T. gondii* strain | $IC_{50}$ Value (μM) |
|---|---|
| pRH | 22.4 ± 5.0 |
| $ARP^R$ | 18.7 ± 2.9 |
| $ANO^R$ | 17.7 ± 2.2 |

**Figure 3.5.** Sensitivity of pRH, $ARP^R$ and $ANO^R$ *T. gondii* lines to arprinocid (0 - 50 μM). Drug treatment occurred 1 h before infection of Vero cells. $^3$H-uracil incorporation was measured during a 4 h period 48 h post-infection. $IC_{50}$ values for arprinocid are shown in the table below graph. Data points are the mean of 3 experiments. Error bars have been omitted for clarity.

$^{3}$H-Uracil uptake by pRH *T. gondii* tachyzoites grown within Vero cells was reduced significantly ($P < 0.05$) by treatment with arprinocid-1-N-oxide at 350 times lower concentration than arprinocid (Figure 3.6). The $IC_{50}$ concentration of arprinocid-1-N-oxide on pRH *T. gondii* tachyzoites was calculated to be 61 ± 28 nM (n = 3). The *T. gondii* strains, $ARP^{R}$ and $ANO^{R}$, were found to be 14 fold less sensitive to arprinocid-1-N-oxide, having $IC_{50}$ values of 832 ± 71 nM and 875 ± 74 nM, respectively.

Adenosine-N-oxide (0 - 20 μM) exhibited similar toxicity to arprinocid against pRH ($P < 0.05$), $ARP^{R}$ ($P < 0.001$) and $ANO^{R}$ ($P < 0.05$) strains of *T. gondii* with $IC_{50}$ values of 12.5 ± 1.39 μM, 11.8 ± 2.4 μM and 8.1 ± 2.4 μM, respectively (Figure 3.7). Further analysis of adenosine-N-oxide (0 - 50 μM) showed that parasite growth was reduced by 74.2 ± 5.0 % ($P < 0.001$) in the presence of 50 μM drug. The calculated $IC_{50}$ of adenosine-N-oxide using this wider dose range was 14.5 ± 2.1 μM (n = 3; data not shown).

The ionophore, monensin, inhibited pRH growth by 96.9 ± 2.45 % at 5 nM with an $IC_{50}$ value of 0.14 ± 0.02 nM (n = 3) (Figure 3.8).

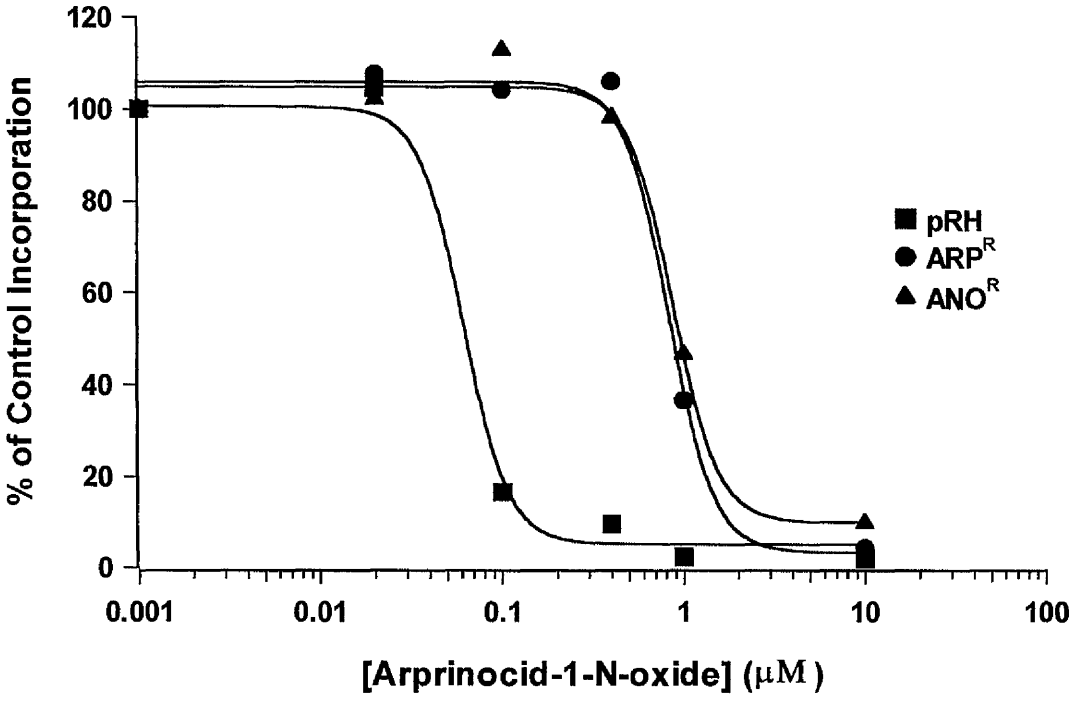
### 3.4.4 Effect of arprinocid, arprinocid-1-N-oxide, adenosine-N-oxide and monensin on growth of Vero cells

In order to determine whether the effect of arprinocid, arprinocid-1-N-oxide, adenosine-N-oxide and monensin on *T. gondii* was secondary to an effect on the host cells a $^{3}$H-thymidine assay was performed on drug-treated uninfected Vero cells (Figure 3.9). Monensin (5 nM) had no significant effect on $^{3}$H-thymidine incorporation ($P > 0.05$)

and did not alter the growth of Vero cells (visual observations). Arprinocid-1-N-oxide (400 nM) had no significant effect $^{3}$H-thymidine incorporation ($P > 0.05$) but reduced uptake by 99.5 ± 0.1 % at 1 μM ($P < 0.005$). At this concentration of arprinocid-1-N-oxide (1 μM), Vero cells became rounded and detached from the surface of the well. In contrast, neither arprinocid (0 - 50 μM) nor adenosine-N-oxide (0 - 50 μM) had any noticeable effect on the Vero cells, although both compounds resulted in a significant increase ($P < 0.05$) in $^{3}$H-thymidine incorporation (Figure 3.9).

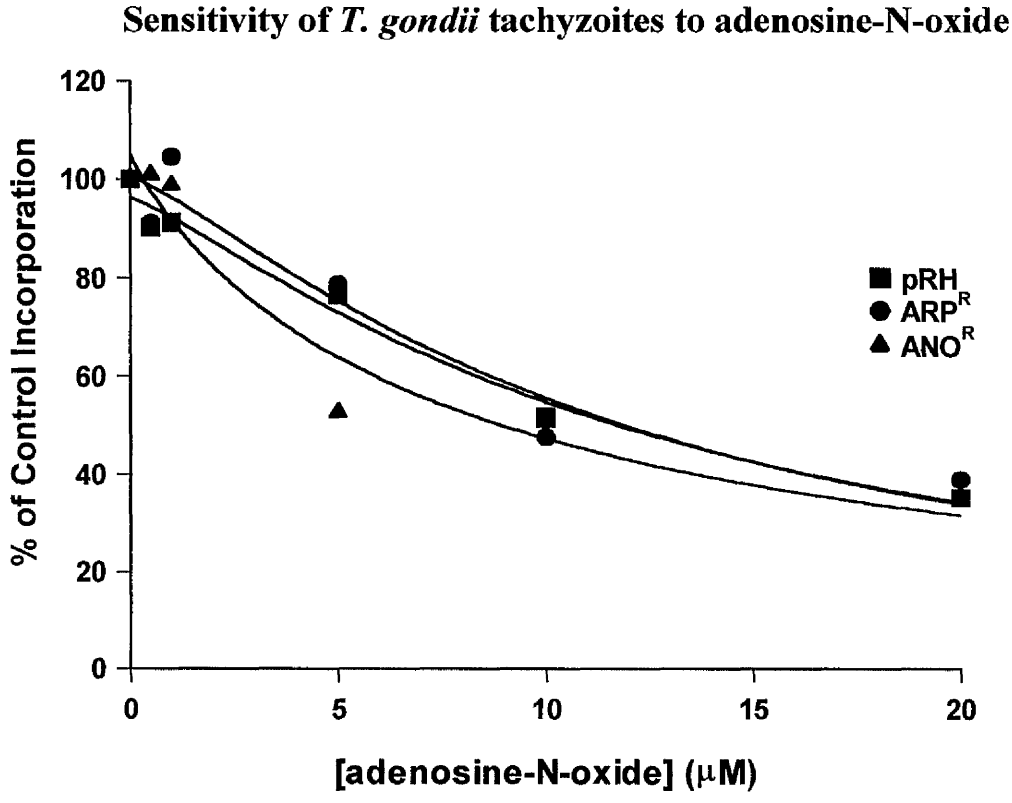
### 3.4.5 Efficacy of arprinocid and arprinocid-1-N-oxide on growth of RH-HX⁻ *T. gondii*

A mutant *T. gondii* isolate lacking the gene encoding for HXGPRT was used to test the possibility that the effect of arprinocid and arprinocid-1-N-oxide was dependent on the HXGPRT-mediated salvage pathway. A $^{3}$H-uracil experiment was performed using S48 *T. gondii* as a control because this strain has a similar growth rate to RH-HX⁻, and therefore any difference in drug efficacy would not be masked by differences in growth rate. $^{3}$H-Uracil incorporation by RH-HX⁻ *T. gondii* tachyzoites was significantly reduced by treatment with arprinocid ($P < 0.001$) and by treatment with arprinocid-1-N-oxide ($P < 0.005$). Calculated $IC_{50}$ values for arprinocid on RH-HX⁻, S48 and pRH were 18.3 ± 1.6 μM, 20.3 ± 2.2 μM and 17.8 ± 1.5 μM, respectively (Figure 3.10) correlating with values previously determined (Section 3.4.3). The $IC_{50}$ values for arprinocid-1-N-oxide on RH-HX⁻, S48 and pRH were 28 ± 21 nM, 10 ± 12 nM and 47 ± 18 nM (Figure 3.11) correlating with values previously determined (Section 3.4.3). Therefore, the mechanisms of action of arprinocid and arprinocid-1-N-oxide are unrelated to hypoxanthine transport.




| *T. gondii* strain | $IC_{50}$ Value (μM) |
|---|---|
| pRH | 0.061 ± 0.028 |
| $ARP^R$ | 0.832 ± 0.071 |
| $ANO^R$ | 0.875 ± 0.074 |

**Figure 3.6.** Sensitivity of pRH, $ARP^R$ and $ANO^R$ *T. gondii* lines to arprinocid-1-N-oxide (0 - 10 μM). Drug treatment occurred 1 h before infection of Vero cells. $^3$H-uracil incorporation was measured during a 4 h period 48 h post-infection. $IC_{50}$ values for arprinocid-1-N-oxide are shown in the table below graph. Data points are the mean of 3 experiments. Error bars have been omitted for clarity.




| *T. gondii* strain | $IC_{50}$ Value (μM) |
|---|---|
| pRH | 12.5 ± 1.3 |
| $ARP^R$ | 11.8 ± 2.4 |
| $ANO^R$ | 8.1 ± 2.4 |

**Figure 3.7.** Sensitivity of pRH, $ARP^R$ and $ANO^R$ *T. gondii* strains to adenosine-N-oxide (0 - 20 μM). Drug treatment occurred 1 h before infection of Vero cells. $^3$H-uracil incorporation was measured during a 4 h period 48 h post-infection. $IC_{50}$ values for adenosine-N-oxide are shown in the table below graph. Data points are the mean of 3 experiments. Error bars have been omitted for clarity.

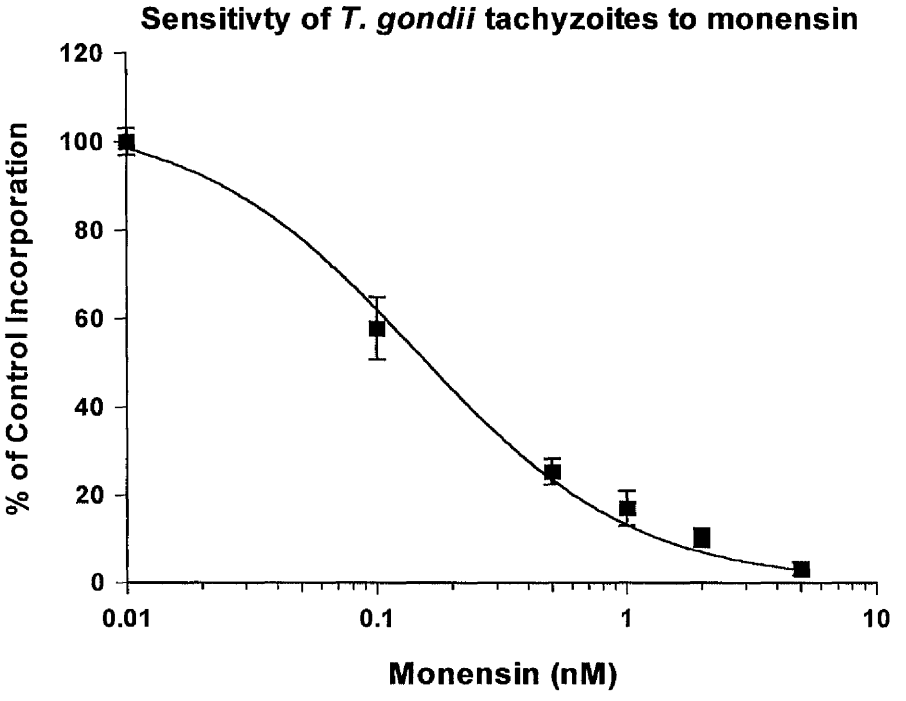



**Figure 3.8.** Sensitivity of pRH *T. gondii* to monensin (0 - 5 nM). Drug treatment occurred 1 h before infection of Vero cells. $^{3}$H-uracil incorporation was measured during a 4 h period 48 h post-infection. Data points are the mean of 3 experiments ± s.d.

**Sensitivity of Vero cells to arprinocid, arprinocid-1-N-oxide, adenosine-N-oxide and monensin**

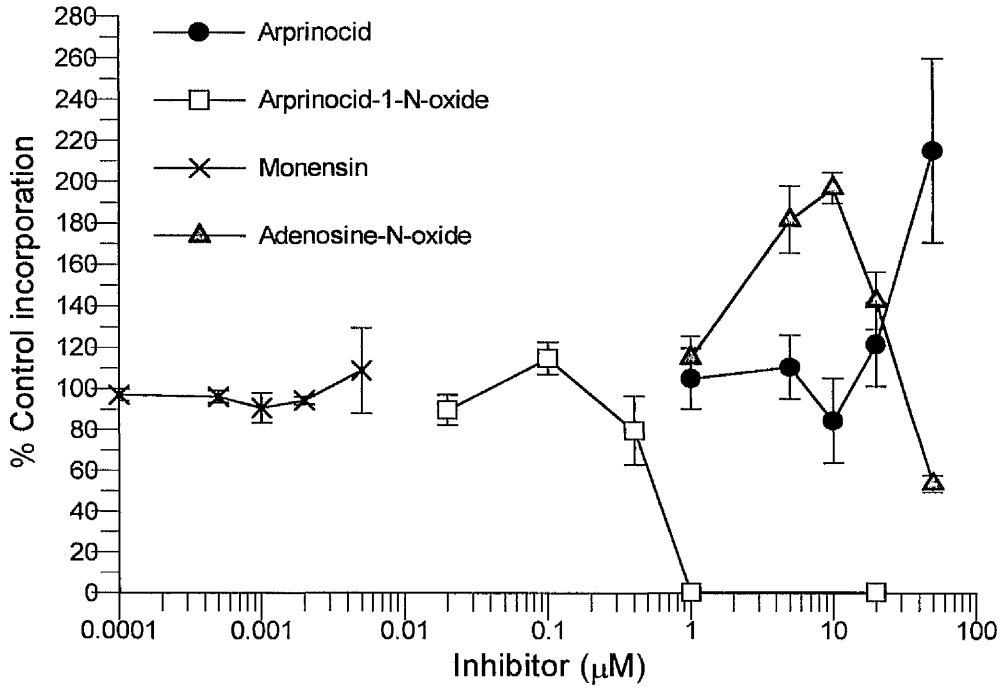
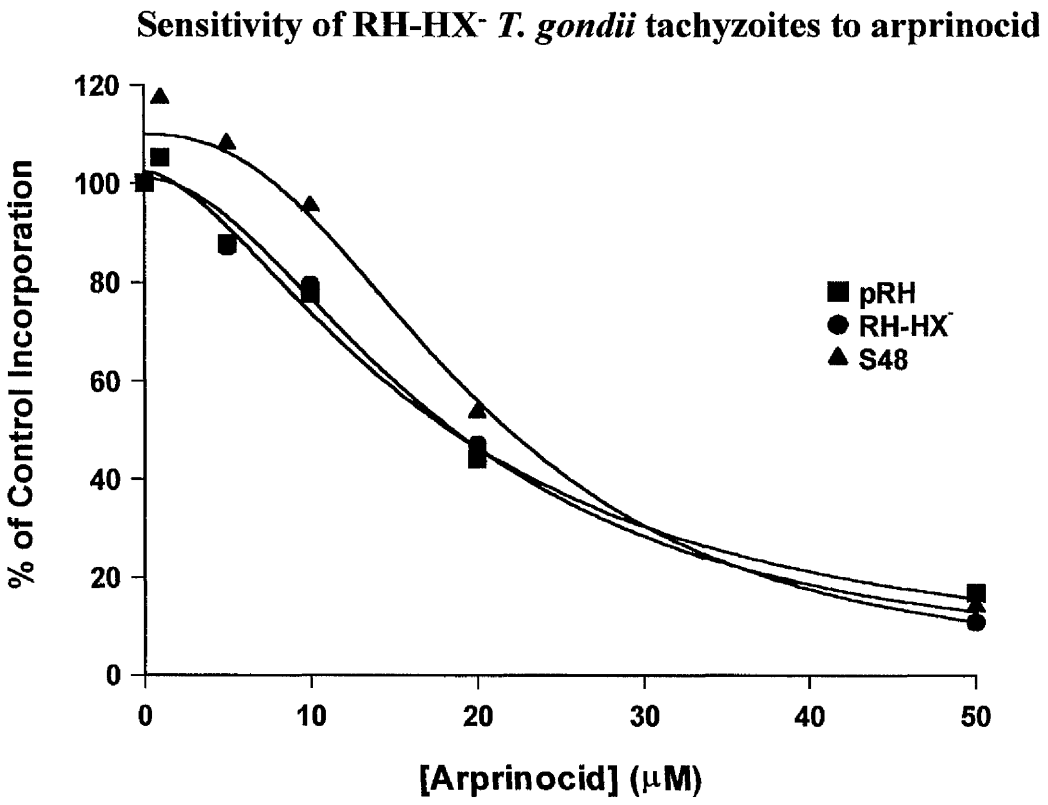
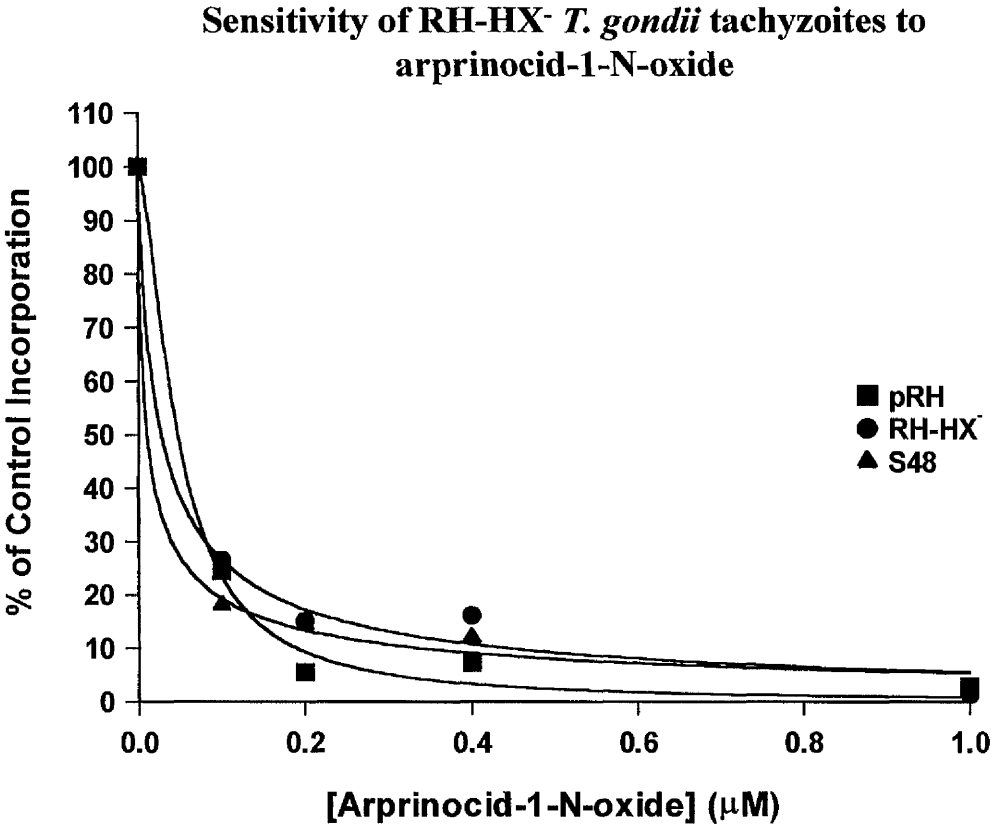



**Figure 3.9.** Sensitivity of host Vero cells to arprinocid (0 - 50 μM), arprinocid-1-N-oxide (0 - 20 μM), adenosine-N-oxide (0 - 50 μM) and monensin (0 - 5 nM). Drug treatment occurred at same time as seeding of vero cells. $^{3}$H-thymidine incorporation was measured during a 6 h period 48 h post-infection. Data points are the mean ± s.d. of 3 experiments.




| *T. gondii* strain | $IC_{50}$ Value (μM) |
|---|---|
| pRH | 17.8 ± 1.5 |
| RH-HX$^-$ | 18.3 ± 1.6 |
| S48 | 20.3 ± 2.2 |

**Figure 3.10.** Sensitivity of RH-HX$^-$ *T. gondii* to arprinocid (0 - 50 μM). pRH and S48 *T. gondii* strains were used as controls. Drug treatment occurred 1 h before infection of Vero cells. $^3$H-uracil incorporation was measured during a 4 h period 48 h post-infection. $IC_{50}$ values of arprinocid against each strain are shown in the table below the graph. Data points are the mean of 3 experiments. Error bars have been omitted for clarity.




| *T. gondii* strain | $IC_{50}$ Value (μM) |
|---|---|
| pRH | 0.047 ± 0.018 |
| RH-HX⁻ | 0.028 ± 0.021 |
| S48 | 0.010 ± 0.012 |

**Figure 3.11.** Sensitivity of RH-HX⁻ *T. gondii* to arprinocid-1-N-oxide (0 - 1 μM). pRH and S48 *T. gondii* strains were used as controls. Drug treatment occurred 1 h before infection of Vero cells. $^3$H-uracil incorporation was measured during a 4 h period 48 h post-infection. $IC_{50}$ values of arprinocid-1-N-oxide against each strain are shown in the table below the graph. Data points are the mean of 3 experiments. Error bars have been omitted for clarity.

### 3.4.6 Dose treatment strategies

Both arprinocid and arprinocid-1-N-oxide were shown to be effective anti-*Toxoplasma* compounds inhibiting tachyzoite replication 24 h and 48 h post-infection. Consequently, experiments were conducted to investigate the effects of the drug within shorter periods of time and the efficacy of the compounds using different treatment regimes.

The effects of arprinocid and arprinocid-1-N-oxide on extracellular tachyzoites were investigated. Tachyzoites were incubated at 37°C for 2 h in the presence of arprinocid (0 - 50 μM), arprinocid-1-N-oxide (0 - 10 μM), or monensin (0 - 5 nM). Thereafter, drug was removed from tachyzoites by washing three times in 5 ml IMDM and centrifugation at 1500 x g for 10 min at 4°C. Vero cells were infected with these drug-treated tachyzoites and $^{3}$H-uracil incorporation measured 48 h later, as described previously. There was no significant difference in CPMs between the lowest and highest concentrations of arprinocid ($P > 0.05$) or arprinocd-1-N-oxide ($P > 0.05$) (Figure 3.12). However, when tachyzoites were treated with monensin (0 - 5 nM) growth was reduced by 37.4 ± 7.2 % (Figure 3.12).

A second experiment was performed to investigate the effects of treating tachyzoites (and host cells) with arprinocid or arprinocid-1-N-oxide for a limited period only. Vero cells were incubated for 1 h in the presence of either arprinocid (0 - 50 μM) or arprinocid-1-N-oxide (0 - 10 μM) and then infected with *T. gondii* tachyzoites (pRH), as described previously. However, drug was removed 24 h post-infection by washing cell monolayers three times in 1 ml IMDM. Cells and parasites in fresh IMDM (no drug) were incubated for a further 24 h and $^{3}$H-uracil incorporation measured, as described

previously. Arprinocid (50 μM) and arprinocid-1-N-oxide (1 μM) decreased $^{3}$H-uracil incorporation by 64.2 ± 9.1 % ($P < 0.05$) and 99.4 ± 9.1 % ($P < 0.005$), respectively (Figure 3.13). Calculated $IC_{50}$ values for arprinocid and arprinocid-1-N-oxide were 13.2 ± 3.1 and 24 ± 6 nM, respectively.

To investigate further how quickly arprinocid and arprinocid-1-N-oxide exerted their effects, an experiment was conducted in which the treatment of tachyzoites was delayed by up to 48 h post-infection. Results showed that both arprinocid (20 μM and 50 μM) and arprinocid-1-N-oxide (0.1 μM and 0.5 μM) were effective whether the tachyzoites were treated for 48 h prior to the measurement of $^{3}$H-uracil incorporation or just during the 4 h period of $^{3}$H-uracil incorporation (Figure 3.14). Arprinocid (50 μM) and arprinocid-1-N-oxide (0.5 μM), when added to culture medium 48 h after infection, reduced $^{3}$H-uracil uptake by 59.7 ± 0.8 % ($P < 0.05$) and by 91.1 ± 1.1 % ($P < 0.005$), respectively.

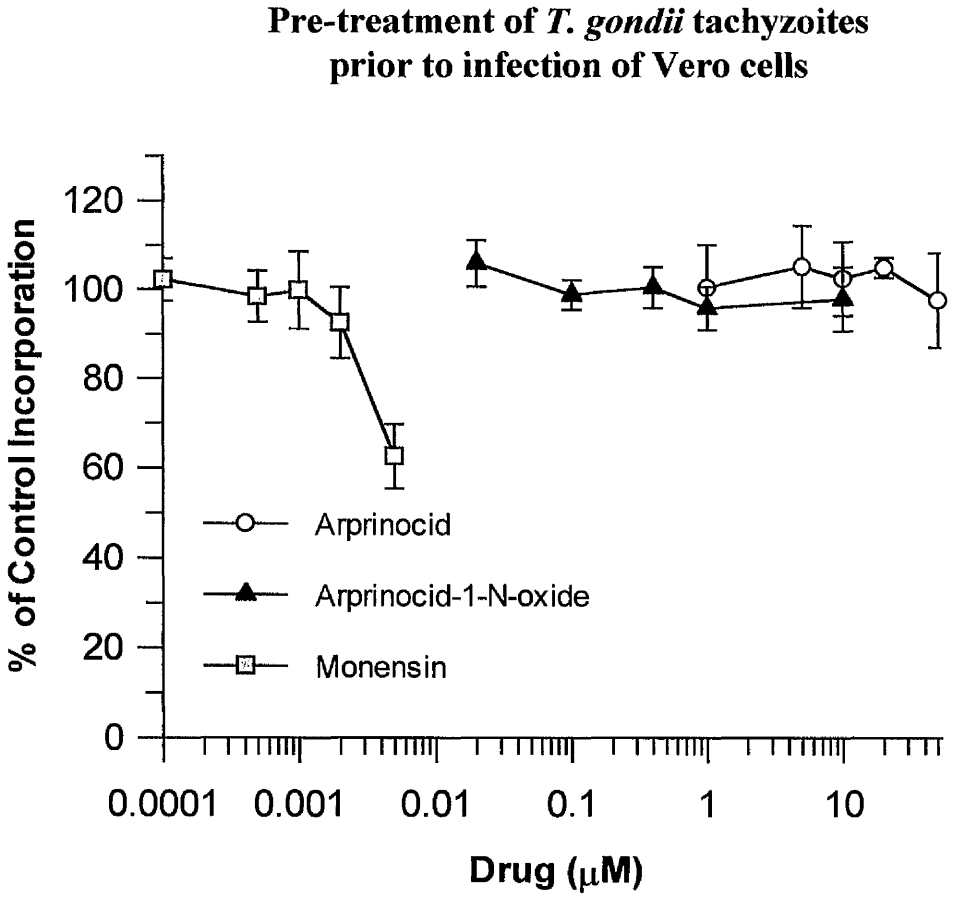
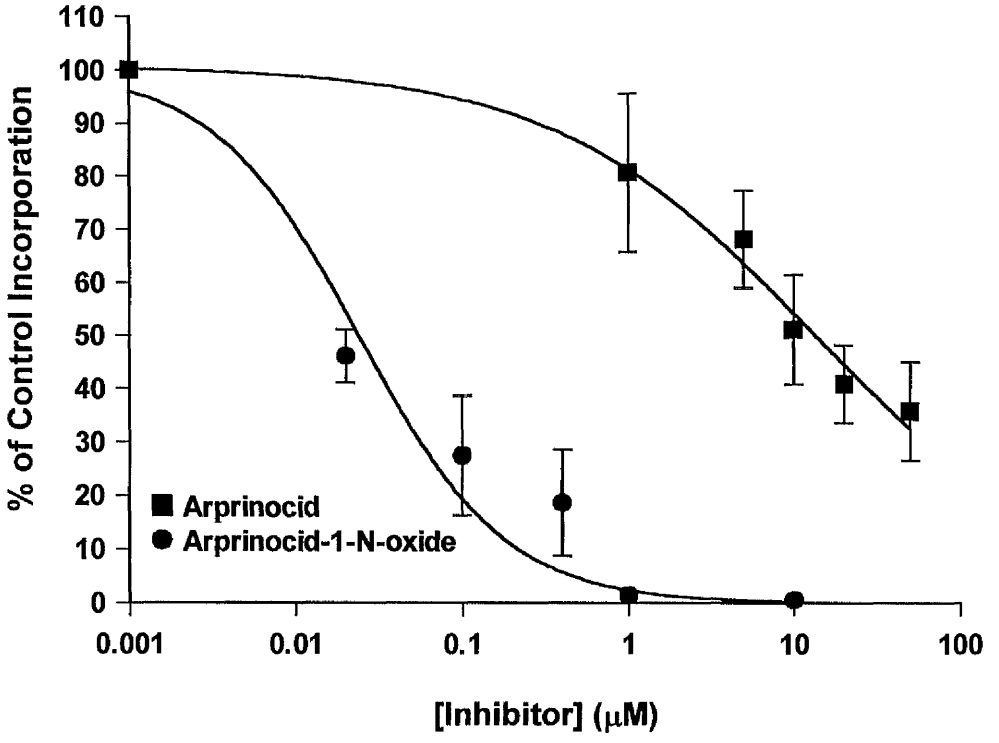



**Figure 3.12.** *T. gondii* tachyzoites (pRH) were incubated at 37°C for 2 h with arprinocid (0 - 50 μM), arprinocid-1-N-oxide (0 - 10 μM) or monensin (0 - 5 μM). Drug was removed from the parasites by washing them three times in 5 ml IMDM. The drug-pretreated tachyzoites were subsequently used to infect Vero cells. $^{3}$H-uracil incorporation was measured during a 4 h period 48 h post-infection. Control assays were in the absence of drug. Data points plotted are the mean ± s.d. of 3 experiments.

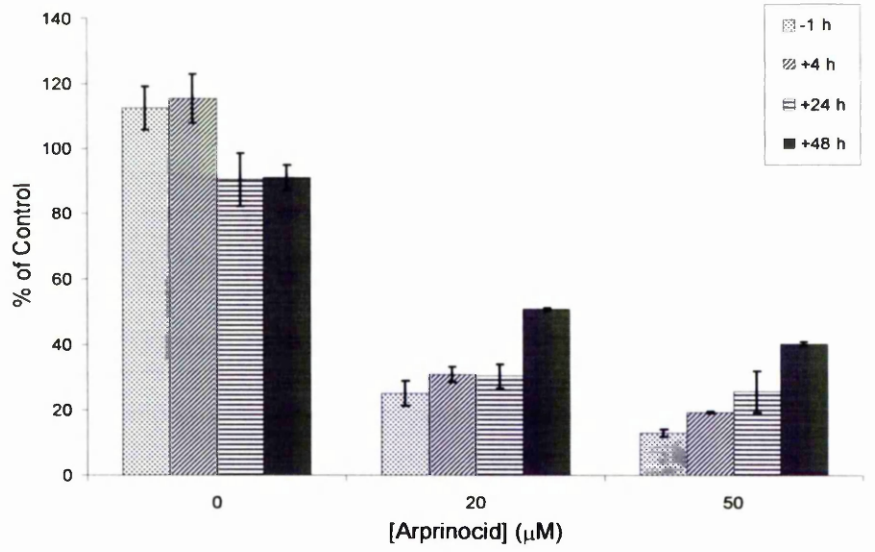
**Efficacy of arprinocid and arprinocid-1-N-oxide after limited treatment of *T. gondii***




| Drug | $IC_{50}$ Value (μM) |
|---|---|
| Arprinocid | 13.2 ± 3.1 |
| Arprinocid-1-N-oxide | 0.024 ± 0.006 |

**Figure 3.13.** Sensitivity of *T. gondii* (pRH) to arprinocid (0 - 50 μM) and arprinocid-1-N-oxide (0 - 10 μM) after limited treatment. Drugs were added to culture medium 1 h before infection of Vero cells and removed 24 h after infection by washing cultures three times in IMDM . $^{3}$H-uracil incorporation was measured during a 4 h period 48 h post-infection. $IC_{50}$ values for arprinocid and arprinocid-1-N-oxide are shown in the table below graph. Data points are the mean of 3 experiments.

(a)




(b)

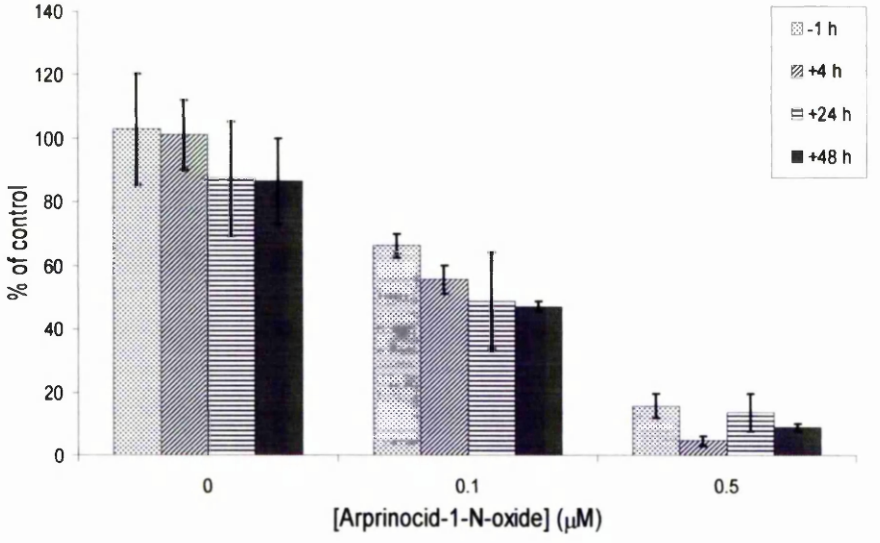



**Figure 3.14.** Sensitivity of *T. gondii* tachyzoites to arprinocid and arprinocid-1-N-oxide at -1 h, +4 h, +24 h, and +48 h relative to time of infection of Vero cells. *T. gondii* tachyzoites (pRH) treated with (a) arprinocid (0 - 50 μM) or (b) arprinocid-1-N-oxide (0 - 0.5 μM). $^{3}$H-uracil incorporation was measured during a 4 h period 48 h post-infection. Data points are the mean ± s.d. of 3 experiments.

## 3.5 Discussion

Both arprinocid and arprinocid-1-N-oxide inhibited the *in vitro* growth of *T. gondii* tachyzoites when measured at 24 h or 48 h post-infection; arprinocid-1-N-oxide displayed greater toxicity against *T. gondii* tachyzoites having an $IC_{50}$ over 350 fold lower than that of arprinocid. The efficacies of both these compounds were in good agreement with previously published data for *T. gondii* (Pfefferkorn *et al.*, 1988; Ricketts and Pfefferkorn, 1993).

The activity of anticoccidial drugs can vary between different organisms and thus activity against *T. gondii* does not necessary translate into similar toxicity against other coccidia. For example, ten-fold higher concentrations of both arprinocid and arprinocid-1-N-oxide were needed for a 50 % growth inhibition of *E. tenella* sporozoites (Wang and Simashkevich, 1980), whilst arprinocid had approximately a 1.5-fold higher $IC_{50}$ against *Neospora caninum* (Lindsay *et al.*, 1994). The relatively small difference in $IC_{50}$ of arprinocid against *N. caninum* compared with *T. gondii* is unlikely to be significant since the 1-N-oxide metabolite is the most active compound. In addition to drug efficacy differences between species, drugs can show markedly different efficacies between two different *in vitro* screens used on the same organism. Results are largely dependent on the type of cells used and the type of medium. For example, arprinocid is more active in chick liver cells than chick kidney cells due to metabolism to the more potent arprinocid-1-N-oxide (Latter and Wilson, 1979). It is unlikely that Vero cells can metabolise arprinocid. Using chick kidney cells, the activity of lasalocid, but not monensin, has been shown to vary over 1000 fold between two types of medium. Since lasalocid and monensin are both ionophores, and ionophores exert their action by

altering the ionic balance across membranes, it is conceivable that this activity would be affected by the ion composition of media (Latter and Wilson, 1979).

It is possible that arprinocid and arprinocid-1-N-oxide act *via* distinct mechanisms. Although structurally similar to arprinocid, the nitrogen-bound oxygen group present in arprinocid-1-N-oxide significantly increased the potency of this compound. It would appear that this substitution is responsible for the acquisition of drug-resistance since Pfefferkorn and co-workers (1988) made several attempts using chemical mutagenesis to create an arprinocid-resistant strain of *T. gondii* and failed on each occasion. By contrast, two successive attempts to create a diclazuril-resistant *T. gondii* mutant by chemical mutagenesis failed and was only achieved by growing parasites in progressively increasing concentrations of drug (Lindsay *et al.*, 1995). There has been no report on whether growing wild-type *T. gondii* under drug pressure could produce an arprinocid-resistant mutant. Further work using analogues of arprinocid-1-N-oxide would be valuable. Perhaps these analogues could include different substitutions of the oxygen group. Most importantly these structure-activity studies would provide vital information regarding the structure-activity relationships and inhibitory effects of purine analogues against *T. gondii*. Similarly, a structure-activity study using a $^{3}$H-hypoxanthine incorporation assay (Chulay *et al.*, 1983) has recently been performed to assess the ability of purine derivatives to inhibit the *in vitro* growth of *P. falciparum* (Harmse *et al.*, 2001). Other studies have assayed activities of structurally similar quinolones and fluoroquinolones against *T. gondii* (Gozalbes *et al.*, 2000; Khan *et al.*, 1999b). Similarly structure-activity assays were performed to assess binding of nucleosides to *T. gondii* adenosine kinase (Iltzsch *et al.*, 1995) and binding to *T. gondii* uridine phosphorylase (el Kouni *et al.*, 1996).

The two mutant strains ($ARP^R$ and $ANO^R$), compared with pRH, were less sensitive to arprinocid-1-N-oxide but equally sensitive to arprinocid. If the presence of an oxygen group at N9 contributes to increased toxicity against *T. gondii* then it might have been expected that adenosine-N-oxide would be more potent. Adenosine-N-oxide, which also has the oxygen group in the same position, was only slightly more toxic to *T. gondii* (there was no difference in efficacy between sensitive and resistant strains) than arprinocid, but not of the magnitude of toxicity of arprinocid-1-N-oxide. Although adenosine-N-oxide has some structural similarity to adenosine-N-oxide, it is possible that the presence of a pentose group could dramatically change its binding capabilities or membrane transport and thus alter its efficacy.

The anti-*Toxoplasma* activities of arprinocid (0 - 50 μM), adenosine-N-oxide (0 - 20 μM) and monensin (0 - 5 nM) are not likely to be secondary effects dependent on adverse effects on host cells. Although arprinocid and adenosine-1-N-oxid resulted in an increase in $^3$H-thymidine incorporation, the cells showed no signs of stress. This increase in $^3$H-thymidine incorporation may be related to a prolonged S-phase of the cell cycle, such that more thymidine is incorporated during the period of analysis (Pfefferkorn *et al.*, 1988). Electron microscopy might reveal more information about the effects of the compounds, particularly arprinocid-1-N-oxide, on the structure of intracellular organelles. Arprinocid-1-N-oxide at concentrations above 0.4 μM resulted in a significant reduction in $^3$H-thymidine incorporation and at concentrations above 1 μM arprinocid-1-N-oxide, host cells became rounded and detached from the surface of the culture flask. Over 80 % of tachyzoites were killed at 0.1 μM arprinocid-1-N-oxide, which is below the concentration at which thymidine incorporation was affected and thus below the concentration which is toxic to Vero cells. It is certain that any effect of

arprinocid-1-N-oxide at concentrations greater than 1 μM is related to an adverse effect on the host cells. This implies that, although arprinocid-1-N-oxide is a potent (and potential) anti-*Toxoplasma* drug, it may have a low therapeutic range that could be used safely and without side effects. Whether this *in vitro* host cell toxicity translates to *in vivo* toxicity remains to be elucidated. Many unknown factors not applicable in *in vitro* screens influence drug activity *in vivo*, such as absorption, distribution and bioavailability of the compounds.

It has been suggested that arprinocid inhibits hypoxanthine transmembrane transport in HeLa cells (Wang *et al.*, 1979a) and in *E. tenella* schizonts grown in chick kidney epithelial cells (Wang and Simashkevich, 1980). Interpretation of how this translates to activity on extracellular *T. gondii* tachyzoites is difficult since any effect on host cells does not necessarily indicate a similar effect on *T. gondii*. Arprinocid (0 - 50 μM) and arprinocid-1-N-oxide (0 - 1 μM) were tested against a null mutant of HXGPRT (RH-HX$^-$; Donald *et al.*, 1996). Although, the 1-N-oxide appeared to be slightly more toxic against RH-HX$^-$ it was concluded that there was no change in efficacy. Hence, the HXGPRT-mediated salvage pathway is not involved in the mechanism of action of either arprinocid or arprinocid-1-N-oxide. This is in agreement with Pfefferkorn *et al.*, (1988) who also concluded that hypoxanthine transport was not involved in their mechanisms of action, since concentrations of hypoxanthine that inhibit parasite growth did not alter the efficacy of either arprinocid or arprinocid-1-N-oxide. Studies on the membrane transport of purines and inhibition by arprinocid and arprinocid-1-N-oxide are discussed in Chapter 6.

Neither arprinocid nor arprinocid-1-N-oxide inhibited the invasion of host cells, since intracellular tachyzoites were present even at the highest concentrations of either compound. Moreover, neither compound was toxic to extracellular tachyzoites since normal invasion and replication occurred following incubation of parasites in the presence of drug for 2 h prior to them being used for infection of Vero cells (Figure 3.12). In contrast, monensin reduced the ability of parasites to invade cells and replicate. This observation is not surprising since monensin, being an ionophore, alters the ionic balance across the parasite membrane thereby reducing the ability of extracellular parasites to invade cells. A similar observation was made by (Melton and Sheffield, 1975) contrasting the activity of monensin against lasalocid, a polyether antibiotic.

Parasiticidal compounds are ideal for short-term therapies since they act to completely inhibit parasite growth even after withdrawal of drug, whereas parasitistatic compounds inhibit parasite growth in the presence of drug but allow continued replication once the drug is removed. The anti-*Toxoplasma* effect of arprinocid and arprinocid-1-N-oxide appears to be parasiticidal, since the removal of drug after 24 h did not significantly alter their efficacies (based on $IC_{50}$ values). However, it may be argued that the compounds are parasitistatic and not parasiticidal. Even though arprinocid and arprinocid-1-N-oxide were washed from the culture medium it is possible that (i) the compounds had already been internalised into the host cell and could still exert an effect on the parasite (ii) the compounds were bound to host cell/parasite proteins and were not completely removed by washing or (iii) the compounds coated the surface of the host cell and, despite washing, damaged the parasite during invasion thereby inhibiting normal replication. This type of observation has been reported previously for other anti-*Toxoplasma* drugs, for example paclitaxel (Estes *et al.*, 1998). It would be interesting to

repeat this work using much shorter incubation times to obtain more information on how early during infection, arprinocid and arprinocid-1-N-oxide, exert their effects.

Treatment of tachyzoites with arprinocid-1-N-oxide (0.5 μM) as late as 48 h after infection was just as effective as when treatment occurred before infection of host cells. In contrast, arprinocid (50 μM) was slightly less effective 48 h post-infection compared with 1 h prior to infection. Since, it is the 1N-oxide metabolite that is active *in vivo* this data is consistent with the findings of Luft (1986) who demonstrated that treatment of *T. gondii* infected mice with arprinocid 72 h after infection resulted in complete protection of mice. Wang and co-workers (1979a) noted that radiolabelled arprinocid was incorporated into HeLa cells and reached equilibrium within the first minute of incubation. Such a rapid uptake by mammalian cells would explain the expeditious efficacy against intracellular *T. gondii* tachyzoites.

Efficacy against bradyzoites/tissue cysts is central to the development of novel anti-*Toxoplasma* drugs. Published data indicate that arprinocid-1-N-oxide is active *in vitro* against intact tissue cysts and bradyzoites (Huskinson-Mark *et al.*, 1991), although whether this activity translates into *in vivo* efficacy remains to be elucidated. Arprinocid has been shown to be effective in murine models of *Toxoplasma* and although *Toxoplasma* was noted in the brains of treated mice, the author does not make clear whether this refers to tissue cysts. Formation of cysts seams unlikely since the mice were infected with the non-cyst forming RH strain of *T. gondii*. Whilst it was part of the original objectives of this project, time limitations have prevented the further investigation of the efficacy of arprinocid and arprinocid-1-N-oxide against a cyst-forming strain using a mouse model. This information would be invaluable for future

testing of these compounds against toxoplasmosis. There has been no published data on the pharmacokinetic profile of arprinocid or arprinocid-1-N-oxide.

In conclusion both arprinocid and arprinocid-1-N-oxide are toxic to intracellular parasites, although the metabolite is significantly more potent. Both compounds act apparently irreversibly and within a short period of time, and are specific to *T. gondii* at therapeutic concentrations. This data provide the groundwork for the continued investigation of the mode of action of arprinocid and arprinocid-1-N-oxide, and the mechanism of acquisition of drug-resistance. Specifically, this comprised of an investigation into the protein expression differences between arprinocid-1-N-oxide-sensitive and -resistant parasites (Chapters 4 and 5) and an investigation of the mode of entry of these compounds into *T. gondii* tachyzoites (Chapter 6).

# CHAPTER 4

# Proteomics 1: Two-dimensional electrophoresis of *T. gondii* proteins

## 4.1 Introduction

### 4.1.1 Proteomics

Complete genome sequence or extensive expressed sequence tag (EST) databases are now available for many important medical and veterinary parasitic protozoa and are poised to revolutionise the study of gene expression in these organisms. In the past, analysing the expression of more than a handful of genes at any one time presented considerable practical difficulties. Much effort was previously concentrated on identifying parasite genes of interest by conventional cloning and sequencing as a first step to elucidating function. Technological breakthroughs such as the development of reverse transcription-polymerase chain reaction (RT-PCR) allowed analysis of mRNA expression between biological samples, but only for a few genes per assay. Now, many thousands of genes can be monitored in parallel to characterise the molecular events that occur during key biological processes such as parasite growth or differentiation. Genomics presents us with a new challenge: obtaining gene sequence is now less of a limiting step; rather, developing and employing methods for identifying which of the many thousands of genes expressed by an organism are relevant to a particular biological question has become a priority. The compelling desire for answers to

fundamental biological questions, a better understanding of disease processes and ultimately the need for more specific, less toxic therapeutic agents has led to the more recent development of "post-genomic technologies". The use of gene microarrays allows the quantitative measurement of global gene expression and how this varies between two samples. Such microarray technologies provide detailed information on gene expression in biological systems but, because there is not always a direct correlation of mRNA abundance with protein levels (Gygi *et al.*, 1999), microarrays do not allow definitive predictions about protein abundance. Furthermore, the 'one gene one protein' hypothesis is not supportable because of extensive alternative splicing, RNA editing and post-translational modification of proteins (Graveley, 2001). Although little information currently exists for *T. gondii* on such mechanisms and what they might mean for protein expression, it seems likely that they will also be important features in the control of protein function in this organism. Moreover, for very many genes the reliable attribution of function based on DNA sequence alone is simply not possible at present. Thus for *T. gondii*, as for most organisms, global analysis of mRNA, although extremely powerful, has limitations. Direct studies at the level of the functional proteins themselves are required to support mRNA analyses.

Analysis of the 'PROTEin complement expressed by a genOME', termed proteomics (Wasinger *et al.*, 1995), has become a key technology (or collection of technologies) and a method of choice for the investigation of expressed gene products. For example, it has applications in the analysis of protein expression changes during cellular processes such as cell differentiation, the detection of disease markers perhaps in cancer studies, the validation and characterisation of novel therapeutic targets with the investigation of the mode-of-action of drugs (Araki *et al.*, 2000; Jungblut *et al.*, 1999; Page *et al.*, 1999). Proteomic methods have evolved from established protein separation techniques such as

2-dimensional electrophoresis (2-DE) to encompass both the rapid identification of polypeptides by mass spectrometry (MS) and the development of bioinformatic methods developed to correlate enzymatic digest mass fingerprints and peptide fragmentation data with genome sequence (Parker, Garrels, et al., 1998 153 /id; Parker, Garrels, et al., 1998 153 /id; Patterson *et al.*, 2001). A schematic representation of these three areas is illustrated in Figure 4.1. The combination of physical protein data from mass spectrometry with genome data enables unambiguous identification of proteins from 2-DE gels, and characterisation of post-translational protein modifications, much faster and many times more economically than conventional peptide sequencing, such as Edman degradation.

### 4.1.2 History of two dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) is a powerful technique for the analysis of complex mixtures of proteins from tissues, cells or other biological samples. Proteins are separated in two discrete steps. Smithies and Poulik (1956) performed the first 2-DE separation using paper electrophoresis and starch gel electrophoresis in succession to analyse serum proteins. The subsequent development of isoelectric focussing (IEF) for separation of proteins in the first dimension based on their charge properties and the introduction of polyacrylamide gel electrophoresis (PAGE) together with the discontinuous SDS gel system (Laemmli, 1970) resulted in an enhanced 2-DE technique. This method development eventuated nearly two decades later in the electrophoretic separation, with increased resolution and reproducibility, of exocrine pancreatic proteins (Scheele, 1975), mouse tissue and serum proteins (Klose, 1975) and notably over 1100 proteins of *E. coli* (O'Farrell, 1975). Although this type of 2-DE was first used in 1975, further improvements in the solubilisation of proteins and the

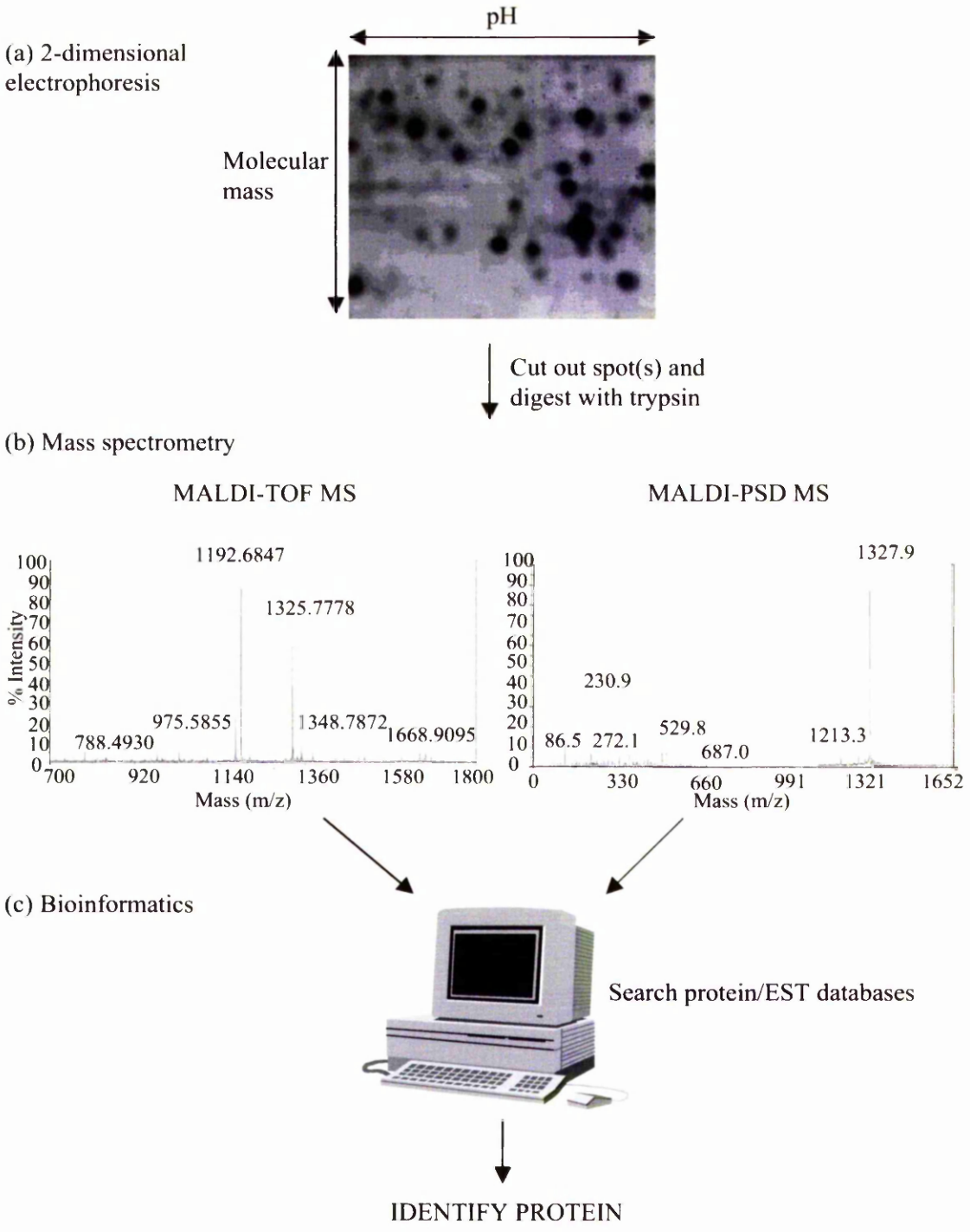



**Figure 4.1.** Schematic representation of the key areas of proteomics. Proteomics consists of three distinct areas: (a) separation of proteins by 2-dimensional electrophoresis in terms of their isoelectric points and molecular masses; (b) mass spectrometry, for example MALDI-TOF to obtain a peptide mass fingerprint (PMF) profile and MALDI-PSD to obtain peptide fragmentation data; (c) PMF and peptide fragmentation data may be used to search protein/EST databases for matching theoretical digest patterns (bioinformatics).

reproducibility and resolving power of the electrophoretic steps have together enabled co- and post-translational modifications to be detected.

There is a tremendous wealth of information 'locked' into a 2-DE gel and the 'key' is to access, understand and store these data. Information on individual proteins such as pI and molecular mass, the numbers of expressed proteins and their level of expression may be obtained from a relatively simple analysis of gels. Further analysis, often with the aid of specialised computer software, allows more detailed examination of the protein complement.

These enhanced methods, coupled with mass spectrometric analysis of in-gel digested proteins, can now be used to characterise the complete proteome of some organisms including *Saccharomyces cerevisiae* (Shevchenko *et al.*, 1996) and *Haemophilus influenzae* (Fountoulakis *et al.*, 1998a; Fountoulakis *et al.*, 1998b). The application of proteomics to parasitological problems is now a real possibility (Barrett *et al.*, 2000; Ashton *et al.*, 2001). Although far less advanced, proteome analysis in some parasitic organisms has been already performed, e.g. *Trypanosoma brucei* and *Plasmodium falciparum* (Huang *et al.*, 2001; Rabilloud *et al.*, 1999; http://www.ebi.ac.uk /parasites/proteomes.html).

2-DE now normally comprises, in the first dimension, isoelectric focussing (IEF) using the recently developed immobilised pH gradient (IPG) strips to separate proteins according to their isoelectric points (pI) and, in the second dimension, SDS-PAGE to separate proteins according to their molecular weights (refer to Sections 4.1.3 and 4.1.4, respectively).

### 4.1.3 The first dimension: Immobilised pH Gradients

Proteins are amphoteric molecules having positive, negative or zero charge depending on the pH of their surroundings. The net charge of a protein, i.e. the sum of all the charges of their constituent amino acids, is zero when at the isoelectric point (pI). Isoelectric focussing (IEF) relies on the presence of a pH gradient to separate proteins. Under the influence of an electric field, a positively charged protein will move to where its net charge is zero, in this case towards the cathode. The opposite is true, a negatively charged protein will move towards the anode until its net charge is zero. Once proteins have migrated to their pI value they will stay there until the electric field is removed. The degree of resolution of protein separation depends on two aspects: (i) the pH gradient in the separation medium; (ii) the size of the electric field. Throughout the late 1970's and early 1980's carrier ampholytes were used to generate pH gradients in rod gels. Carrier ampholytes are small, soluble amphoteric molecules that move towards their pI value in the same fashion as described for proteins. However, carrier ampholytes were not well characterised and suffered from batch-to-batch variations during synthesis. Since the molecules were not fixed within the IEF gel, prolonged focussing resulted in the migration of water (electroendosmotic flow) and a shift in the pH gradient and, therefore, a shift in proteins' positions. In addition, the difficulty in handling rod gels without breakage or stretching meant that 2-DE using this technology was neither reproducible nor practical for separation of thousands of proteins (Dunn and Görg, 2001). These difficulties were overcome by the introduction of fixed (immobilised) pH gradient (Bjellqvist *et al.*, 1982) based on acrylimido buffers (Amersham Pharmacia Biotech Immobine™ chemistry). In contrast to carrier

ampholytes, Immobine™ buffers are well-characterised molecules, each having an acidic or basic group bound to an acrylamide monomer (Figure 4.2).

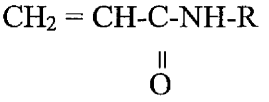


**Figure 4.2.** Structure of acrylamido buffer (Immobine™ buffer). Each molecule has a defined acidic or basic group (R) attached to an acrylamide monomer.

Mixtures of these buffers are combined during the polymerisation of the immobilised pH gradient (IPG) gels. The advantages of IPG strips are: (i) gels are polymerised onto a plastic backing thereby preventing stretching of the gel; (ii) the gel gradient is fixed and thus not susceptible to the effects of electroendosmosis; and (iii) different pH ranges may be cast. IPG strips, supplied as dehydrated gels to allow long-term storage, are rehydrated, usually in the presence of a protein sample, and the proteins focussed using a flatbed electrophoresis unit under large voltages, typically 5000 V or more. A potential difference of this magnitude is required to obtain sharply focused bands. The number of volt-hours (Vh, a standardised method to describe the length of time of IEF) used for IEF depends on the type of IPG strip used, typically 32,500 Vh for pH 4 -7 and longer for narrow and basic pH ranges. The advantage of using a flatbed apparatus is that strips may be kept at a constant temperature (usually 20°C, since urea starts to crystallise at lower temperatures) using Peltier cooling chips. A good review of 2-DE

using IPG strips (Gorg *et al.*, 2000) describes in more detail the advantages of this new technology.

### 4.1.4 The second dimension: SDS-PAGE

Following IEF, the IPG strips and proteins are equilibrated in SDS buffer containing DTT. Proteins must be bound to SDS to negate the effect of protein charge during protein separation in the second dimension. DTT is a charged molecule and is ionised during IEF causing migration of DTT to the anode. Thus, DTT disperses from the proteins during IEF. Replacement of this DTT ensures disulphide bonds remain broken and improves transfer to the second dimension (Herbert *et al.*, 1998). However, DTT causes point streaking that is often observed subsequent to silver staining. Thus a second equilibration is performed in the presence of SDS and iodoacetamide. Iodoacetamide alkylates excess DTT reducing the transfer of DTT into the gel (Gorg *et al.*, 1987). Proteins are separated in the second dimension according to molecular weight by SDS-PAGE using either flatbed or vertical formats. Vertical systems are more convenient for running multiple gels simultaneously. In addition vertical units allow casting (or ready-made) gels of reduced thickness, typically 0.5 - 1 mm. Higher voltages may be applied resulting in increased resolving power of spots due to less diffusion.

### 4.1.5 Visualisation of proteins

Subsequent to separation on the 2$^{nd}$ dimension, proteins are visualised by traditional methods such as Coomassie- or silver-staining or alternatively by the recently introduced fluorescent stains such as Sypro Ruby® (Molecular Probes). The advantages

and disadvantages of these staining techniques will be discussed in this Chapter, in relation to the sensitivity of detection of proteins, and in Chapter 5, in relation to mass spectrometry.

## 4.2 Aims

Many parameters affect the resolution, separation and reproducibility of 2D-gels. The most critical are the choice of equipment, sample solubilisation procedures, running conditions (e.g. duration of IEF, pH separation range) and method of protein staining. The main objectives of this chapter were to optimise the 2-DE separation of proteins from *T. gondii* tachyzoites and to answer the following questions:

(i) How suitable is 2-DE for determining protein expression differences that are potentially associated with parasite virulence. For example, can it be used to characterise an attenuated vaccine line, such as S48 *T. gondii*?

(ii) The function of a gene can be investigated by creating gene knockout strains. For example, analysis of the gene encoding for HXGPRT was demonstrated to be non-essential for *T. gondii* survival by creating a null mutant (RH-HX$^-$). However, there are consequences at the protein level of gene manipulation. Can 2-DE provide useful information on changes in protein expression as a result of gene manipulation?

(iii) The mechanism(s) of acquisition of drug resistance in ARP$^R$ *T. gondii* is not known; it is possible resistance may be mediated by the up-regulation of a detoxification pathway or a modified drug transporter or changes in the drug target itself. The latter introduce a new question; a point mutation resulting in an amino acid change may cause the dysfunction of a drug transporter or lack of binding to the drug target. Can 2-DE detect protein modifications that relate to drug resistance?

Bioinformatics is a major aspect of this work since the ability to identify proteins of interest is paramount to exploiting the full potential of proteomics. This work, in conjunction with the development of strategies for the identification of *T. gondii* tachyzoite proteins by mass spectrometry (see Chapter 5), was intended for the production of a preliminary 2D-map of the *T. gondii* proteome.

## 4.3 Methods

Two methods for 2-DE were used. The first method used the Investigator™ 2D gel system from Genomic Solutions (GS) and the second method used the IPGhor IEF unit and Hoefer DALT tank system purchased from Amersham Pharmacia Biotech (APB).

### 4.3.1 2D gel electrophoresis using the Investigator™ system (GS)

#### *4.3.1.1 Preparation of tachyzoites for 2-DE*

Frozen tachyzoite pellets were disrupted in 40 µl of sample buffer I (0.3 % SDS, 200 mM DTT, 50 mM Tris-HCl, pH 8.8). Proteins were denatured at 95°C for 5 min and incubated on ice for 10 min after the addition of 4 µl sample buffer II (500 mM Tris-HCl, 50 mM $MgCl_2$, 2000 units/ml DNAse I and 750 units/ml RNAse A). Where indicated, proteins were sonicated and/or subjected to three cycles of freezing in liquid nitrogen and thawing in a gloved clenched fist. Solubilisation was enhanced further by the addition of 160 µl loading buffer (8 M urea, 4 % CHAPS, 40 mM Tris base, 6.5 mM DTT and 0.01 % bromophenol blue) to each sample. Rehydration buffer (8 M urea, 2 % CHAPS, 10 mM DTT, 2 % ampholytes and 0.01 % bromophenol blue) was then combined with sample to a total volume of 400 µl. Samples were vortexed vigorously for 1-2 min at each step. Finally, insoluble proteins were removed by centrifugation at 16,000 x g at 4°C for 3 min.

*4.3.1.2 Isoelectric focussing and SDS-PAGE*

Proteins were separated in the first dimension using the pHaser™ Isoelectric Focusing System (Genomic Solutions, USA). Solubilised proteins (400 µl) were pipetted into each groove of the pHaser tray and an 18 cm IPG strip (Amersham Pharmacia Biotech) placed gel-slide down over the sample. IPG strips were rehydrated for 24 h and the proteins focussed to their isoelectric points using the following parameters: max voltage, 5000 V; duration, 24 h 30 min; holding voltage, 124 V; and 80 µA/gel. Following isoelectric focusing, gels were equilibrated for 15 min in 10 ml equilibration buffer I (GS) and for 15 min in 10 ml equilibration buffer II (GS). Proteins were separated in the second dimension using the 2-DE Running System (GS). The lower tank (anode) was filled with 11 L of buffer containing 25 mM tris/acetate pH 8.3 and the upper tank was filled with 3 L of buffer containing 0.2 M tris, 0.2 M tricine and 0.4 % SDS. During electrophoresis, the tank buffers were maintained at 10°C by peltier cooling. Focussed IPG strips were loaded on the top surface of precast 10 % homogeneous gels (GS), of dimensions 22 cm width x 22 cm length x 1 mm gap thickness. Proteins were separated according to molecular weight at 500 V (20 W maximum per gel) for 4.5 h.

**4.3.2 2D gel electrophoresis using the IPGphor™ & Hoefer™ DALT (APB)**

*4.3.2.1 Preparation of tachyzoites for 2-DE*

Two methods (i and ii) of solubilisation were utilised.

(i) Protein from 1 x $10^8$ tachyzoites was initially solubilised in 40 µl of 0.1 % SDS in 40 mM Tris and subjected to 3 cycles of rapid freezing using liquid nitrogen and thawing in a gloved clenched fist followed by a denaturing step at 95°C for 5 min. Solubilisation was further improved by the addition of 40 µl lysis buffer containing 8 M urea, 4 % CHAPS and 40 mM Tris. Solubilisation was aided by sonication at room temperature for 5 min in a water bath sonicator (Ultrasonics Ltd, UK) and the addition of rehydration buffer, to a final sample volume of 350 µl, containing 8 M urea, 2 % w/v CHAPS, 22 mM DTT (added fresh), 0.5 % v/v IPG buffer (Amersham Pharmacia Biotech) and bromophenol blue. At the end of the procedure, insoluble material was removed by centrifugation at 16,000 × g for 3 min at 4°C.

(ii) Frozen tachyzoite pellets (1 x $10^8$) were disrupted by the addition of 40 µl lysis buffer (described above) and subjected to 3 cycles of rapid freezing using liquid nitrogen and thawing in a gloved clenched fist, followed by sonication at room temperature for 5 min in a water bath sonicator (Ultrasonics Ltd). Solubilisation was aided by the addition of 310 µl rehydration buffer (described above). Insoluble material was removed by centrifugation at 16,000 × g for 3 min at 4°C, as described.

Since the total amount of protein that can be rehydrated into an IPG strip depends upon the pH range to be analysed and also the method of visualising, protein loading was first optimised. For pH 4-7, pH 6-11 and narrow range pH 5.5-6.7 linear IPG strips, 5 x $10^7$ tachyzoites (approximately 100 µg protein) were used for each gel stained with silver and 1 x $10^8$ tachyzoites for each Coomassie Blue-stained gel. Preparative Coomassie Blue-stained gels for mass spectrometry were loaded with 2 x $10^8$ tachyzoites.

*4.3.2.2 Multiple casting of large-format gels*

Gels (23 cm width x 19 cm length x 1 mm gap thickness) were cast using the Hoefer™ DALT Multiple Gel Caster (Amersham Pharmacia Biotech). All apparatus and gel cassettes were thoroughly cleaned using 18 MΩ water before gel casting was begun. The gel caster was assembled as described in the manufacturer's manual inserting the recommended number of filler blocks and separator sheets to enable 12 gels to be cast simultaneously. A feed tube and funnel were connected to the bottom of the balance chamber, which was filled with 150 ml of displacing solution (see below for recipe). The gel solution was prepared as outlined in Table 4.3.1 (without APS and TEMED) and degassed using a vacuum pump for 15 minutes whilst being stirred slowly. The appropriate volumes of TEMED and APS were added to the gel solution to begin polymerisation and gel mix slowly poured into the funnel (taking care to minimise air bubbles) and thus into the gel casting unit. Once pouring was complete, the feed tube was removed and the displacing solution allowed to 'push' the remaining gel solution into the casting block. If necessary, more displacing solution (up to 50 ml) was added to the balance chamber in order to raise the level of the gel solution in the gel cassettes to the appropriate height. Immediately after pouring, the surface of each gel was overlaid with 0.75 ml of water-saturated *n*-butanol resulting in a level gel surface. The gel casting block was covered with damp towels and the gels left to polymerise overnight (tests using small amounts of gel solution indicated polymerisation was complete in approximately 1 hour). After polymerisation, the surface of each gel was washed with ddd$H_2O$ to remove *n*-butanol and unpolymerised acrylamide. The gel cassettes were also cleaned in ddd$H_2O$ and stored in a polystyrene box at 4°C. Gels, covered with

damp towels to prevent drying out and cracking, were stored for no longer than 14 days before use.

**Table 4.3.1.** Recipe for casting 12 large format gels. Gels have dimensions 23 cm width x 19 cm length x 1mm thickness and are cast using Hoefer™ DALT Multiple Gel Caster (Amersham Pharmacia Biotech).

| Solution | Volume (ml) |
|---|---|
| Acrylamide 40 % | 192.8 |
| N-N'-methylenebisacrylamide 2% | 102.9 |
| 1.5 M Tris-HCL pH 8.8 | 190.2 |
| 18 MΩ $H_2O$ | 265.9 |
| 10 % SDS | 7.71 |
| 10 % APS* | 7.71 |
| 10 % TEMED* | 1.35 |

*added immediately before gel pouring

**Displacing Solution:** 0.375 M Tris-HCl, 50 % glycerol, trace bromophenol blue.

**Overlay solution:** 50 ml *n*-butanol, 5 ml ddd$H_2O$.

*4.3.2.3 Isoelectric focussing and SDS-PAGE*

Except where stated, all electrophoretic equipment, gel analysis equipment and reagents for 2-DE were supplied by Amersham Pharmacia Biotech Ltd, UK. Proteins were separated in the first dimension using the IPGhor™ Isoelectric Focusing (IEF) system employing immobilised pH gradient (IPG) strips. *T. gondii* proteins were focused to their isoelectric points using 18 cm Immobiline DryStrips™ according to the conditions in Table 4.3.2. Rehydration and IEF were performed at 20°C. Following IEF, the proteins were reduced and bound to SDS by equilibrating each strip for 15 min in 10 ml of SDS Equilibration Buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % v/v glycerol, 2

% w/v SDS) containing 100 mg DTT (added fresh before use). A second equilibration step in SDS Equilibration Buffer containing 250 mg iodoacetamide (added fresh before use) instead of DTT was performed in order to prevent protein re-oxidation and to minimise reactions of cysteine residues. After equilibration, the IPG strips were loaded onto 10 % homogeneous acrylamide gels (1 mm thickness x 24 cm width x 19 cm length), sealed with 0.5 % agarose (SeaKem) and proteins electrophoresed for 20 h at 100 V and 11°C.

**Table 4.3.2.** Isoelectric focusing steps used to focus 4-7 linear (50 μA/strip) and 6-11 linear (30 μA/strip) IPG strips.

| IPG | Step | Voltage | Duration (h) | Gradient type |
|---|---|---|---|---|
| 4-7 linear | Rehydration | - | 12 | - |
| | 1 | 500 | 1 | Stepped |
| | 2 | 1000 | 1 | Stepped |
| | 3 | 8000 | 4* | Stepped |
| 6-11 linear | Rehydration | 30 | 12 | Stepped |
| | 1 | 500 | 1 | Stepped |
| | 2 | 1000 | 1 | Stepped |
| | 3 | 8000 | 0.5 | Linear |
| | 4 | 8000 | 6* | Stepped |

*These steps were extended as necessary until the final volt-hours reached for the 4-7 linear and 6-11 linear IPG strips were a minimum of 32500 Vh and 55000 Vh, respectively.

### 4.3.3 Visualisation of proteins

#### *4.3.3.1 Coomassie-Blue staining*

Gels were soaked in 350 ml Coomassie stain overnight and destained using several washes of Coomassie destain (350 ml) until the background was clear.

**Coomassie stain (1 L):** 0.5 g Coomassie Brilliant Blue R250, 100 ml glacial acetic acid, 125 ml isopropanol.

**Coomassie destain:** 10 % glacial acetic acid, 12.5 % methanol, 77.5 % $dddH_2O$.

*4.3.3.2 Silver stain kit (APB, Heukeshoven and Dernick, 1985)*

Gels were stained using the Plusone™ Protein Silver Staining Kit (Amersham Pharmacia Biotech) and Hoefer™ Automated Gel Stainer (Amersham Pharmacia Biotech). Proteins were first fixed for 30 min in Fixative Solution and then sensitised for 30 min in Sensitiser Solution. Gels, washed 3 x 5 min in 18 MΩ water, were soaked in Silver Solution for 20 minutes and then washed 2 x 1 min in 18 MΩ water. Gels spots were visualised by soaking the gel in Developer Solution for between 4 and 5 min depending on the quantity of protein present and presence/absence of glutaraldehyde. When developing was finished, gels were soaked in Stop Solution for 10 min and washed 3 x 15 min in 18 MΩ water. All solutions (325 ml per gel) were made using 18 MΩ water. This protocol is tabulated in more detail, for comparison with other stains used, in Table 4.4.3.

For mass spectrometry of silver-stained protein spots, gels were washed twice in fixative and glutaraldehyde was omitted from Sensitiser Solution. Gels, which were to be subsequently analysed by mass spectrometry, were stored in 1 % acetic acid until protein extraction was performed. Other analytical gels for long-term storage were soaked in preserving solution, heat-sealed in plastic wrap and stored at 4°C.

*4.3.3.3 Silver stain kit (Genomic Solutions, (Rabilloud, 1992)*

Proteins were fixed, for 1 h, in Fixative Solution and then sensitised for 60 min or overnight in Sensitiser Solution. Gels, washed 4 x 15 min in 18 MΩ water, were placed in 500 ml Silver Solution for 30 min and excess silver removed by washing 1 x 1 min in 18 MΩ water. Development of gels typically took between 5 and 10 min and was stopped by placing each gel into Stop Solution for 10 min. All solutions (500 ml per gel) were made using 18 MΩ water. Gels were placed into 20 % glycerol preservative for 10 min and stored as described in section 4.3.3.2. This protocol is tabulated in more detail in Table 4.4.3.

*4.3.3.4 Morrisey silver stain (Morrisey, 1981)*

Proteins were fixed in Fixative Solution for 1 h, washed 3 x 15 min in 18 MΩ water to remove SDS and fixative, and then soaked in DTT Solution for 20 minutes. Gels were placed in Silver Solution for 20 min and rinsed in 18 MΩ water for 5 min. Subsequently gels were washed in 300 ml of Developing Solution for 30 s and then immersed in fresh Developer Solution. Development of gels typically took between 5 and 10 min and was stopped by placing each gel into Stop Solution for 5 min and then rinsing the gels in 18 MΩ water for 10 min. All solutions (500 ml per gel) were made using 18 MΩ water. Gels were preserved and stored as described in section 4.3.3.2. This protocol is tabulated in more detail in Table 4.3.3.

**Table 4.3.3.** Silver-staining protocols for visualisation of proteins.

| Method / Step | PlusOne™ silver stain Kit[1] | Genomic Solutions Kit[1] | Morrisey stain[1] |
|---|---|---|---|
| **Fixative Solution** | 40 % ethanol<br>10 % glacial acetic acid<br><br>*30 min* | 40 % ethanol<br>10 % glacial acetic acid<br><br>*60 min* | 50 % methanol<br>10 % glacial acetic acid<br><br>*60 min* |
| **Sensitiser Solution** | 30 % ethanol,<br>5 ml 25 % glutaraldehyde<br>40 ml 5% sodium thiosulphate<br>68 g sodium acetate<br><br>*30 min* | 10 ml 50 % glutaraldehyde<br>30 % ethanol<br>2.5 g potassium tetrathionate<br>68 g sodium acetate<br><br>*60 min. or overnight* | |
| **Wash, 18 MΩ $H_20$** | 3 x 5 min | 4 x 15 min | 3 x 15 min |
| **DTT Solution** | | | 5 mg DTT<br><br>*20 min* |
| **Silver Solution** | 10 ml 2.5 % silver nitrate<br>0.4 ml 37 % formaldehyde<br><br>*20 min* | 2 g silver nitrate<br>0.25 ml formaldehyde<br><br>*30 min* | 2.04 g silver nitrate<br><br>*20 min* |
| **Wash, 18MΩ $H_20$** | 2 x 1 min | 1 x 1 min | 1 x 5 min |
| **Developer Solution** | 25 g sodium carbonate<br>0.2 ml 37 % formaldehyde<br><br>*approx. 4 - 5 min* | 30 g potassium carbonate<br>0.15 ml formaldehyde<br>7.5 mg sodium thiosulphate<br><br>*approx. 5 - 10 min* | 34.7 g sodium carbonate<br>0.5 ml formaldehyde<br><br>300 ml for 30 s and then replace with fresh 500 ml<br><br>*approx 5 - 10 min* |
| **Stop Solution** | 14.6 g EDTA-$Na_2 \cdot 2H_2O$<br><br>*10 min* | 50 g Tris base<br>20 ml glacial acetic acid<br><br>*10 min* | 30 ml glacial acetic acid<br><br>*5 min* |
| **Wash, 18MΩ $H_20$** | 3 x 15 min | | 1 x 10 min |

[1] Reagents required for 1 L.

### 4.3.4 Image analysis

Images of gels were acquired at 300 dpi using a Labscan v3.0 software (Amersham Pharmacia Biotech) on a Umax flatbed scanner (OD max 3.4) with integrated transparency adapter. Images were analysed using ImageMaster® 2D v3.01 software (Amersham Pharmacia Biotech).

## 4.4 Results

### 4.4.1 Sample preparation and resolution of 2-DE separations (GS)

In retrospect of the quality of the gels run using the Amersham Pharmacia Biotech (APB) system and regardless of the staining technique, gels run using the Genomic Solutions (GS) system contained fewer protein spots. Moreover, the resolution, in terms of how rounded spots were, was comparatively inferior.

Sample preparation is critical to the outcome of 2D-gels and was therefore investigated by comparing the manufacturers' (GS) recommended sample preparation protocol with the non-chemical lysis methods, such as sonication and/or freezing in liquid nitrogen and thawing (Figure 4.3 a-c). The GS sample preparation protocol (and silver staining using the GS silver stain kit) resulted in the detection of just 161 protein spots on a pH 3 - 10 non-linear gel (Figure 4.3a). Automatic and manual detection of spots using ImageMaster® 2D was made more difficult because of the overall poor quality of the gel. Several well-resolved proteins may be seen in the acidic region of the gel, although there were increased numbers of low molecular weight proteins and better resolution of spots between pH 7 and 10. With the intention of further improving protein solubilisation and thus enhancing separation, the sample preparation protocol was modified to include three cycles of rapid freezing in liquid nitrogen and thawing (Figure 4.3b). Although the quality of the gel appears to have improved because the proteins were more strongly stained, the total number of protein spots (165 spots) was similar to the previous gel (Figure 4.3a). Accurate and consistent developing of gels using an automatic gel stainer was not available at the time of this analysis. As with the

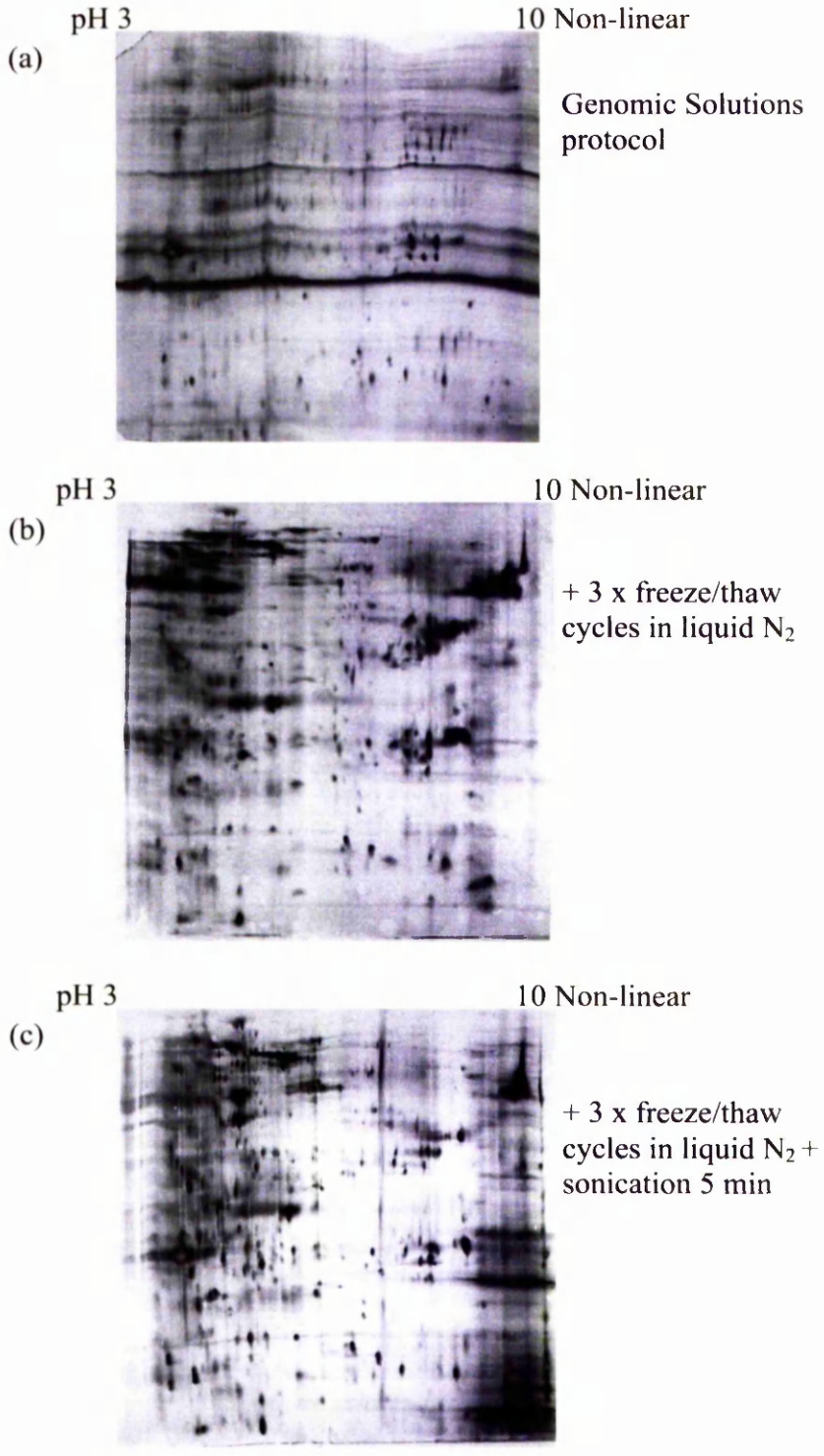



**Figure 4.3.** 2-DE separation of S48 *T. gondii* tachyzoites ($1.2 \times 10^8$) using three different solubilisation protocols (Genomic Solutions equipment). (a) Standard procedure (refer to Section 4.3.1.1). (b) Samples were subjected to 3 cycles of freezing in liquid nitrogen and thawing subsequent to the addition of lysis buffer. (c) Samples were sonicated in a water bath at 22°C for 5 min in addition to the method in (b).

GS sample solubilisation method (Figure 4.3a), spots using this modified method were poorly resolved, particularly in the high molecular weight and acidic regions of the gel. The procedure for sample preparation was subsequently modified to include sonication of the protein sample in addition to three cycles of freezing in liquid nitrogen and thawing (Figure 4.3c). In this experiment, a larger number of resolved proteins (194 spots) were visualised, including several in the high molecular weight area of the gel. The overall quality of the gel was improved. Other variations of sample solubilisation methods were tested, including osmosis of cells using $dddH_2O$ and omission of SDS from the lysis buffer (data not shown). These gels were not significantly improved compared with the gel shown in Figure 4.3c.

### 4.4.2 Assessment of three silver-staining protocols and the reproducibility of 2-DE gels (GS)

It was considered that the limited number of proteins detected might be as a consequence of lack of sensitivity of the silver stain. Therefore, three staining methods were compared: (i) The Genomic Solutions Silver-Stain Kit (used above) resulted in low background gels with a good intensity of proteins staining (Figure 4.4a); (ii) As a cost-saving initiative, the Genomic Solutions Silver-Stain Kit, based on Rabilloud's stain (1992), was attempted using 'home-made' chemical solutions. There was no increase in the number of proteins detected compared to the GS Kit (Figure 4.4b). The developing stage lasted 45 minutes whereas the GS Kit takes approximately 5 min; (iii) The Morrissey stain (1981) afforded no increase in sensitivity of detection of proteins (Figure 4.4c).

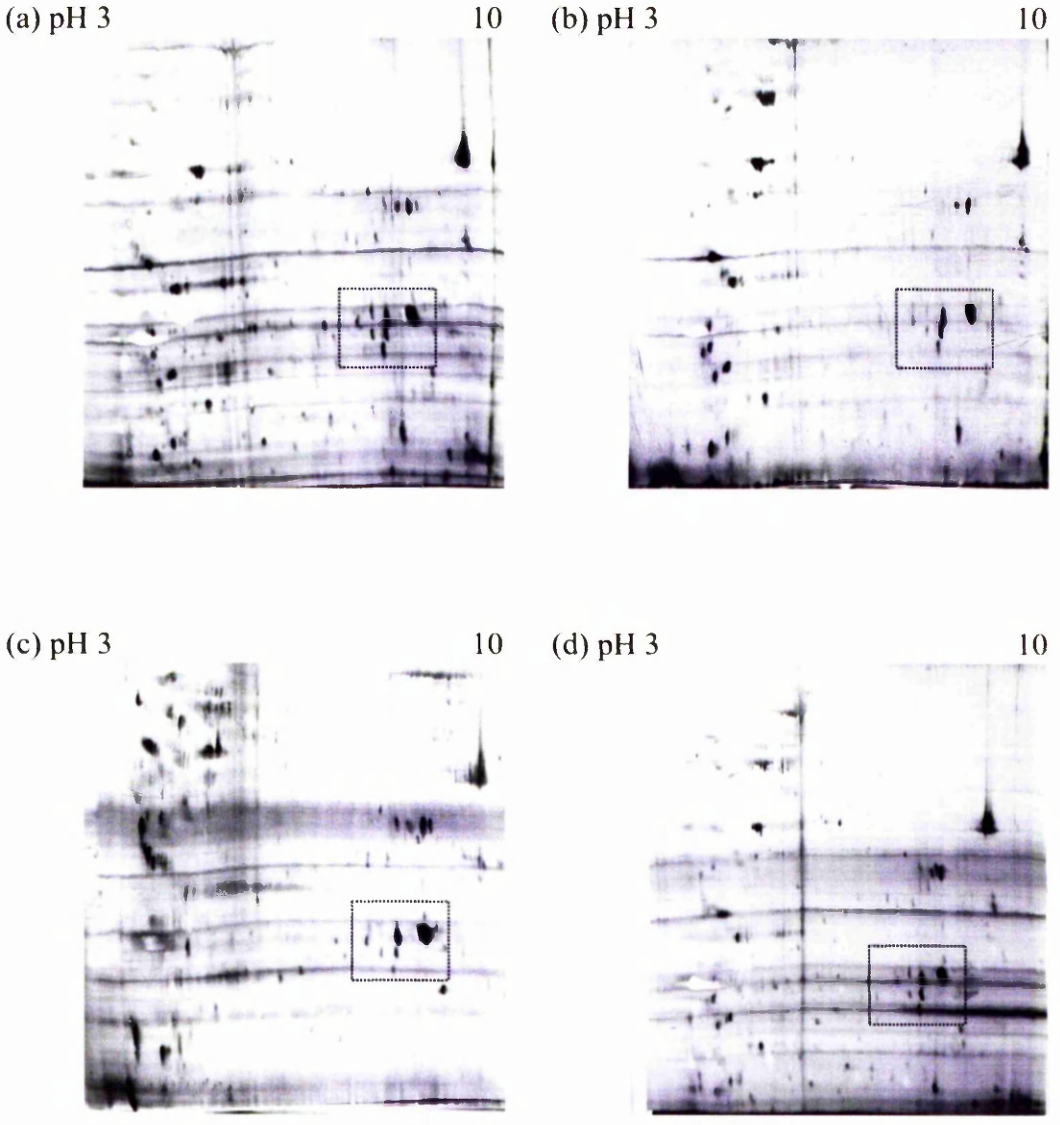



**Figure 4.4.** Comparison of three different silver stain methods for visualisation of proteins separated by 2-DE (Genomic Solutions equipment). (a) Genomic Solution Kit (b) 'Home-made' preparation of chemicals used in 'a' (c) Morrisey stain. The reproducibility of the Silver-Stain Kit was tested using a different preparation of cells (d). The dotted box represents an area which was found to be reproducible between the gels shown in a, b, c and d.

The reproducibility of gel separation and silver-staining using the GS Silver Stain Kit was assessed by analysing a second batch of *T. gondii* proteins prepared from a subsequent passage. Although the quality of the gel photograph and scanned image is less good, *in silico* analysis of the gels highlighted some areas of high reproducibility between gels (Figure 4.4d, boxed area).

The inability to separate and visualise large numbers of *T. gondii* proteins together with the apparently low resolving power of the GS system was of concern. Therefore, the equipment was changed to that supplied by Amersham Pharmacia Biotech (APB).

### 4.4.3 Sample preparation, resolution and sensitivity of 2-DE separations (APB)

Two methods of sample preparation (described in Section 4.3.2.1) were compared using the APB equipment (Figure 4.5). Analysis of proteins solubilised by the first method (i) and separated using pH 3-10 non-linear IPG strips detected 436 proteins whilst method (ii) detected 592 proteins. Protein spots separated using each method (i and ii) were highly resolved and significantly better than the 2-DE separations performed using the GS equipment. For all subsequent analyses, method ii was chosen since the greater numbers of spots were obtained using this solubilisation protocol.

The sensitivity of 2-DE analysis of *T. gondii* tachyzoites was investigated by determining the number of resolved protein spots detected on Coomassie- and silver-stained gels over a range of pH separations (Table 4.4.1). Figure 4.6(a) shows typical gels of tachyzoite proteins separated over pH ranges 4-7 linear (panel i) and 6-11 linear

(panel ii) and stained using the APB Silver-Stain Kit. Gels were analysed by a combination of automatic spot detection using digital images processed by ImageMaster® 2D software and manual detection. As expected, silver-staining proved more sensitive than Coomassie staining (Figure 4.7 a and b); but these differences could in part be lessened by increasing the number of tachyzoites loaded onto Coomassie-stained pH 4 - 7 linear gels. For instance, an average of 514 polypeptide spots were resolved on Coomassie gels loaded with 2 x $10^8$ tachyzoites (416 μg protein) compared with an average of 630 spots on equivalent silver-stained gels which had been loaded with 0.5 x $10^8$ parasites (104 μg protein) (Table 4.4.1). Although silver-stained gels were generally significantly more sensitive than Coomassie-stained gels, in a small number of instances Coomassie staining detected proteins not seen with silver staining. With both silver- and Coomassie-stained gels, further increases in the number of tachyzoites added to each gel resulted in some loss of resolution of the more abundantly expressed proteins, although weaker protein spots were detected more readily. The most striking increase in sensitivity and resolution was achieved when proteins were separated on narrow range IPG strips. For example, over 500 protein spots were resolved on a silver-stained gel over just a single pH unit using the pH 5.5-6.7 IPG strip (Figure 4.8).

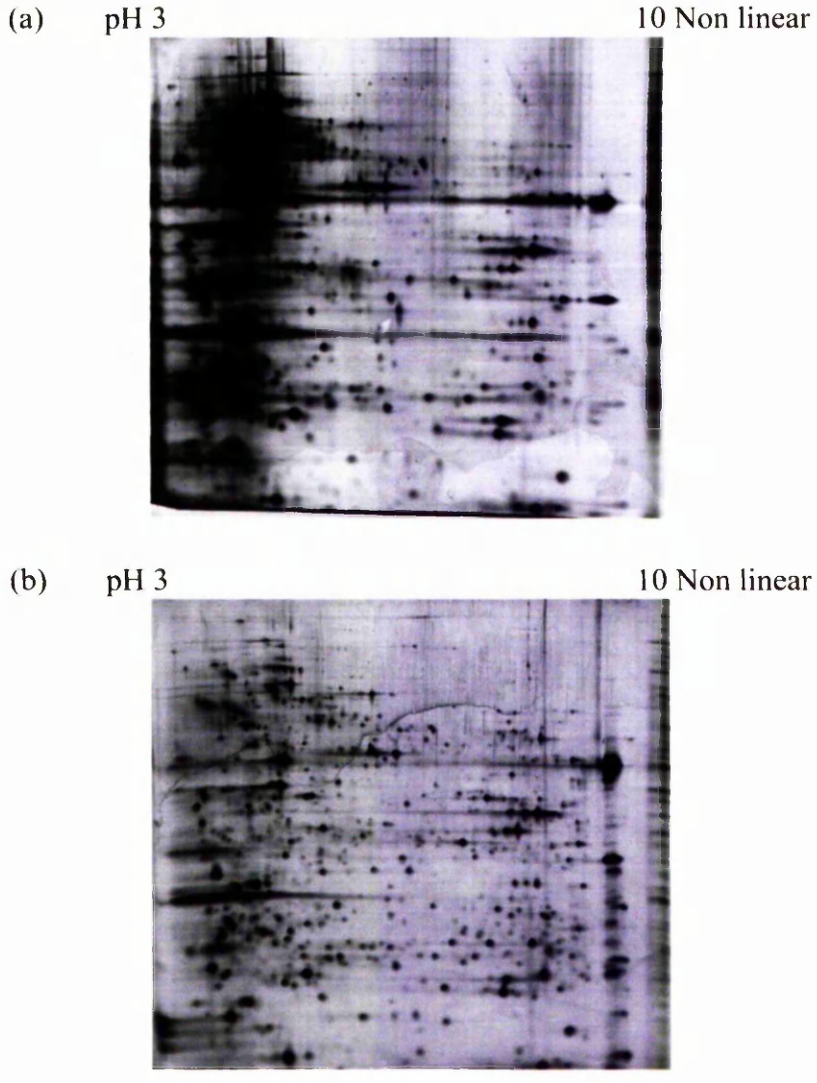



**Figure 4.5.** Comparison of two sample solubilisation protocols (Amersham Pharmacia Biotech equipment). (a) Manufacturer's recommended protocol (refer Section 4.3.2.1i). (b) Same method, except solubilisation was enhanced by freezing in liquid nitrogen and thawing, and sonication in a water bath (refer Section 4.3.2.1 ii).

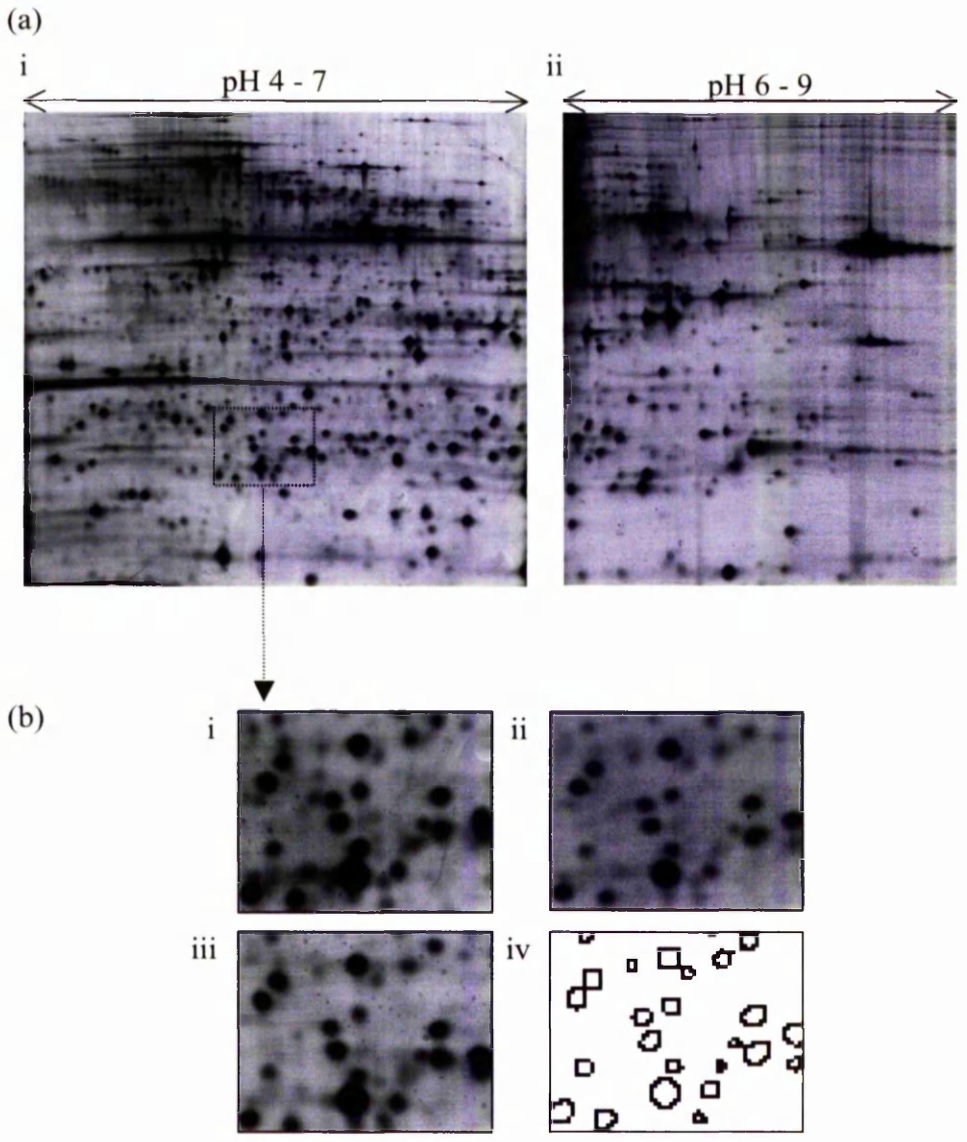



**Figure 4.6.** (a) 2-DE gel separations of proteins from 5 x $10^7$ *T. gondii* RH strain tachyzoites. Proteins were focused to their isoelectric points using 18 cm Immobiline pH Gradient strips. Panels (i) and (ii) show typical pH 4-7 linear and pH 6–11 linear gels, respectively. Proteins were separated in the second dimension according to molecular mass using a 10 % (w/v) homogeneous SDS-PAGE gel (24 cm x 19 cm). The proteins were visualised by silver staining. (b) Reproducibility of the sample preparation, gel electrophoresis and staining. Each panel (i-iii) represents the same enlarged region (dotted box) of replicate silver-stained gels. Each of the 3 gels were run independently on different days using different batches of prepared tachyzoites and using different batches of poured gels. A digital composite image of all three gels created using ImageMaster® 2D software is shown in panel (iv) using only spots present in all three replicate gels to produce an "averaged gel".

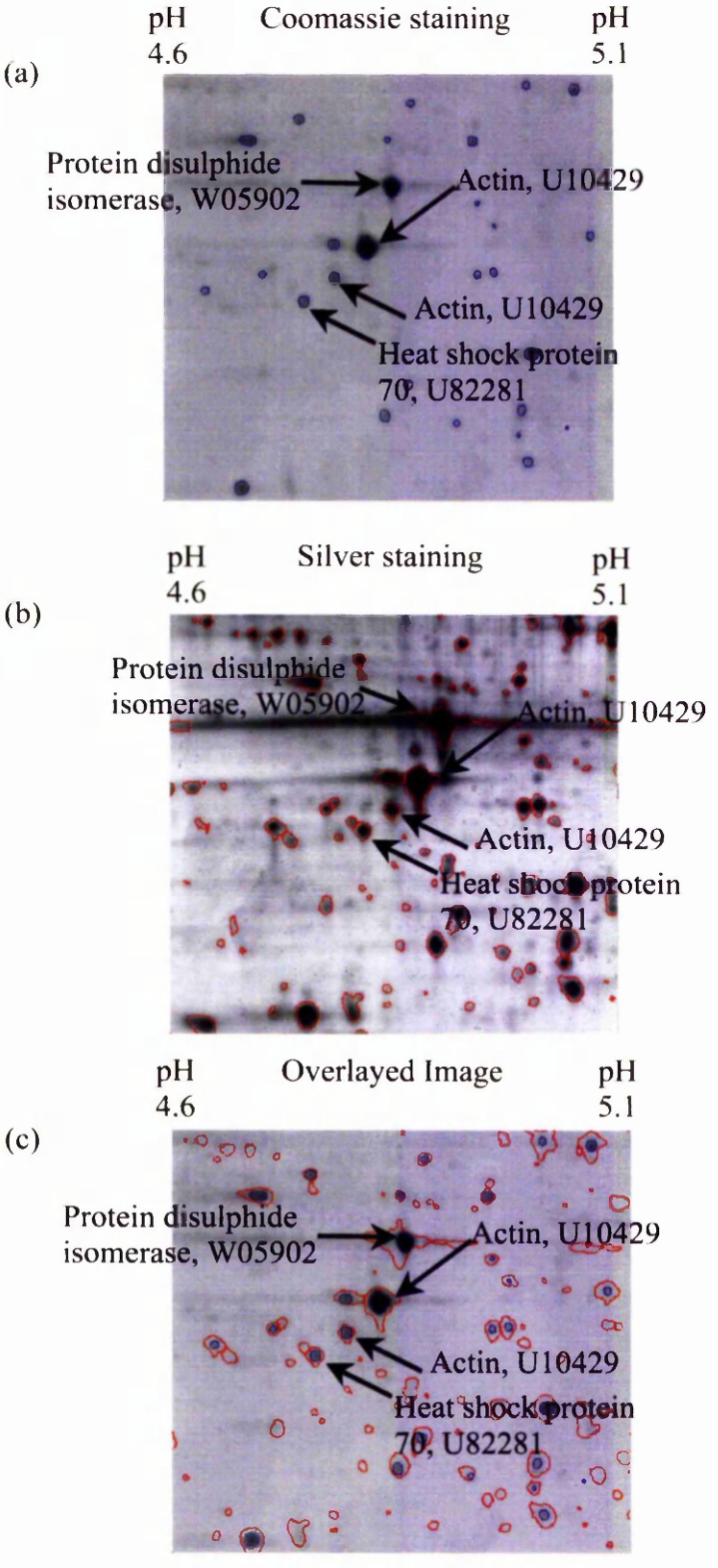



**Figure 4.7.** Spot detection using ImageMaster® 2D. (a) Coomassie blue stained gel loaded with 1 x $10^8$ pRH tachyzoites and (b) silver-stained gel loaded with 0.5 x $10^8$ pRH tachyzoites. Spots detected by ImageMaster ® 2D are highlighted in blue and red, respectively. (c) Gel stained with silver was overlaid on Coomassie-stained gel, clearly showing those spot detected by both staining choices and the enhanced sensitivity of silver staining.

**Table 4.4.1.** The sensitivity of 2-DE analysis of *T. gondii* tachyzoites. The number of resolved protein spots for Coomassie- and silver-stained gels using a range of pH separations. Gels were analysed by a combination of automatic spot detection using digital images processed by ImageMaster® 2D software and manual detection.

| IPG pH range | No. tachyzoites | Protein (μg) | Stain | No. resolved protein spots | | |
|---|---|---|---|---|---|---|
| | | | | Average | Range | Sample size |
| 4 - 7 linear | $0.5 \times 10^8$ | 104 | Silver | 630 | 591 - 685 | 3 |
| 4 - 7 linear | $1 \times 10^8$ | 208 | Coomassie | 193 | 176 - 217 | 3 |
| 4 - 7 linear | $2 \times 10^8$ | 416 | Coomassie | 514 | 452 - 576 | 2 |
| 6 - 11 linear | $1 \times 10^8$ | 208 | Silver | 373 | 340 - 414 | 4 |
| 6 - 11 linear | $2 \times 10^8$ | 416 | Coomassie | 123 | - | 1 |
| 5.5 - 6.7 linear | $1 \times 10^8$ | 208 | Silver | 513 | - | 1 |

### 4.4.4 Reproducibility of 2-DE separations (APB)

The reproducibility of sample preparation, gel electrophoresis and gel staining was investigated by running sequential replicate gels with protein prepared from different batches of parasites on different days. Most variability between replicate gels occurred at the very periphery of the gel i.e. at very high or low molecular weights and at the extremes of the pH gradient. When these data were excluded from the analysis, the majority of protein spots could be resolved with a very high degree of reproducibility. The outcome of a typical experiment is shown in Figure 4.6(b). Each panel (i-iii) represents a magnified inset of replicate silver-stained gels from three different batches of parasites run on different days, thus introducing the maximum number of potential variables. A digital composite image of all three gels created using ImageMaster® 2D

software is shown in Figure 4.6(b) panel (iv) using only spots present in all three replicate gels to produce an 'averaged gel'.

With the aid of computer software, high abundance proteins visualised using Coomassie Blue were also reproducibly detected on replicate gels stained using silver. For example, protein from 1 x $10^8$ *T. gondii* tachyzoites visualised using Coomassie Blue were outlined by ImageMaster® 2D in blue and similarly protein from 0.5 x $10^8$ *T. gondii* tachyzoites visualised using silver-staining were highlighted in red (Figure 4.7a and b, respectively). Using ImageMaster® 2D, proteins were matched and overlaid, clearly indicating those proteins visualised in both staining techniques (Figure 4.7c).

### 4.4.5 Standardisation of sample preparation (APB)

Since *T. gondii* tachyzoites are obligate intracellular parasites, great care was taken to minimise any host cell contamination in the tachyzoite protein preparations. Filtration through 3 μm polycarbonate membranes and differential centrifugation resulted in the removal of most contaminating host cell material. This was initially confirmed microscopically by examining tachyzoite preparations before protein solubilisation. Moreover, none of the polypeptide spots subsequently excised from the 2-DE gels and analysed by mass spectrometry were shown to be of host cell origin (Chapter 5).

To determine whether the length of time the parasites had been grown in host cells and whether the ratio of intracellular:extracellular tachyzoites had any influence on the protein expression profiles, parasites were harvested at 2, 3, 4 and 5 days after invasion of the parasites into cells. In general, the protein expression profiles between different

pH 5.5 pH 6.7

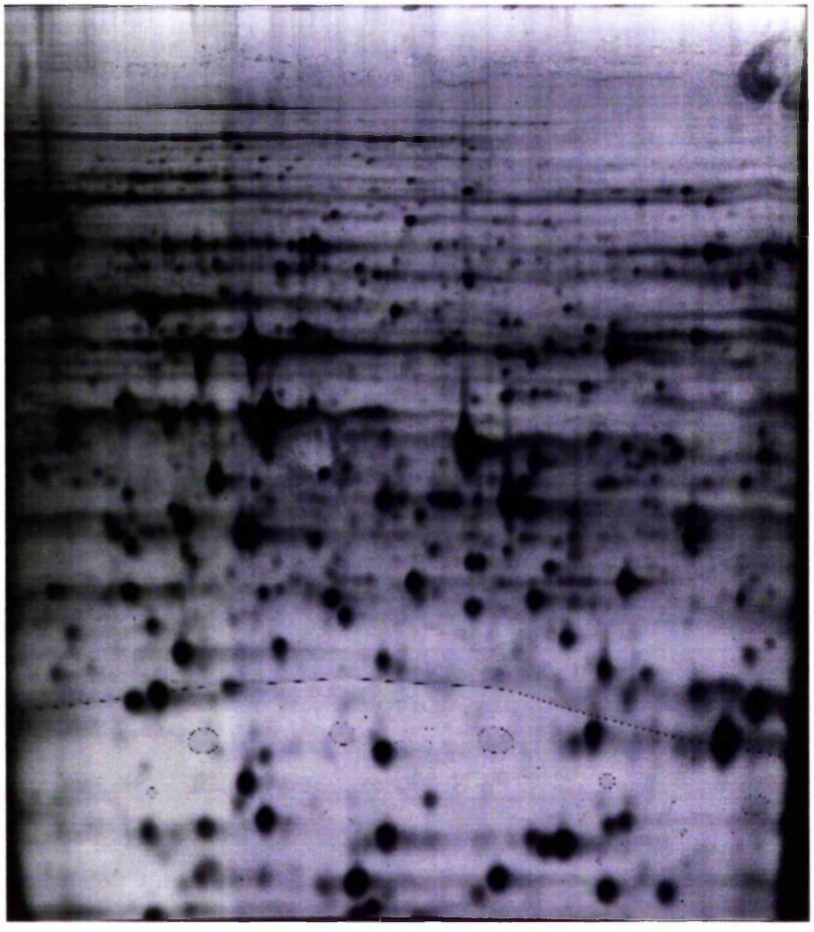


**Figure 4.8.** 2-DE separation of proteins from pRH *T. gondii* tachyzoites ($1 \times 10^8$) using a narrow range IPG strip. Proteins spots (513) were visualised using silver staining.

days were comparable with areas of the gels showing good reproducibility. However, some protein expression differences were observed between each time point (Figure 4.9). Further analysis of these gels, together with additional repeat experiments would be required to detect subtle changes, such as gradual increases/decreases in protein expression over the period of 5 days. This is a large undertaking and not within the scope of this study. Therefore to remove this variable, all parasite harvests were standardised at 72 h after infection of host cells.

### 4.4.6 2-DE analysis of S48 *T. gondii* vaccine line

The power of 2-DE as a technique for determining protein expression differences between vaccine and non-vaccine lines of *T. gondii*, and thus for identification (in combination with mass spectrometry) of proteins that are potentially associated with virulence factors was investigated. Proteins from pRH and S48 tachyzoites ($0.5 \times 10^8$) were separated by 2-DE in the pH range 4 - 7 and stained using silver. Image analysis, performed using ImageMaster® 2D, identified several areas where protein expression was identical between these two lines. One such area, magnified, is shown in Figure 4.10. After performing detailed spot detection (automatic and manual) and overlaying images of each gel, several proteins were detected that were potentially differentially expressed (Figure 4.11a). However, visual analysis of replicate gels indicated that the majority of these protein differences were not real (refer to Discussion). Interestingly, five proteins were detected as present in pRH tachyzoites but absent from the vaccine line, and two proteins were found in the S48 line but not in pRH tachyzoites (Figure 4.11a). These proteins expression differences were reproduced in one other replicate gel. Of these differences, two proteins present in pRH and absent in S48 (Figure 4.11a,

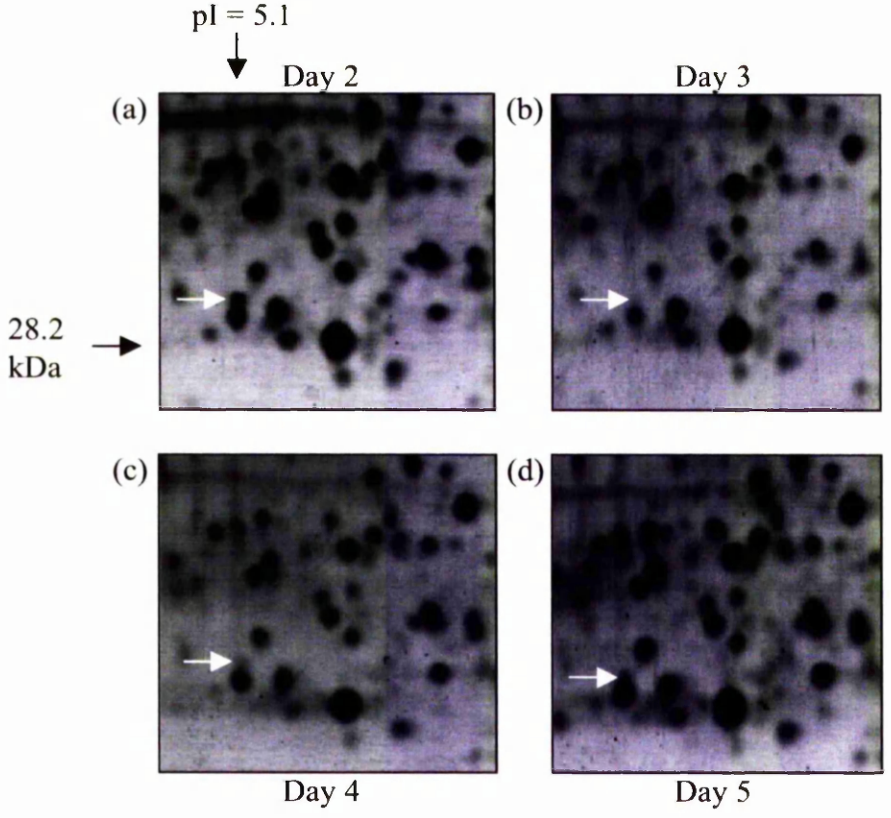



**Figure 4.9.** Selected region of a 2-DE gel of *T. gondii* tachyzoites harvested at varying times during the *in vitro* cycle of infection and lysis of host cells. The white arrow indicates a protein that is differentially expressed during the 4-day period. Tachyzoites were harvested at 48 h, 72 h, 96 h and 120 h after infection of host cells (panels a - d). Protein from 5 x $10^7$ tachyzoites was separated on each gel and visualised by silver staining.

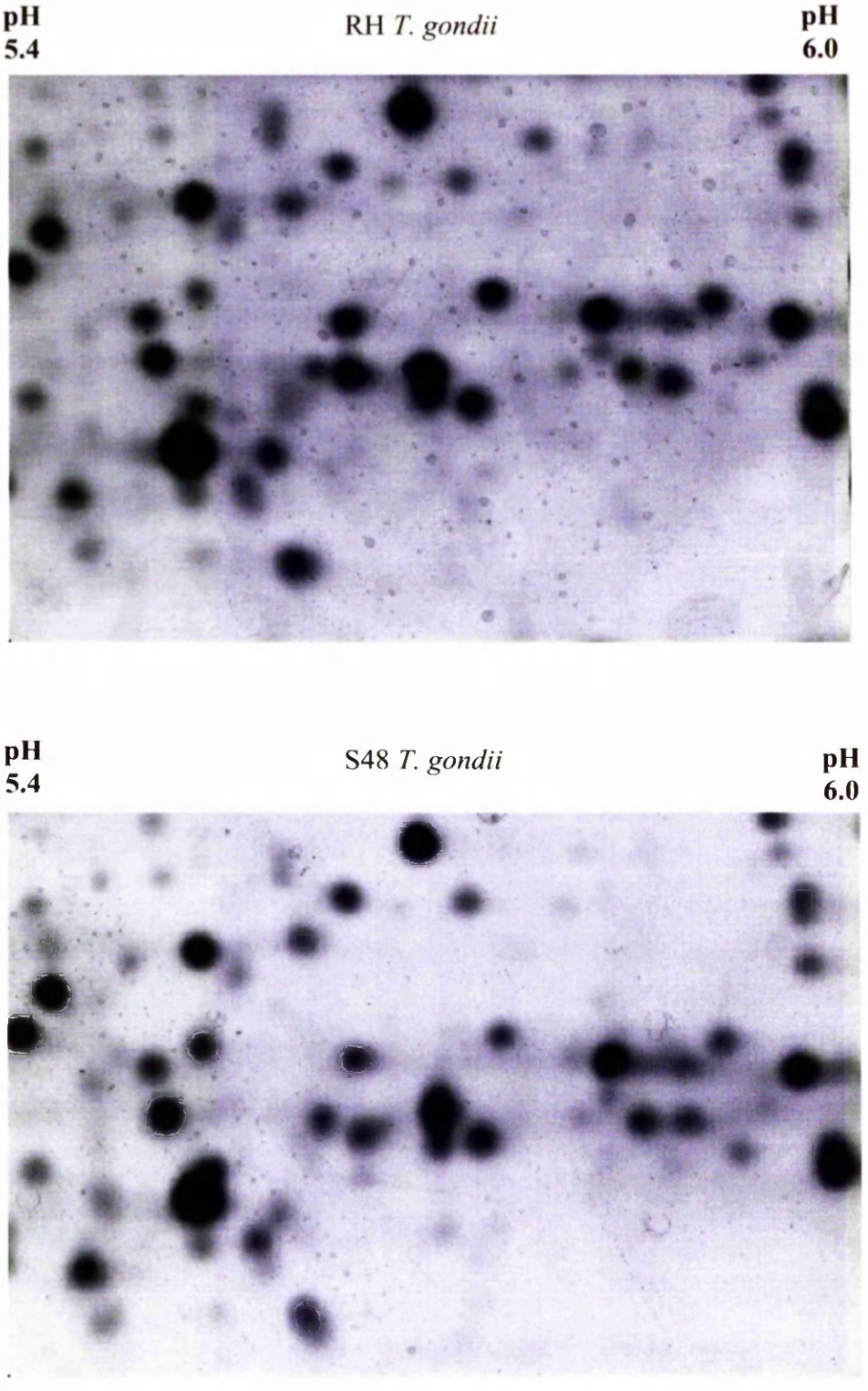



**Figure 4.10.** Conservation of protein expression between pRH and S48. Each panel represents the same enlarged region of a 2-DE gel of pRH and S48. Gels were stained with silver.

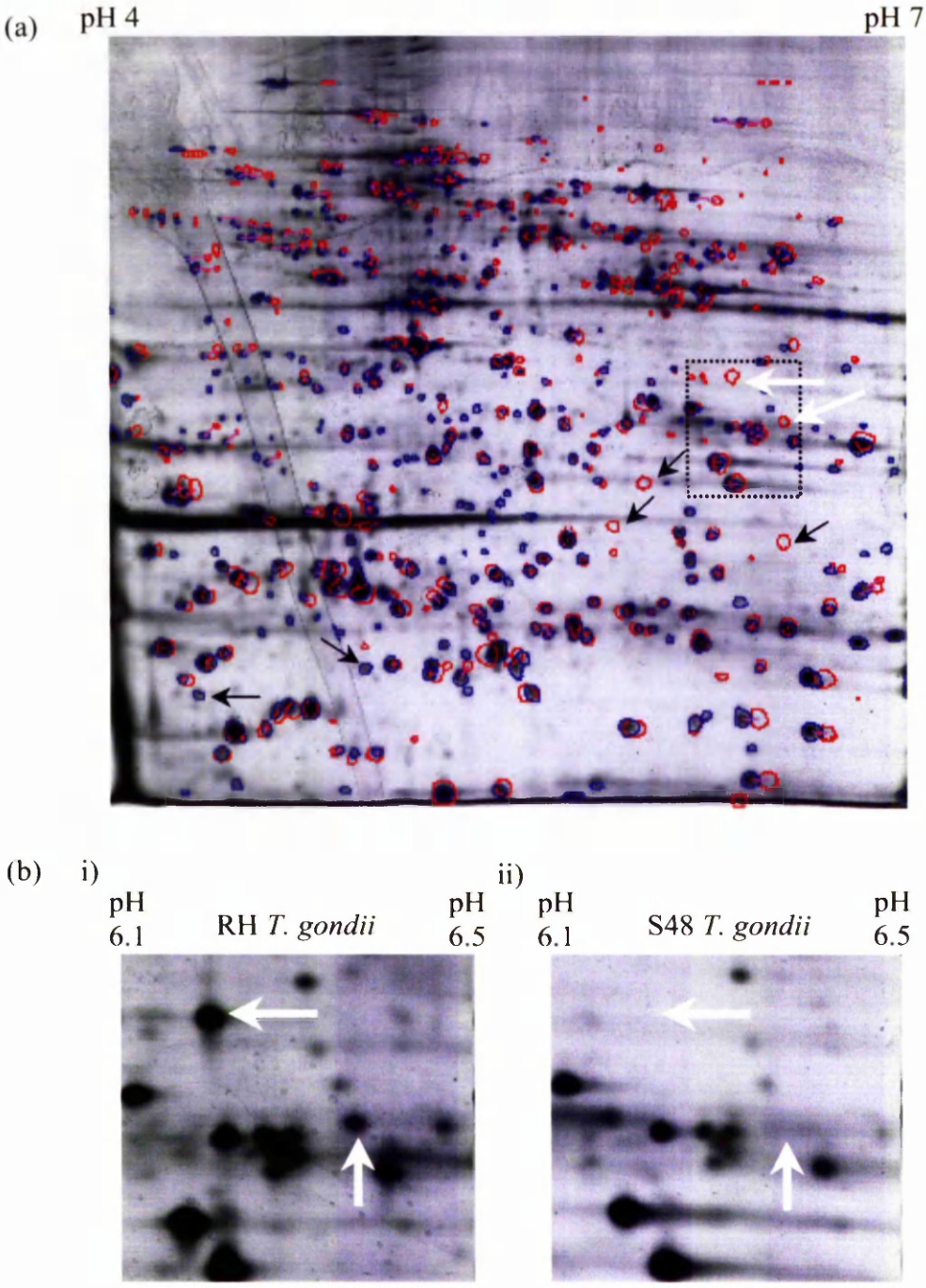



**Figure 4.11.** Comparison of RH and S48 *T. gondii* lines. (a) S48 *T. gondii* proteins (5 x $10^7$ tachyzoites) separated by 2-DE and visualised using silver. S48 proteins detected by ImageMaster® 2D Elite and manual detection are outlined in blue. A 2-DE separation of RH *T. gondii* proteins (5 x $10^7$ tachyzoites) visualised using silver was also analysed using ImageMaster® 2D Elite. RH protein spots, outlined in red, are shown overlaid on the S48 2D gel. Two proteins present in RH and absent in S48 are indicated by white arrows. The dotted box region has been enlarged clearly demonstrating differential protein expression between RH and S48 *T. gondii* (b(i) and (ii), respectively). Other proteins that may be differentially expressed between these lines are indicated by black arrows (a). The vast majority of unmatched S48 proteins (blue) are expressed in low quantities and careful analysis of gels suggests that these are not 'real' differences.

white arrows) were reproducible differences detected in three replicate experiments. The same region of the S48 and pRH gels containing these protein expression differences is shown, enlarged, demonstrating the presence of two proteins found in pRH and their absence in S48 (Figure 4.11b).

### 4.4.7 2-DE analysis of HXGPRT knockout line

Protein expression changes as a result of genetic manipulation of *T. gondii* were investigated using 2-DE. Similar analyses to that described for S48 and pRH (Section 4.4.6) was performed for analysis of protein expression (pH range 4 - 7) differences between a mutant *T. gondii* line, RH-HX$^-$, lacking the gene encoding for HXGPRT and the parental line, SRH. As with S48 and pRH, areas of high protein similarity between SRH and RH-HX$^-$ were demonstrated. Digital images of each gel were processed using ImageMaster® 2D, overlaid and proteins in each line matched (Figure 4.12a). Several proteins appeared to be differentially expressed, although detailed visual analysis of replicate gels indicated that many were not reproducible. However, one protein present in 3 of 4 gels of SRH line but present in only 1 of 4 gels of RH-HX$^-$ was discovered (Figure 4.12a, b).

It was expected that the protein HXGPRT would be present in SRH but absent from RH-HX$^-$ *T. gondii* tachyzoites. Two sequences encoding for HXGPRT were found in GenBank (accession numbers AAD44365 and AAB60214) and using MS-Product (http://prospector.ucsf.edu/ucsfhtml3.4/msdigest.htm) estimated isoelectric points and molecular weights were obtained (pI = 5.89, MW = 26.4; and pI = 6.10, MW = 31.5,

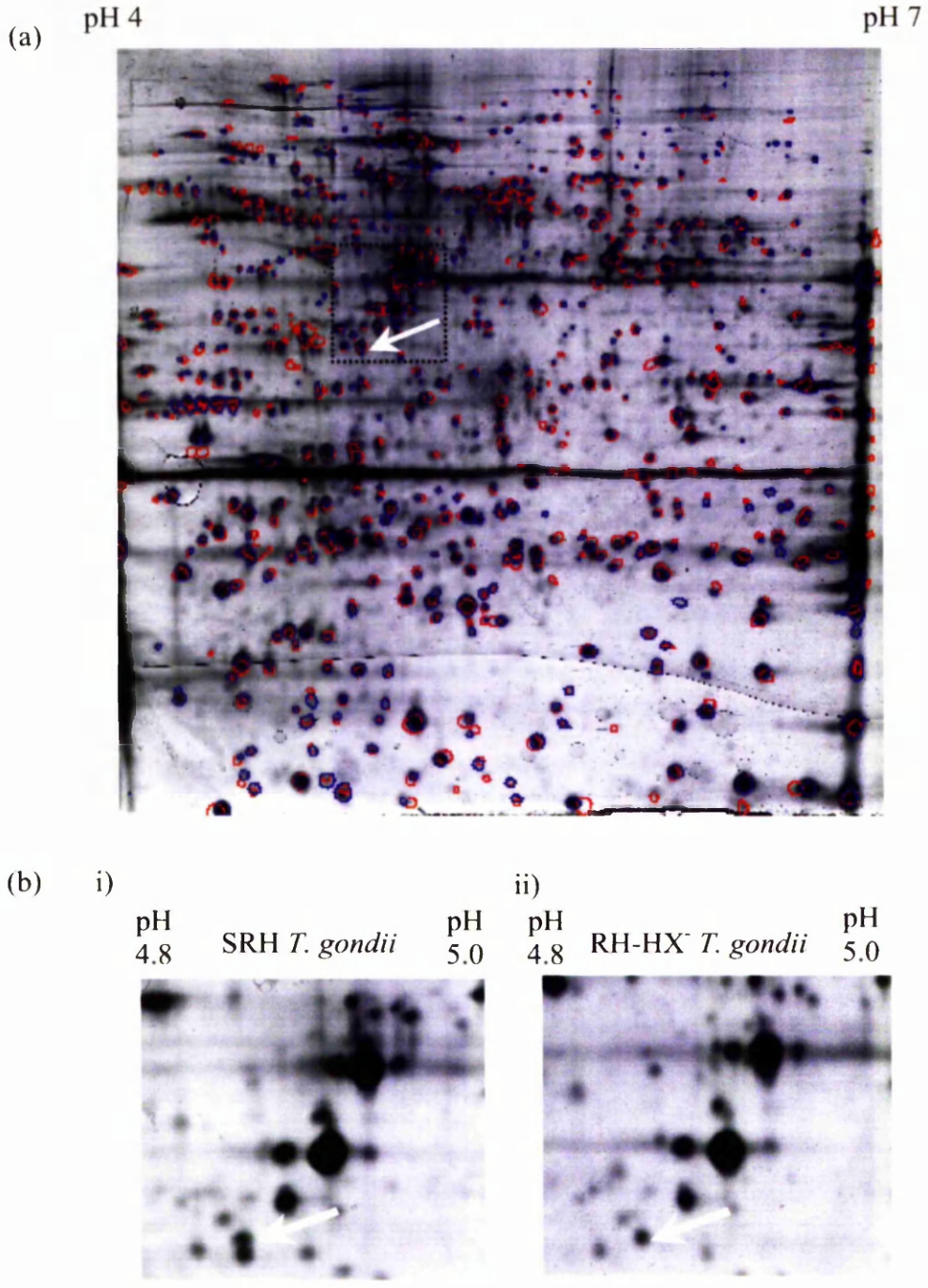



**Figure 4.12.** Comparison of SRH and RH-HX$^-$ *T. gondii* lines. (a) RH-HX$^-$ *T. gondii* proteins (0.5 x $10^8$ tachyzoites) separated by 2-DE and visualised using silver. RH-HX$^-$ proteins detected by ImageMaster® 2D and manual detection are shown outlined in blue. A 2-DE separation of parental SRH *T. gondii* proteins (0.5 x $10^8$ tachyzoites) was also visualised using silver and analysed using ImageMaster® 2D. SRH protein spots, outlined in red, are shown overlaid on the RH-HX$^-$ 2D gel. A protein present in SRH and absent in RH-HX$^-$ is indicated by the white arrow. The dotted box region has been enlarged to indicate more clearly this differentially expressed protein (b(i),(ii)).

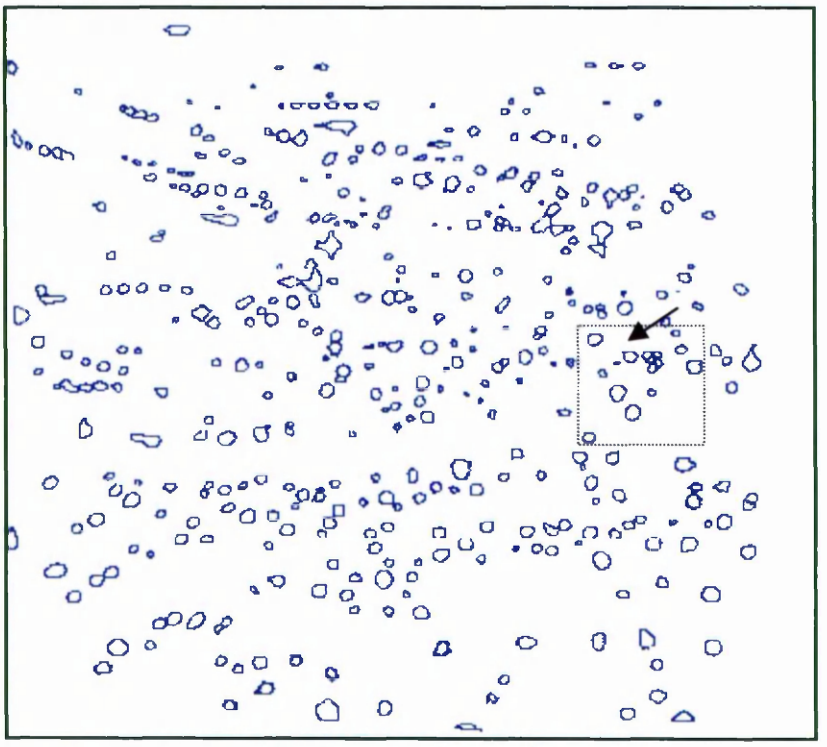
respectively). Analysis of the 2D-gels in these regions failed to identify any protein expression differences.

### 4.4.8 2-DE analysis of an arprinocid-1-N-oxide -resistant *T. gondii* mutant

The application of 2-DE for the detection of proteins that may be related to the acquisition of drug-resistance was investigated. Protein expression between arprinocid-1-N-oxide-resistant ($ARP^R$) and -sensitive (pRH) lines was compared using 2-DE and the linear pH ranges 4 - 7, 6 - 11 and 5.5 - 6.7. No protein expression differences between these lines could be identified using pH 4 - 7 linear and pH 6 - 11 linear IPG strips and Coomassie Blue staining (data not shown). Consequently, in all subsequent experiments proteins were visualised by sensitive silver staining.

Replicate 2D gels of protein from pRH and $ARP^R$ *T. gondii* tachyzoites using the pH range 4 – 7 ($0.5 \times 10^8$ tachyzoites) and 6 – 11 ($0.5 \times 10^8$ tachyzoites), and 5.5 – 6.7 ($1 \times 10^8$ tachyzoites) and stained with silver were analysed using both visual analysis and ImageMaster® 2D software. Image analysis using ImageMaster® 2D consisted of automatic spot detection followed by extensive manual editing of spots to remove those not considered to be real (such as background noise from silver-staining) and incorporate spots not detected by the software. Four replicate gels of pRH and three replicates of $ARP^R$ in the pH range 4 -7 were processed and an average gel of pRH created, whereby a protein spot must be present in at least three of the four replicate gels (Figure 4.13a). Similarly an average gel of $ARP^R$ was created, whereby a protein must be present in all three replicate gels (Figure 4.13b). A protein present in only 2 out of 3 gels was not considered reproducible so the stringency for the $ARP^R$ average gel was

(a) pH 4 Average pRH *T. gondii* pH 7



(b) pH 4 Average ARP$^R$ *T. gondii* pH 7

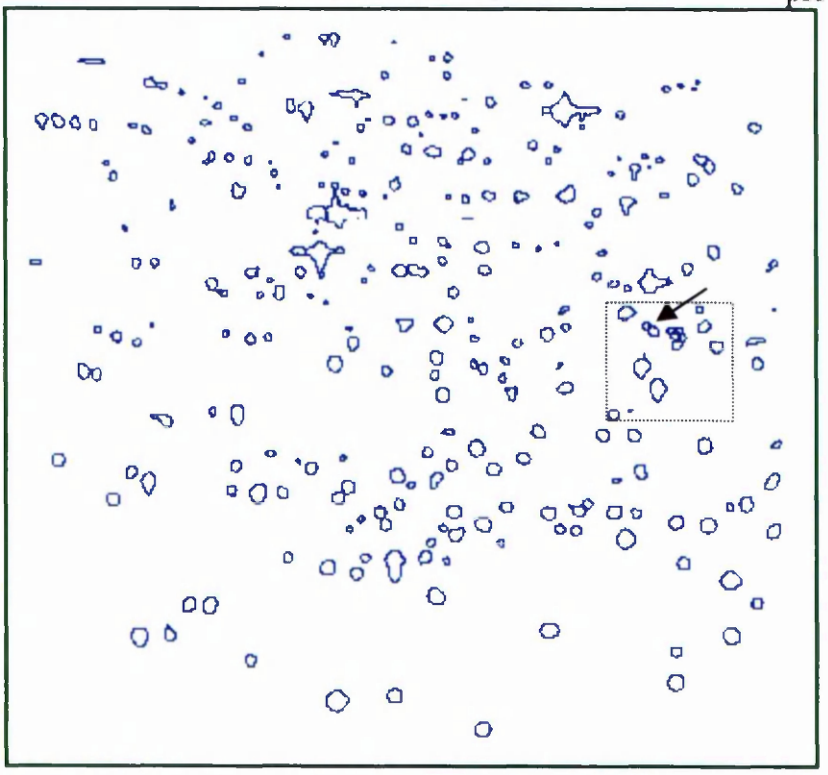


**Figure 4.13.** Composite protein gels of pRH and ARP$^R$ *T. gondii*. (a) pRH *T. gondii* proteins present in a minimum of 3 of 4 replicate silver-stained 2-DE gels. (b) ARP$^R$ *T. gondii* proteins present in 3 of 4 replicate silver-stained 2-DE gels. The arrow indicates a protein present in ARP$^R$ and absent from pRH.

increased. Analysis of the two average gels for pRH and $ARP^R$ indicated that approximately 20 - 25 proteins differed in their expression between the drug-resistant and -sensitive lines. However, visual analysis of each individual gel showed that only one of these protein spots was a reproducible difference (Figure 4.13, arrow). This protein was reproducibly expressed in 3 replicate gels of $ARP^R$ and was consistently absent from 4 replicate gels of pRH (each gel was run on a different day using a different batch of parasites, as discussed in Section 4.4.4). The same enlarged region from representative pRH and $ARP^R$ gels highlighting this protein expression difference is shown in Figure 4.14.

Proteins in the pH range 6 - 11 were analysed by the same process as for 4 - 7 gels, described above. Average gels were created for both pRH and $ARP^R$ using the criterion that a protein must be present in at least 2 of the 3 replicate gels for each line (Figure 4.15a and b, respectively). As was demonstrated with the analysis of the pH 4 – 7 linear 2-DE gels, the creation of pH 6 – 11 linear average gels resulted in many false positive protein expression differences between the drug-sensitive and -resistant lines. None of these differences were confirmed as reproducible by visual analysis of the gels.

The single reproducible protein expression difference that was detected between pRH and $ARP^R$ using the pH 4 – 7 linear range was investigated further by zooming into that region of the gel. The estimated pI of this differentially expressed protein was 6.3 and therefore pH 5.5 – 6.7 IPG strips were used. Duplicate 2-DE gels each loaded with 1 x $10^8$ tachyzoites were run for the drug-sensitive (Figure 4.16) and -resistant lines, and analysed using ImageMaster® 2D. Although, overlaid images of each gel indicated the presence of many differentially expressed proteins, no reproducible changes were

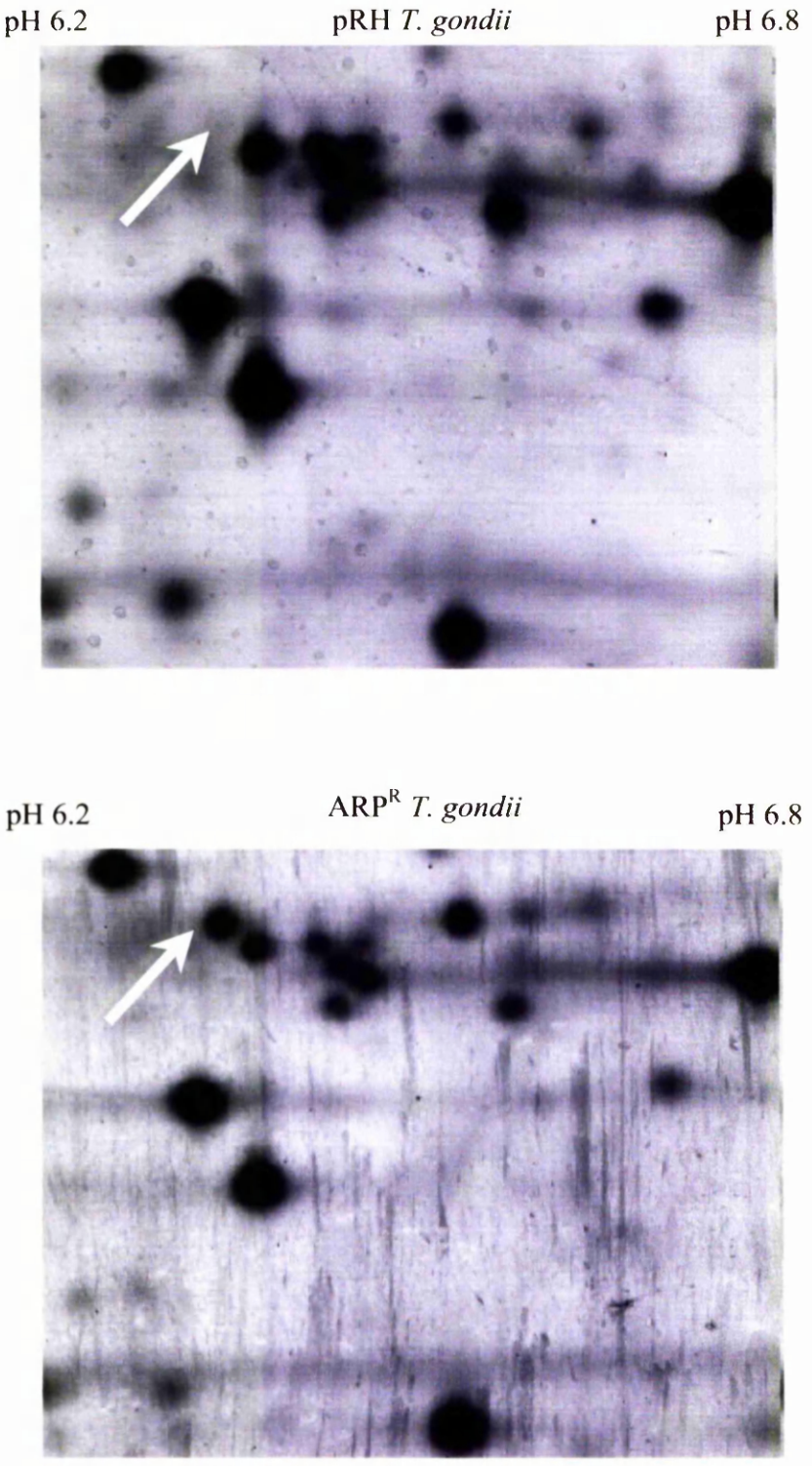
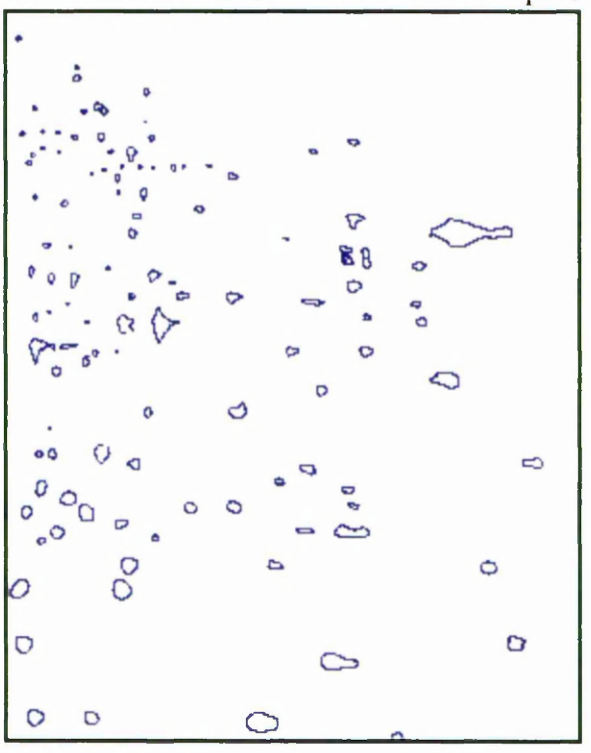



**Figure 4.14.** Differentially expressed protein reproducibly expressed in ARP$^{R}$ *T. gondii* tachyzoites and not in pRH *T. gondii* tachyzoites. Enlarged region of gel shown as boxed area in Figure 4.13.

(a) pH 6 Average pRH *T. gondii* pH 9



(b) pH 6 Average ARP$^R$ *T. gondii* pH 9

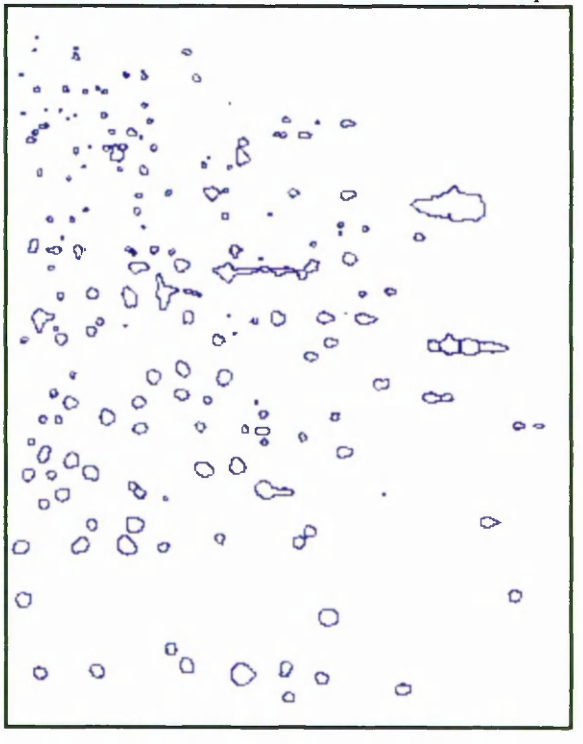


**Figure 4.15.** Composite protein gels of pRH and ARP$^R$ *T. gondii*. (a) pRH *T. gondii* present in a minimum of 3 of 4 replicate 2-DE gels. (b) ARP$^R$ *T. gondii* proteins present in 3 of 3 replicate 2-DE gels.

pH 5.5 pH 6.7

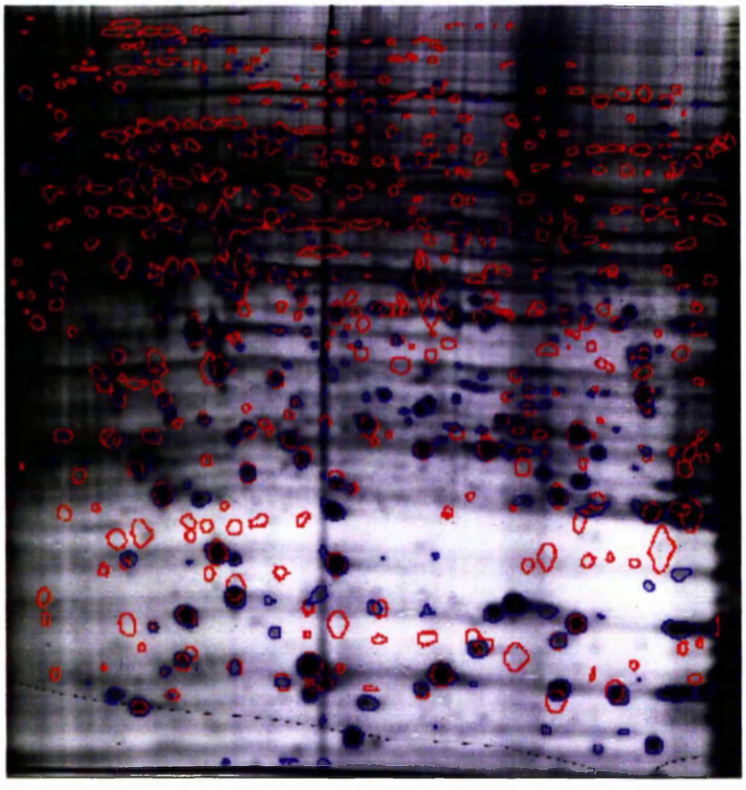


**Figure 4.16.** Comparison of pRH and ARP$^{R}$ *T. gondii* lines using pH 5.5 - 6.7 linear IPG strips. *T. gondii* proteins (1 x $10^{8}$ tachyzoites) were separated by 2-DE and visualised using silver. pRH proteins detected by ImageMaster® 2D and manual detection are shown outlined in blue. ARP$^{R}$ protein spots, outlined in red, are shown overlaid on the pRH 2D gel.

detected. In addition, more gels are needed to confirm any protein expression differences. The protein difference found using pH 4 – 7 linear IPG strips could not be found using the pH 5.5 - 6.7 linear IPG strip. Indeed, because of the large numbers of proteins (> 500) that were separated in approximately 1 pH unit, it was difficult to correlate these proteins with those protein separated on a pH 4- 7 gel.

## 4.5 Discussion

Two-dimensional electrophoresis is one of the most widely accepted methods for the separation and comparison of complex protein mixtures and has become the main platform from which to base proteomic studies. 2-DE offers greater protein resolution potential than any other current separation technique and has recently been used to map large numbers of proteins from different organisms and tissues and also to identify changes in protein expression (reviewed by (Jensen and Pennington, 2001). The considerable progress that has been made in the speed and accuracy of protein identification from individual spots by mass spectrometry means that the mapping of the entire proteome is now an achievable and economically viable prospect for some organisms. In this study it was demonstrated that 2-DE gel analysis is capable of resolving over a thousand individual polypeptide spots from as few as 1 x $10^8$ *T. gondii* tachyzoites using a combination of 4-7 linear and 6-11 linear IPG strips with silver staining. Moreover, a narrow pH range gel (one pH unit, 5.5-6.7) resolved almost as many spots (>500) as a pH 4-7 linear gel, suggesting that the full resolving potential of 2-DE analysis for tachyzoite proteins would be even greater if multiple single pH unit analyses were conducted. The data presented suggest that the total number of tachyzoite polypeptides resolvable by single pH unit 2-DE and silver-staining is 3000-4000, assuming 500 spots per pH unit over the range pH 4-8, but fewer at the high and low extremes of pH. Despite the considerable resolution of these 2-DE analyses, it is important to note that the data will not represent all tachyzoite proteins; there are some well-established technical limitations with 2-DE such as solubilisation of basic proteins and detection of low copy number proteins (Celis and Gromov, 1999).

In the early phase of this study many difficulties were encountered in setting up the techniques to achieve great resolution, sensitivity and reproducibility. Clearly sample preparation and staining choice are important and are discussed below, but also equally significant, and perhaps less often considered, is the choice of equipment. Several manufacturers produce different machines and different formats, for example horizontal $2^{nd}$ dimension systems versus vertical $2^{nd}$ dimension systems. The critical consideration is the transfer of proteins from the first dimension IPG strip to the second dimension gel. In the 1980's flatbed SDS-PAGE systems suffered due to troublesome 'edge-to-edge' contacts that resulted in gel shrinkage and swelling leading to incomplete migration of proteins from the $1^{st}$ to the $2^{nd}$ dimension (Görg et al., 1985). Since both the GS and APB systems used in this work were vertical, these factors would not influence the numbers or resolution of protein spots. The markedly different gels obtained by each system (GS and APB) is perhaps related to the IEF system used and the numerous technical differences in the equipment from each supplier. For example, the GS and APB systems have maximum voltages for isoelectric focussing (IEF) of 5000 V and 8000 V, respectively. In order to achieve the same volt-hours with both sets of equipment, longer focussing times were required using the GS system and thus electroendosmotic effects or increased protein precipitation may have occurred. In addition, higher voltages enable sharper focussing of proteins. It seems unlikely that voltage differences alone contributed to the gel differences between the two systems. The type of IPG strip holder and the cooling system employed in both these systems, together with the increased user manipulation of IPG strips during IEF in the GS system compared to the APB system, all may contribute towards the overall quality of separation of proteins. A recent publication compared three different commercially available IEF units (the Multiphor, IPGphor and the Protean IEF cell) and found that the

number of spots generated, reproducibility and staining intensity all varied between machines independent of sample preparation (Choe and Lee, 2000). Generally the IPGphor performed least well. However, as stated in the article, optimisation of protocols for specific machines would have yielded better results. Since the resolution and sensitivity of the 2-DE was higher using the APB system, this was the equipment chosen for the remainder of the study.

The issue of reproducibility needed to be carefully addressed since the ability to run consistent gels is paramount when comparing samples. Good reproducibility could be achieved for the *T. gondii* tachyzoite protein separations even with samples prepared from different batches of parasites on different days. In addition, 2D-gels were not bought precast from the manufacturer but were manually cast in batches of 12. This added another variable to the ability to run reproducible gels, but was not found to be a major issue. Newer systems now available, such as the DALT II (Amersham Pharmacia Biotech), have precast gels on fixed plastic supports available. This should enhance reproducibility between gels further, stop gel shrinkage/expansion during staining processes, and most importantly enable the high-throughput analysis of gels in combination with automatic spot pickers and digesters.

In order to obtain reproducible results, it was important to standardise both sample preparation and the source of material. The proteome of an organism is not a stable entity and will change rapidly as the functional requirements of the organism change, for example during differentiation. Comparison of the proteome of tachyzoites harvested at different times after cell infection (Figure 4.8) demonstrated that differential protein expression occurred in tachyzoites during the *in vitro* cycle of invasion and lysis of cells. This highlighted the need to standardise the time of harvest

of tachyzoites for 2-DE analysis. Studies involving the comparison of different *T. gondii* isolates would need to address this issue carefully when comparing protein expression profiles between different *T. gondii* strains that may grow at different rates in tissue culture.

A further consideration with 2-DE is the sensitivity, linearity and dynamic range of the stains used to visualise the proteins. Although silver staining proved more sensitive than Coomassie-Blue staining, quadrupling the amount of protein loaded onto a Coomassie gel enabled over 500 spots to be visualised in the pH 4-7 range (compared to an average of 630 with silver staining). Further increases in protein loading, however, resulted in the loss of resolving power, particularly with high abundance proteins. Whilst silver staining proved more sensitive than Coomassie staining, it has two important disadvantages. Firstly, the non-linearity of silver staining and limited dynamic range can make assessment of the level of protein expression difficult. Secondly, low detection limits often occur in MS because the stain modifies cysteine residues and the glutaraldehyde and formaldehyde used in the procedure alkylate protein amino groups (Patton, 2000). Whilst glutaraldehyde may be omitted from the staining, formaldehyde may not as it is required to reduce silver nitrate to silver. In this project, a modified silver stain, omitting the use of glutaraldehyde, was used on some gels and a limited number of high abundance proteins stained by this method were identified by MS (Chapter 5).

The molecular weight and pI range of proteins that can be analysed on a single gel is limited and a complete proteome analysis for an organism is thus unlikely to be possible using a single gel. One of the aims of this study was to identify protein changes between a drug-resistant and -sensitive strain of *T. gondii*. Only one reproducible difference was

detected and given the overall reproducibility of the gels it is tempting to suggest that this protein may be related to or even responsible for acquisition of resistance. It would be interesting to investigate whether or not this same protein is expressed in the other arprinocid-1-N-oxide strain, $ANO^R$ and also whether this protein is expressed in the drug-resistant *Eimeria spp* identified by Chapman (1983). It is possible that other proteins varying between the drug-sensitive and -resistant *T. gondii* lines, despite extensive visual and software analysis of the gels, were not noticed. For example, a subtle change in protein position or a small increase/decrease in expression of a protein are not easy to detect (limitations in software analysis is discussed below). The advantage of newer stains such as the Sypro Ruby is that this stain has the same sensitivity of detection as silver but, in contrast to silver staining, is linear, thus allowing more accurate measurements of protein expression (Patton, 2000). It must also be considered that drug-resistance may occur due to a modified drug transporter and that this protein, being membrane bound, may not have been solubilised. Membrane proteins are notoriously difficult to solubilise for 2-DE due to their hydrophobicity and visualisation of membrane transporters requires more emphasis to be placed on the method of solubilisation. Recently a number of methods have been developed that have dramatically improved the solubility for 2-DE analysis for this important group of proteins (Rabilloud *et al.*, 1999). Other techniques are also available for improving protein solubilisation, such as the use of non-ionic reducing agents like tributyl phosphine (Herbert *et al.*, 1998), the addition of chaotropes including thiourea and the use of amidosulfobetaine detergents such as ASB-14 and C8$\phi$ (Phadke *et al.*, 2001). The relatively simple extraction methods used in this study by Phadke *et al.* appeared to give good solubility for many inner and outer cell membrane proteins (for instance, transport and receptor proteins). However, the issue of *T .gondii* membrane proteins will need to

be addressed more fully in further work. Alternative methods to 2-DE for protein separation for proteomic analysis could be considered in the future, including the development of "arrays" or "chips" for rapid, high-throughput analysis similar to that in use for mRNA microarrays (Jensen and Pennington, 2001), or the use of liquid-phase chromatographic separations coupled to MS (LC-MS) (Keough *et al.*, 2000). However, these technologies are as yet some way from being generally applicable. Moreover, the results of this work demonstrate that high-resolution 2-DE separations of parasite protein populations can be achieved with consistent reproducibility and these should be sufficient for many analyses.

The problem of eliminating gel-to-gel variation was aided by the production of composite gels derived from averaging computer-generated images. This made comparisons between different protein populations easier. For example, proteins differentially expressed in tachyzoites harvested from tissue culture at different times after cell invasion were identifiable by comparison with the standardised gels. Of particular advantage, is the ability to open two linked windows, each containing a 2D gel, so that by scrolling through the same (linked) areas of two gels, the 'human eye' can easily detect protein differences between the two gels. Automatic spot detection is an important facility central to the function of the software. However, there are limitations and further improvements in the encoding algorithms are needed for the software to be completely beneficial. Currently, the extremes of spot sizes are not readily detected and detection of background noise can be problematic. Extensive manual editing and matching of spots is required, thus reducing the efficiency of the software (Voss and Haberl, 2000). Because of the failure of the software to detect all spots and the inability to detect manually all remaining proteins accurately, the average gels that are created

contain errors. For example, a protein manually incorporated into one gel may have inadvertently been missed on a replicate gel. Consequently, many proteins appear to be differentially expressed between lines but are in fact false positives. Confirmation of these potential differences is essential. The software can work though, in that the reproducible protein difference between pRH and $ARP^R$ was also found using average gels.

The criteria applied in the creation of average gels were strict. In general, for a protein to be included in the analysis it must be present in at least all but one of the gels. Reducing this parameter to allow for more errors reduced the frequency of false positive protein expression differences. One danger with gel averaging, however, may be that more subtle protein changes could be missed due to elimination from the composite images. For gel-to-gel comparisons, a more attractive approach may be to include spots from all gels and apply multivariate statistical analysis to the data (Vohradsky, 1997). This would enable not only subtle statistically significant protein changes to be detected, but also help identify groups of polypeptides whose expression is altered in a linked manner. For example, the up- or down-regulation of components in a metabolic pathway as a result of gene-manipulation could be identified in this way.

Further improvements in software are needed for the full potential of 2-DE analysis to be realised. The task at hand is very difficult and ultimately 'intelligent' software is needed. The new release version of ImageMaster® claims to have improved spot detection and matching algorithms (http://www.nonlinear.com/ news_press_releases/10-8-2001.htm) and a similar program, Progenesis, claims to be automated 'hands free' analysis software for batch processing large numbers of gels (http://www.nonlinear.com /news_press_releases/23-3-2001.htm). The most exciting prospect is the recently

developed CyDye™ technology and fluorescence detection software (Amersham Pharmacia Biotech). Fluorescence 2-D difference gel electrophoresis (DIGE) allows samples (A and B), labelled with different fluorescent dyes, to be co-separated on the same gel, thereby eliminating gel-to-gel variation (Beaumont, *et al.*, 2001). Proteins from sample A are visualised by excitation at wavelength 1, and proteins from sample B are visualised by excitation at wavelength 2. Dedicated DeCyder 2D analysis software (Amersham Pharmacia Biotech) accurately detects and matches proteins from the two samples with minimal user input (20 images may be analysed in 15 min) and allows differences to be easily visualised by simultaneous excitation at both wavelengths (Beaumont, *et al.*, 2001). Moreover, spot intensities may be viewed in 3-D and specialised algorithms enable statistical analysis to be performed. The technology is also compatible with existing staining technologies, for example Sypro Ruby, and is designed to function with automatic spot pickers and digesters. Whilst this technology is affordable to limited research groups, its predicted ease of use and high-throughput facilities will make proteomics an essential tool for the investigation of many biological questions. As was demonstrated in this study, 2-DE has the capabilities to detect protein changes associated with virulence, genetic modifications and for investigation of acquisition of drug resistance.

It is not known how many proteins are to be expected in the proteome of *T. gondii* tachyzoites. Based on gene densities from *Plasmodium falciparum* (Bowman *et al.*, 1999), there may be as many as 17,000 genes present in *T. gondii* (Ivens, 2000). However, only a proportion of these will be expressed in tachyzoites and not all will encode proteins. Nevertheless, this number of genes seems high for a relatively simple organism, given a recent estimate for the human genome of just 30,000 genes (Claverie,

2001). The latest release of the *T. gondii* EST database contains just over 5,000 consensus sequences, but EST databases almost certainly exclude a sizeable number of low, or transiently expressed, genes. Moreover, the "one gene one protein" hypothesis is unlikely to hold for *T. gondii.* Our 2-DE analysis showed that several clearly resolved protein spots appeared to be encoded by the same gene, for example actin and HSP70. These proteins presumably result from different isoforms of the same protein or from post-translational modifications of that protein. Complex patterns are seen for structural molecules such as actin in *Drosophila*, where two isoforms of actin and precursor molecules are clearly resolved by 2-DE (An and Mogami, 1996). Further characterisation of the *T. gondii* proteome is likely to reveal many other such examples. Recent work suggests that alternative splicing in *T. gondii* may account for two isoenzymes of the purine salvage enzyme, hypoxanthine-guanine phosphoribosyl-transferase (White *et al.*, 2000). These observations imply that the functional complexity of *T. gondii* will be significantly greater than would appear from its primary gene sequence and highlights the need for further proteomic studies.

# CHAPTER 5

# Proteomics 2: Identification of *T. gondii* proteins using mass spectrometry

## 5.1 Introduction

### 5.1.1 Genome and protein databases

The output of DNA sequencing projects, including the human genome map (McPherson *et al.*, 2001), during the last few years has resulted in a massive increase in the size of protein, nucleotide and expressed sequence tag (EST) databases. Notably the DNA databases have expanded not just for higher eukaryotes, but also for lower eukaryotes and prokaryotes including those for important pathogenic micro-organisms. These advanced databases have been used for analysing proteomes. For instance, proteome studies on *Helicobacter pylori* (Jungblut *et al.*, 2000), and *Mycobacterium bovis* (Jungblut *et al.*, 1999) have resulted in the identification of proteins from 2-DE gels which include virulence factors and novel antigens. Currently over 5 million EST sequences exist within Genbank, which can be utilised to correlate protein data with genomic information. For example, a recent proteome study of the human spliceosome complex identified protein spots not just from full gene sequences, but also from expressed sequence tags. This confirmed that where there is limited genomic sequence

information, EST sequence databases are sufficient to identify samples (Neubauer *et al.*, 1998).

The recent completion of the *Caenorhabditis elegans* genome-sequencing project (ftp://ftp.sanger.ac.uk/pub/databases/C.elegans_sequences) has been a major stimulus for studies on nematodes. The genomes of several protozoan parasites, including *Plasmodium falciparum* (Anonymous, 2001), *Cryptosporidium parvum* (Piper *et al.*, 1998; Liu *et al.*, 1999; Strong and Nelson, 2000) and *Trypanosoma brucei* (ftp:/ftp.ebi.ac.uk/pub/database/parasites/brucei) are in the final stages of genome sequencing. For others such as the coccidia *Toxoplasma gondii*, *Neospora caninum* and *Eimeria* species genome information is more limited. However, expressed sequence tag (EST) databases exist, some of them being highly developed containing several thousand sequences (http://www.ebi.ac.uk/parasites/paratable.html). The analysis of gene expression in both the tachyzoites and bradyzoites of *T. gondii* has been significantly advanced by the creation of EST libraries for these stages (Ajioka *et al.*, 1998; Ajioka, 1998). Currently there are about 16,000 ESTs available for *T. gondii* tachyzoites including 3,000 for bradyzoites (http://ParaDB.cis.upenn.edu/toxo/index.html). Construction of a non-redundant consensus EST database (approximately 5200 sequences) has helped to characterise tachyzoites and bradyzoites genes (Wan *et al.*, 1997).

### 5.1.2 Mass spectrometry

Proteomics is very much a technology driven science. Advances in 2-DE and staining chemistries have driven the ability to separate and visualise thousands of proteins. Similarly, improvements in the resolution of mass spectrometers together with vastly

expanding genome and protein databases have enabled the identification of these proteins. In the 1980's, the introduction of automated sequencers (Hewick *et al.*, 1981) allowed Edman degradation to become the major technology for sequencing proteins/peptides. This approach employed a step-wise chemical degradation of peptides from the *N*-terminus, separation by reverse phase HPLC and identification of derivatised amino acids based on their retention times. Edman degradation suffers from many drawbacks that limit its use for sequencing of small quantities of gel-separated proteins. For example, the presence of gaps in the sequence due to low abundance amino acids or modified residues and blockage of the *N*-terminus due to acetylation or reactions of amino acid groups with chemical buffers, acrylamide or stains used to visualise the proteins. The recent developments in mass spectrometry (MS) have surpassed *N*-terminal degradation techniques and have since become the mainstay of protein/peptide sequencing, especially as MS approaches are readily automatable (Gevaert and Vandekerckhove, 2000).

Mass spectrometry relies on the ability to create charged gas-phase ions in a vacuum and has advanced significantly since the introduction of two 'soft' ionisation processes in the 1980s - (1) electrospray ionisation (ESI) (Fenn *et al.*, 1989) and (2) matrix assisted laser desorption/ionisation (MALDI, Karas and Hillenkamp, 1988). They are referred to as 'soft' ionisation methods since during the ionisation process the molecules are not degraded. The three commercially available types of mass spectrometer are MALDI-time-of-flight (TOF) with/without post source decay (PSD), ESI triple quadrupole and the ESI ion trap. Reviews of these mass spectrometers and protein/peptide identification have been published extensively (Patterson *et al.*, 2001; (Borchers *et al.*, 2000; Chaurand *et al.*, 1999; Mann *et al.*, 2001; Pappin, 1997;

Shevchenko *et al.*, 2000; Spengler, 1997; Yates, III, 2000). A brief description of MALDI-TOF/PSD is given here, since it is most relevant to the work presented.

MALDI-TOF/PSD mass spectrometers consist of three major components - (i) a MALDI ionisation source (ii) a mass analyser and (iii) a detector. Peptides are co-crystallised with a matrix onto a stainless-steel target plate, which is inserted into the vacuum chamber ($10^{-7}$ - $10^{-8}$ Torr) of the mass spectrometer. The matrix, a small weakly acidic molecule, absorbs energy from a laser (337 nm) and emits this energy as heat which results in desorption of the sample. Gas-phase ions are generated which are subsequently accelerated at a fixed voltage (usually > 20,000 V) and, through a series of lenses, are directed into the field-free drift region of the mass spectrometer. Since all ions are accelerated with the same potential from a fixed point they will drift according to their mass : charge ratio. Lighter ions travel more quickly and appear at the detector sooner - hence time-of-flight (TOF) analysis.

During the ionisation process a spread of kinetic energies is imparted across the ion cloud (generated from the laser pulse) resulting in a small variation in the kinetic energy of each ion and thus a loss of resolving power. In terms of the peptide mass fingerprint spectra, mass peaks become broader, especially for larger ion masses, and the ability to resolve carbon isotopes of peptides is reduced. Two improvements have been made to this technology to increase the mass resolution of peaks - (i) the introduction of an ion mirror (reflectron) and (ii) delayed extraction. The reflectron, placed at the end of the flight tube, has a potential difference applied to it that is slightly higher than the accelerating voltage. Consequently, ions reaching the reflectron are decelerated to a dead stop and then re-accelerated back to a second detector. Since ions with a higher kinetic energy penetrate the reflectron further than ions with a lower

kinetic energy, slower ions 'catch up' with quicker ions. Hence, reducing the initial spread of energy and increasing the mass resolution. The second improvement was the introduction of delayed extraction by Vestal *et al.,* (1995) and Brown and Lennon (1995) based on a technique pioneered by Wiley and McLaren (1953). In this process, ions are allowed to spread out *before* the voltage is applied (a second lens placed nearer the sample focuses ions *before* they are accelerated into the flight tube) significantly decreasing the spread of kinetic energies and limits peak broadening.

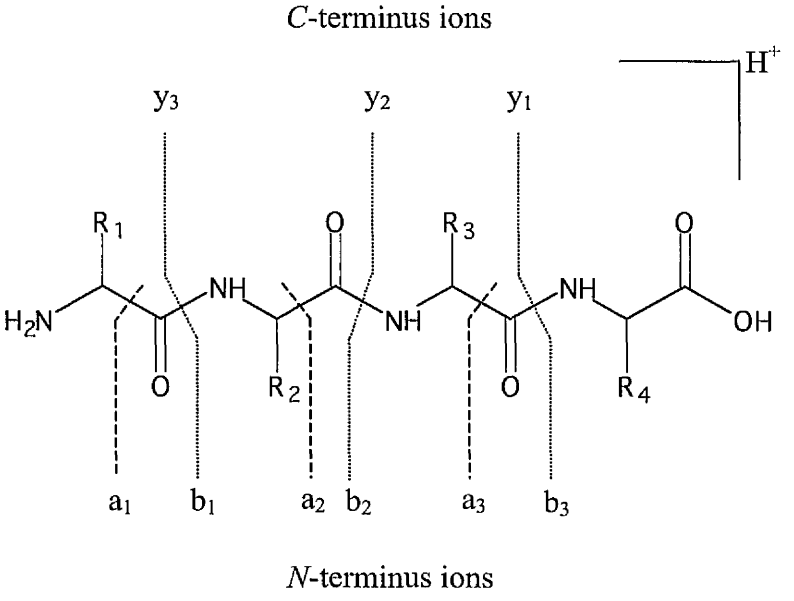
In MALDI-PSD mass spectrometry, as the ions pass through the flight tube they acquire sufficient energy to fragment (energy arises mainly from laser irradiation and gas-phase collisions), i.e. they undergo post source decay (PSD). The daughter ions that are produced have the same velocity as the parent ion but, since they have different masses, they have different kinetic energies. A specific parent ion and related daughter ions are selected by electrostatic beam blanking (an ion gate consisting of a plate and three wires which have a voltage across them that serve as a high voltage switch to deflect all ions except those of a specific mass range). The reflectron used in MALDI-TOF mode is now used as an energy analyser to discriminate between ions of different kinetic energy. Ions with increased kinetic energy penetrate further into the reflectron and arrive later at the second detector thus enabling the measurement of the masses of the daughter ions. The reflectron focuses ions over a limited magnitude of kinetic energies and so in order to focus fragment ions of a wider range, the voltage across the reflectron must be successively dropped, usually over several steps. Computer software is used to generate a complete ion fragmentation pattern by overlapping each individual fragment spectra. The fragmentation of peptides predominantly occurs along the carbon backbone resulting in characteristic ion masses that are sequence-specific (Chaurand *et*

*al.*, 1999). Peptide fragment ions are designated on the basis of the type of bond dissociation and by the type of charge retention. The most common types of ions observed in a post source decay spectra are a, b, y, immonium and internal fragment ions (Figure 5.1a and b). The number of amino acids in a fragment is written in subscript, e.g. $b_3$ contains the three amino acids from the *N*-terminus. Differences in the masses of consecutive ions of a specific series, for example '$b_2$' and '$b_3$', are characteristic of the mass of an amino and therefore may be used to determine the amino acid sequence of a peptide. Immonium ions are low mass ions which indicate the presence of an amino acid but give no sequence information. Internal fragment ions resulting from the partial fragmentation of a peptide may be used to confirm the peptide sequence, but only once the sequence has been interpreted. Other fragment ions that occur in PSD may be due to the loss of ammonia (minus 17 mass units) or the loss of water (minus 18 mass units). As a consequence of the complexity of the spectra, manual interpretation can be a complex process and is often not possible.

### 5.1.3 Identification of proteins

The identification of proteins using mass spectrometry data is simplified by the use of specialised software algorithms that compare actual peptide mass fingerprinting data (MALDI-TOF) or peptide fragmentation data (MALDI-PSD) with theoretical patterns generated from databases (Gevaert and Vandekerckhove, 2000). For example the MS-FIT and MS-TAG programs (ProteinProspector tools, refer to section 5.3.3 for more detail). Protein identifications may be obtained by searching protein/DNA databases. The specificity of the protein information generated by mass spectrometry correlates with the specificity of the search and ultimately the confidence level of a match. This is an important factor to be considered when identifying proteins from EST databases.

(a)




(b)

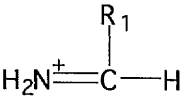



**Figure 5.1.** Examples of sequence ions that result from the cleavage of bonds along the backbone of a protonated peptide. (a) a-type and b-type ions arise from the breakage of a C-C and C-N bonds, respectively, with the charge remaining on the *N*-terminus fragment. 'Y'-type ions arise from the breakage of C-N bonds with the *C*-terminus fragment retaining the charge. (b) Immonium ion, which indicates the presence of a specific amino acid but gives no sequence information.

## 5.2 Aims

The optimisation and construction of a partial proteome map of the tachyzoite stage of *T. gondii* by 2-DE analysis was described in chapter 4. To gain the full potential of proteomics, proteins of interest need to be identified. The objectives of this chapter were:

(i) To create a skeleton 2-DE gel map of *T. gondii* tachyzoite proteins and in doing so evaluate the value of the *T. gondii* DNA sequence data currently available for direct protein identification using matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry. In common with many other microbial pathogens, complete genome sequence for *T. gondii* is not yet available. Accordingly, the value of the substantial *T. gondii* EST database for protein identification by post source decay analysis (MALDI-PSD) was also investigated.

(ii) To identify a protein expressed in the arprinocid-1-N-oxide-resistant *T. gondii* strain ($ARP^R$) but not in the parental drug-sensitive strain (pRH). The differentially expressed spot was visualised using silver staining and so the compatibility of a modified silver stain to MS was also investigated, together with the applicability of fluorescent staining techniques.

## 5.3 Methods

### 5.3.1 Processing of proteins for mass spectrometry.

Protein digestion was performed according to the method of Hellman *et al.*, (1995). Briefly, protein spots were excised from the gel and sliced into small pieces using a clean scalpel blade. Gel pieces were washed for 1 h with 500 µl 100 mM ammonium bicarbonate, for 1 h with 500 µl 100 mM ammonium bicarbonate/50% (v/v) acetonitrile and for 10 min in 50 µl 100% acetonitrile. Remaining solvent was removed by drying the gel pieces for 1-2 min in a vacuum centrifuge (DNA speed Vac, Savant Ltd, UK). Proteins were incubated for 15 min at room temperature in 10 µl 25 mM ammonium bicarbonate containing 0.2 µg sequence-grade modified trypsin (Promega Ltd, UK). Gel pieces were covered with 20 µl buffer and digestion allowed to continue overnight at 37°C on a flatbed shaker at 200 rpm (shaking was found to improve the trypsin autolysis peaks that were subsequently used for internal calibration of peptide mass fingerprint data). Tryptic digests were stored frozen at -20°C until analysis.

### 5.3.2 Mass spectrometry.

The peptide mixtures from the tryptic digests were desalted and concentrated using 10 µl C18 ZipTips™ (Millipore) according to the manufacturer's instructions. Typically, samples were eluted in 1 µl of 50 % v/v acetonitrile/0.1 % trifluoroacetic acid (TFA). Two methods for application of sample to the MALDI target plate were employed. For the dried droplet method (Karas and Hillenkamp, 1988), eluted sample was mixed 1:1 with a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA, Sigma Aldrich

Ltd, UK) in 50 % (v/v) acetonitrile/0.3 % TFA. Samples (0.5 µl) were applied to a stainless steel target MALDI plate and air-dried before analysis in the mass spectrometer. For the solution phase nitrocellulose method (Landry *et al.*, 2000), HCCA matrix was prepared in acetone (40 mg/ml) and, separately, nitrocellulose was dissolved in acetone (20 mg/ml). Matrix, nitrocellulose and 2-propanol were combined in a ratio of 2:1:1. Eluted sample was combined 1:1 with this matrix solution and 0.5 µl spotted onto the target plate and air-dried. After drying, samples were washed for 10 s with 3 µl of 5 % (v/v) formic acid, followed by a wash for 10 s with 3 µl 18 MΩ water. Samples were again air-dried before acquisition of spectra. Unless stated otherwise, the dried droplet method of matrix/sample preparation was used.

All mass spectrometry was performed using a PerSeptive Biosystems Voyager DE-STR equipped with a nitrogen laser (337 nm, 3 ns pulse width, 3 Hz repetition rate). Peptide mass fingerprint spectra were acquired in the reflectron positive mode with delayed extraction (150 ns) using approximately 200 laser shots and calibrated using trypsin autolysis peaks as internal standards (842.5100, 2211.1046 Da). All post source decay (PSD) spectra were acquired in segments, with each segment representing a reduction in the reflectron voltage of 15 %. Depending on the size of the peptide being analysed, 10-14 segments were used. Each segment was the result of averaging up to 6 acquisitions, with each acquisition being the result of accumulating 50 laser shots. P.E. Biosystems Voyager Data Explorer software (version 5.01) was used to generate a single contiguous PSD spectrum from the segments, which was then calibrated using angiotensin I ion fragments as a standard curve. In both reflectron and PSD mode, ions were accelerated at 20 kV.

### 5.3.3 Database searching.

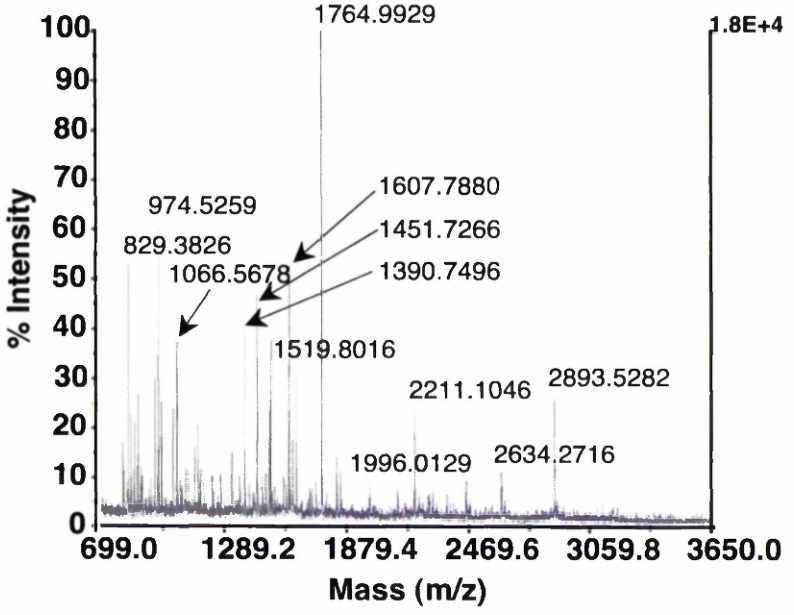
Peptide mass fingerprint (PMF) data from MALDI-TOF and peptide fragmentation data from MALDI-PSD were used to search genomic and protein databases including Genpept translated nucleotide database (http://www.ncbi.nlm.nih.gov/Entrez), Swissprot protein database (http://www.expasy.ch/sprot/sprot-top.html) and pdbEST expressed sequence tag database (http://www.ncbi.nlm.nih.gov/dbEST/index.html). All searches were performed using the MS-FIT or MS-TAG algorithm available from the ProteinProspector search tools developed by P. R. Baker & K. R. Clauser at the University of California, San Francisco (http://prospector.ucsf.edu). Searches were run on the World Wide Web (WWW) or locally on databases (or database subsets) that were either downloaded from the WWW or from the Pfizer Central Research Intranet. Search criteria used were molecular mass between 1–100 kDa (except when searching ESTs) and all isoelectric points. Cysteines were carbamidomethylated and allowed modifications considered were oxidation of methionine and acrylamide-modified cysteine. A maximum of 1 missed cleavage site was allowed and a mass tolerance of ± 50 ppm. For peptide fragmentation data, mass tolerance of ± 50 ppm was allowed for the precursor mass and ± 1500 ppm for fragment ions. Matches to *T. gondii* proteins using PMF data were considered unambiguous when there was a minimum of 5 peptides and 15 % sequence coverage (Mann *et al.*, 2001). Matches to ESTs using PMF data were considered significant with a minimum of 2 peptides. Searches of protein and EST databases using peptide fragmentation data were considered unambiguous when (i) more than 70 % of fragment ions matched to the peptide sequence, and (ii) the majority of these matches corresponded to 'a', 'b' and 'y', or related ions (Chaurand *et al.*, 1999).

## 5.4 Results

### 5.4.1 Preparation of samples for mass spectrometry

Twenty protein spots from a 2-DE gel (6-11 linear IPG) of *T. gondii* tachyzoites were digested with trypsin and analysed by matrix-assisted-laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using both the dried-droplet (DD) and solution phase nitrocellulose (SPN) methods of preparing samples. The SPN and DD methods resulted in very similar spectra with peptide masses in good agreement. The number of peptide peaks seen in each method differed only for those of relatively low intensity, with neither method being obviously better at enhancing sensitivity. The intensities of the highest peaks in each spectrum were similar with both methods, however there were differences in relative intensities of some peaks (Figure 5.2). For example, a peptide of mass 974.5259 was approximately twice the intensity of a peptide having a mass of 2893.5282 using the DD method whereas the intensity of the equivalent peaks using the SPN method differed by 9 fold. Four samples were identified as *T. gondii* proteins (catalase, lactate dehydrogenase, 20 kDa cyclophillin and cytochrome b) by searching Genpept using both DD and SPN methods (Table 5.4.1). PMF data for the remaining unidentified samples was used to search pdbEST. More matches to *T. gondii* ESTs were obtained using the SPN method compared with the DD method. However, despite more than 4 peptides matching in each result, only one sample (Ctoxqual2_2078) gave the same EST match using both techniques. Since the DD method requires less sample manipulation and is quicker, this method was chosen for subsequent MS analysis.

(a) Dried droplet method




(b) Solution phase nitrocellulose method

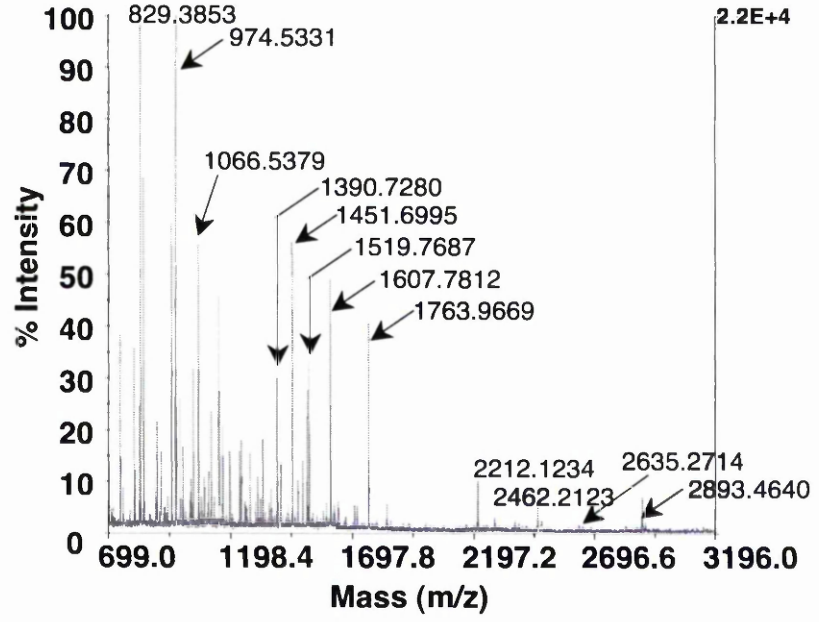



**Figure 5.2.** Comparison of (a) dried-droplet (Karas and Hillenkamp, 1988) and (b) solution phase nitrocellulose (Landry *et al.*, 2000) methods for preparation of mass spectrometry samples. *T. gondii* proteins were separated by 2-DE and visualised using Coomassie Blue, before being tryptically digested and analysed by MALDI-TOF MS. Neither method was found to be significantly better than the other, although some peaks were found to vary in their intensities.

**Table 5.4.1.** Comparison of two methods for preparing sample and matrix for mass spectrometry. Twenty *T. gondii* tachyzoite protein samples from a 2-DE gel were analysed by MALDI-TOF MS and the resulting PMF data used to search Genpept and pdbEST. Samples for MS were prepared using the dried droplet (Karas and Hillenkamp, 1988) and the solution phase nitrocellulose methods (Landry *et al.*, 2000).

| Sample No. | Dried droplet | Solution phase nitrocellulose |
|---|---|---|
| 1 | No match | Ctoxqual2_117 |
| 2 | Ctoxqual2_144 | Ctoxqual2_1032 |
| 3 | No match | EST488511 |
| 4 | Catalase | Catalase |
| 5 | Ctoxqual2_468 | Ctoxqual2_2078 |
| 6 | No match | Ctoxqual2_60 |
| 7 | Lactate dehydrogenase | Lactate dehydrogenase |
| 8 | EST467670 | EST116842 |
| 9 | No match | No match |
| 10 | No match | No match |
| 11 | 20 kDa cyclophillin | 20 kDa cyclophillin |
| 12 | No match | Ctoxqual2_1762 |
| 13 | EST 7586419 | EST117640 |
| 14 | No match | Ctoxqual2_665 |
| 15 | Ctoxqual2_2078 | Ctoxqual2_2078 |
| 16 | Ctoxqual2_312 | Ctoxqual2_25 |
| 17 | EST623360 | Ctoxqual2_573 |
| 18 | EST623404 | Ctoxqual2_2184 |
| 19 | EST622793 | No match |
| 20 | Cytochrome b | Cytochrome b |

### 5.4.2 Construction of a skeleton 2-DE proteome map for *T. gondii* tachyzoites

Having established a standardised and reproducible method for the preparation and separation of *T. gondii* proteins, a skeleton 2-DE map was created for tachyzoite proteins (Figure 5.3). Tachyzoite proteins were separated by using 4-7 linear and 6-11 linear IPG strips and the second dimension gels stained with Coomassie Blue. Polypeptide spots covering a wide range of molecular weights, isoelectric points and relative intensities were excised from the gels and characterised by mass spectrometry. Protein spots (71 in total) were digested overnight with trypsin and analysed by both MALDI-TOF and MALDI-post-source decay (MALDI-PSD) mass spectrometry. The protein identities of over half of the samples (61 %) were readily determined by using the resulting mass spectrometry data to search genomic and protein databases. The results of database searches demonstrated that the *T. gondii* proteins could be identified in one of three ways: (1) from complete gene sequences of *T. gondii* in databases (Table 5.4.2); (2) from single or consensus ESTs of *T. gondii* (Table 5.4.3); (3) based on the homology between the *T. gondii* peptides and peptides of known proteins of other organisms (Table 5.4.4).

### 5.4.3 Identification of *T. gondii* proteins using peptide mass fingerprint data from MALDI-TOF MS to search protein databases

Searching the Genpept and Swissprot protein databases with peptide mass fingerprinting (PMF) obtained by MALDI-TOF analysis data enabled 28 % of the total number of samples (46 % of the identified samples) to be identified from their full gene sequences. For example, three dense granule protein precursors GRA1 (Accession

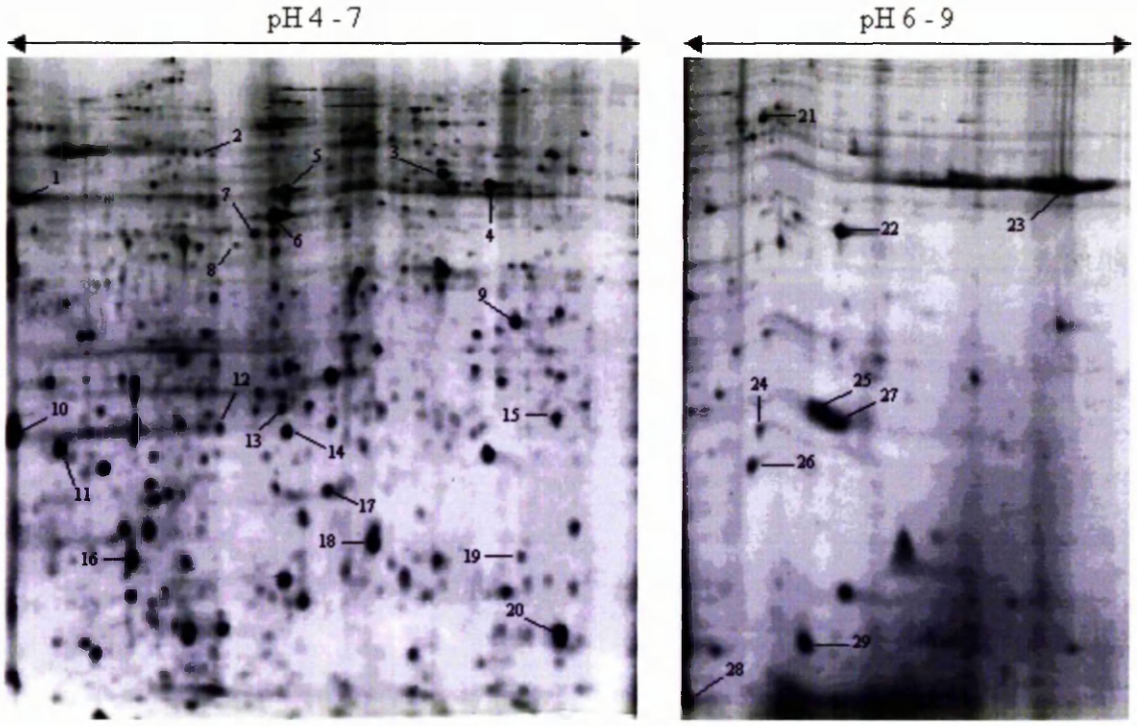



**Figure 5.3.** 2-DE map of *T. gondii* RH strain proteins and putative proteins identified by mass spectrometry. (1) Rhoptry protein precursor, ROP1A (M71274); (2) Ctoxqual2_2195 (no homology); (3) ATP synthetase beta subunit (M12082); (4) Enolase (AW703364); (5) Protein disulphide isomerase (W05902); (6) Actin (U10429); (7) Actin (U10429); (8) Heat shock protein 70, HSP70 (U82281); (9) Guanine nucleotide binding protein (ctoxqual2_1780); (10) Dense granule protein precursor, GRA 1 (Ctoxqual2_2619); (11) Ctoxqual2_257 (homology to a 19kDa *Eimeria* antigen); (12) Dense granule protein precursor, GRA7 (U79158); (13), Triosephosphate isomerase (AW703329); (14) Ctoxqual2_257 (homology to a 19 kDa *Eimeria* antigen); (15) Antioxidant protein (ctoxqual2_195); (16) Heat shock protein 70, HSP70 (U82281); (17) Heat shock protein 30, HSP30 (ctoxqual2_349); (18) Dense granule protein precusor, GRA6 (ctoxqual2_1252); (19) Adenylyl cyclase associated protein (ctoxqual2_871); (20) 18 kDa cyclophillin (U04633); (21) Catalase (AF161267); (22) Fructose bisphosphate aldolase (ctoxqual2_2078); (23) Elongation factor-1-alpha (U69697); (24) Superoxide dismutase (AF029915); (25) 28 kDa antigen (M99392); (26) Peroxiredoxin (AF029915); (27) 28 kDa antigen (M99392); (28) Histone H4 (U65675); (29) Actin depolymerising factor (U62146).

**Table 5.4.2.** Identification of *T. gondii* tachyzoite proteins using peptide mass fingerprint data from MALDI-TOF mass spectrometry to search full gene sequence databases. Matches were confirmed by searching protein and EST databases with peptide fragmentation data from MALDI-PSD analysis.

| **Result of database search with peptide mass fingerprint data** | **Identification confirmed by peptide fragmentation data** | | **Genpept / Swissprot Accession No.** | **Theoretic molecula mass (kDa); pI** |
|---|---|---|---|---|
| **Genpept match** | **Genpept match[a]** | **EST match** | | |
| Dense granule protein precursor, GRA1 | ++ | Ctoxqual2_2619 | A07518 | 28.2; 4.7 |
| Dense granule protein precursor, GRA2 | ++ | Ctoxqual2_1721 | J04018 | 28.0; 9.8 |
| Dense granule protein precursor, GRA7 | + | Ctoxqual2_31 | U79158 | 25.9; 5.1 |
| Lactate dehydrogenase[b] | ++ | Ctoxqual_3845 | U35118 | 35.5; 6.0 |
| Lactate dehydrogenase[b] | ++ | Ctoxqual_3845 | U35118 | 35.5; 6.0 |
| Rhoptry protein precursor, ROP1A | + | Ctoxqual2_1897 | M71274 | 42.7; 5.8 |
| Actin[c] | + | Ctoxqual2_931 | U10429 | 41.9; 5.1 |
| Actin[c] | + | Ctoxqual2_931 | U10429 | 41.9; 5.1 |
| Tubulin beta chain | Not done | Not done | P10878 | 50.1; 4.7 |
| Antigen p28 | Not done | Not done | M99392 | 19.8; 9.0 |
| Catalase | Not done | Not done | AF161267 | 57.3; 6.7 |
| 18kDa Cyclophilin | + | Ctoxqual2_223 | U04633 | 19.7; 6.1 |
| 20kDa Cyclophilin | +++ | Ctoxqual2_743 | U04634 | 38.1; 9.0 |
| Actin depolymerising factor | + | Ctoxqual2_2834 | U62146 | 13.0; 8.4 |
| Peroxiredoxin | + | Ctoxqual2_665 | AF305718 | 19.9; 7.0 |
| Superoxide dismutase | + | Ctoxqual2_197 | AF029915 | 22.6; 6.6 |
| Heat shock protein, HSP70 | + | Ctoxqual2_1265 | U82281 | 72.3; 5.1 |
| Heat shock protein, HSP70 | + | -[d] | U82281 | 72.3; 5.1 |
| Heat shock protein, HSP70 | + | Not done | U82281 | 72.3; 5.1 |
| Heat shock protein, HSP70 | + | Not done | AF023616 | 70.6; 5.2 |

[a] + corresponds to the number of peptides analysed
[b] Two samples analysed from pH overlap region on 4-7 linear and 6-11 linear gels
[c] Two samples analysed from different areas of 4-7 linear gel
[d] No EST match was obtained

**Table 5.4.3.** Identification of *T. gondii* tachyzoite proteins by using peptide mass fingerprint data (MALDI-TOF) and peptide fragmentation data (MALDI-PSD) to search pdbEST. Putative proteins were identified from TBLASTX search of ESTs to known proteins.

| **Result of database search with peptide mass fingerprint data** | **Result of database search with peptide fragmentation data** | **Putative protein** |
|---|---|---|
| **EST match** | **EST match** | |
| Ctoxqual2_349 | Ctoxqual2_349 | Heat shock protein 30 |
| Ctoxqual2_2078[a] | Ctoxqual2_2078 | Fructose bisphosphate aldolase |
| Ctoxqual2_2078[a] | Ctoxqual2_2078 | Fructose bisphosphate aldolase |
| No match | Ctoxqual2_257 | Homology to *Eimeria* antigen |
| No match | AW703329 | Triosephosphate isomerase |
| No match | Ctoxqual2_1252 | P33 protein (Dense granule GRA6) |
| No match | Ctoxqual2_3031 | Enolase |
| No match | Ctoxqual2_247 | GAPDH |
| No match | Ctoxqual2_1516 | Elongation factor |
| No match | Ctoxqual2_2195 | No homology |
| No match | Ctoxqual2_195 | Antioxidant protein 2 |
| No match | TgESTzya25f04.y1 | No homology |
| No match | TgESTzya43e10.y1[b] | No homology |
| No match | Ctoxqual2_349[b] | Heat shock protein 30 |
| No match | Ctoxqual2_195[b] | Antioxidant protein |
| No match | Ctoxqual2_871[b] | Adenylyl cyclase associated protein |
| No match | Toxqual512851(W05902)<br>Toxqual574387(W66308) | Protein disulphide isomerase |
| No match | Ctoxqual2_1780<br>Toxqual623420 | Guanine nucleotide binding protein |

[a] Two samples analysed from different areas of 4-7 linear gel
[b] EST/consensus sequences confirmed by analysis of two peptides

**Table 5.4.4.** Putative protein characterisation using peptide fragmentation data acquired by MALDI-PSD mass spectrometry to identify *T. gondii* proteins based on homology to peptides from proteins of different organisms.

| Protein Homology | Database searched | Species | No. ions matching | Accession number |
|---|---|---|---|---|
| Elongation factor-1-alpha | Genpept | *C. parvum* | ≥ 22 | U69697 |
| Elongation factor-1-alpha | Genpept | *S. cerevisiae*<br>*T. cruzi* | ≥ 30 | X00779<br>L76077 |
| Histone H4 | Genpept | *P. falciparum*<br>*H. sapiens* | ≥ 30<br>≥ 27 | U65675<br>X60482 |
| ATP synthetase β subunit | Genpept | *S. cerevisiae*<br>*M. pneumoniae* | ≥ 17<br>≥ 14 | M12082<br>U43738 |
| Protein phosphatase 2C | pdbEST | *A. thaliana* | ≥ 21 | AB028608 |

number A07518), GRA2 (Accession number J04018) and GRA7 (Accession number U79158), a rhoptry protein (ROP1A) (Accession number M71274) and lactate dehydrogenase (Accession number U35118) were readily identified, along with other proteins, from the Genpept database (Table 5.4.2). In the majority of cases, the theoretical molecular weights and isolectric points matched to the position of the proteins on the 2-DE gels. However, in one sample, rhoptry protein precursor ROP1A, the theoretical and observed values did not correspond. Although this protein did not focus to its theoretical position, time of flight and post source decay analyses of two separate tryptic digest preparations of the protein spot yielded identical results. The discrepancy between actual and theoretical isolectric point may indicate co- and/or post-translational modification of the polypeptide.

### 5.4.4 Using peptide mass fingerprint data (MALDI-TOF) to search EST databases

Peptide mass fingerprint (PMF) data can also be used to search the pdbEST database (or a subset of ESTs). Accession numbers from possible matches may then be used to search the *T. gondii* EST database (http://ParaDB.cis.upenn.edu/toxo/index.html) to identify a corresponding single, or consensus EST sequence. In one third of the samples identified in this survey, PMF data also matched to *T. gondii* EST entries. For example, PMF data identified *T. gondii* dense granule protein precursor (GRA1) from Genpept (A07518) and from a single EST (AW702218) from pdbEST. In some instances, PMF data matched to several ESTs which were subsequently found to belong to the same consensus sequence (Table 5.4.3). For example, PMF data matched to eight EST sequences which were found to belong to the same *T. gondii* consensus sequence, Ctoxqual2_2078. In such cases, matches were confirmed by post-source decay (PSD)

analysis. However, some protein spots gave weak PMF matches to single ESTs and further analysis of the sample by peptide fragmentation using PSD analysis was required to give unambiguous protein identity.

### 5.4.5 Identification of proteins using peptide fragmentation data obtained by MALDI-post-source decay (PSD) analysis

All proteins that were identified by PMF data were subsequently confirmed by PSD analysis of 1 or more peptides and searching Genpept. In each case, the protein match obtained with peptide fragmentation data agreed with that from PMF (Table 5.4.2). Peptide fragmentation data obtained by MALDI-PSD was found to be the most reliable method of protein identification from EST sequences. To test this, all samples which had been unambiguously identified by PMF data were subsequently analysed by PSD and the peptide fragmentation data used to search pdbEST without reference to the protein database. Matching ESTs identified in this way were then used in a BLAST search and in each case found to match the protein identity obtained by PMF (Table 5.4.2). For example, peptide fragmentation data of 3 peptides from the same sample each confirmed the protein identity of a 20 kDa cyclophillin (searching Genpept) and in addition, data from all of the peptides confirmed the corresponding consensus EST sequence for this protein, Ctoxqual2_743 (searching pdbEST).

Of the total number of samples investigated, 25 % (42 % of the identified samples) were identified from searching six-frame translations of ESTs rather than from full gene sequences using peptide fragmentation data (Table 5.4.3). BLAST search homology allowed a putative protein to be assigned to each match. Several categories of results were distinguished: (a) protein spots could be linked to gene sequence using

peptide fragmentation data from peptides that matched to a single EST; (b) protein spots could be identified from peptides that matched several ESTs belonging to the same consensus sequence; (c) protein spots could be identified from peptides that matched different ESTs but were later shown to belong to an contiguous open reading frame. For instance, PSD analysis of two peptides from a single sample matched to two different ESTs (W05902 and W66308) which, at the time of analysis, had not been previously linked in the EST consensus database. As our mass spectrometry suggested these peptides and their corresponding EST matches were from the same gene (since they were derived from the same protein spot), we sought to test this by amplifying by PCR the gene fragment linking the two ESTs (Figure 5.4). Primer sequences designed to EST W05902 and EST W66308 amplified a single band of 580 bp suggesting that the two EST sequences were indeed part of a single open reading frame. The resulting fragment was sequenced from both ends to reveal the linking DNA fragment. BLAST searching of this sequence showed homology to several protein disulphide isomerases (PDI) from different species, with the top match to *C. elegans*. Since this analysis was conducted, a new release of the *Toxoplasma* consensus EST database (version 3) has confirmed that the two ESTs do indeed belong to the same contig (Ctoxqual_695) with high homology to PDI (BLAST score = 2e-31) (Figure 5.5). PCR amplification of gene fragments using primers designed on the two ends of a consensus EST sequence, of which two peptides matched to either end of the contig, was also demonstrated. For example, primer sequences designed from peptides that matched to opposite ends of Ctoxqual2_1780 amplified a single band of 850 bp (Figure 5.4). BLAST searching of this fragment sequence showed high homology to a *Chlamydomonas reinhardtii* guanine nucleotide binding protein (BLAST score = 1e-24).

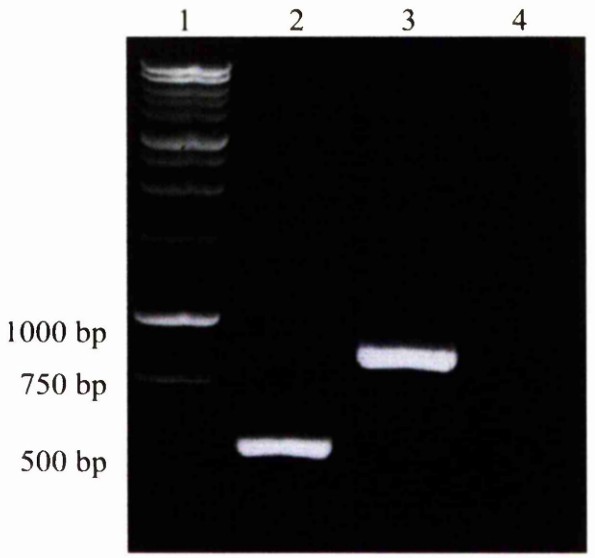



**Figure 5.4.** PCR amplification of gene fragments encoding putative *T. gondii* proteins identified by MALDI-PSD mass spectrometry. Putative protein identities were assigned by searching the pdbEST database with peptide fragmentation data (MALDI-PSD). Primers designed from two matching ESTs (W05902 and W66308), 5'-CGT CGA TGC CAC CAG CGA GA-3' and 5'-CGG GTG TCA CGG AGT TCC TT-3', amplified a 580 bp fragment which was sequenced and subsequently shown to have homology to protein disulphide isomerase (lane 2). Primers designed from either end of a *T. gondii* consensus sequence (Ctoxqual2_1780), 5'-GCA AGC AGG ACG ATA ATC GA -3' and 5'-GAG TTA GAG TCC AGA GAG TA -3', amplified an 850 bp fragment which was sequenced and subsequently shown to have homology to guanine nucleotide binding protein (lane 3). Lane 1, DNA ladder; Lane 4, control.

i) Reading frame +3

```
Query: 15   QAV**REYPTLTLFRKEK**PEKYTGGRTAESNC*VXXXXXXXXXXXXXXXXXXXXXXXXHPIA 194
            Q VREYPTLTLFRKEKPEKYTGGRTAE+                                PIA
Sbjct: 97   QGVREYPTLTLFRKEKPEKYTGGRTAEAIV-EWIEKMTGPAVTEVEGSAEDKVTKEAPIA 155

Query: 195  FVAELASKDSDMAKLFEEXANEVPPAGKGSWLSTGASXEKDXLPSLXGGHLKPSQAKTKD 374
             FVAELASKDSDMAKLFEE ANE    G+      GAS EK        G  +    KTKD
Sbjct: 156  FVAELASKDSDMAKLFEEVANESRQLGR-FLAKYGASDEKIYSLRYEEG-TEAFTGKTKD 213

Query: 375  ELKKFVDTESFPLXG 419
             ELKKFVDTESFPL G
Sbjct: 214  ELKKFVDTESFPLLG 228
```

**Figure 5.5.** Sequence alignment of protein disulphide isomerase from *Toxoplasma gondii*. A gene fragment from a putative *T. gondii* protein identified by MALDI-PSD mass spectrometry was amplified (Figure 5.4.3) and sequenced. The resulting *T. gondii* sequence (query) showed high BLASTX homology to protein disulphide isomerase from *C. elegans* (not shown). Since this study the new release of the *T. gondii* EST database has confirmed the protein identity (sbjct)(score = 2e-31. Sequence alignments for two different reading frames are shown (i and ii). Query sequence highlighted in bold letters indicates the sequence initially identified by mass spectrometry.

Furthermore MS data were found to help in mapping the EST database. For example Ctoxqual2_347 was identified from 2 peptides, the first matched to 2 EST sequences and the second peptide matched to 10 EST sequences. At the time of analysis, version 2 of the *T. gondii* database listed a total of 7 EST sequences as part of the contig. The current version 3 of the database now lists a total of 13 EST sequences to be part of this contig (renamed as Ctoxqual_3057) including the sequences that were identified as part of the MS study.

### 5.4.6 Using peptide fragmentation data to identify proteins based on homology with proteins of other organisms

Of the total number of identified samples, 12 % were characterised by matching peptide fragmentation data of 1 or 2 peptides to peptides from proteins of organisms other than *T. gondii* (Table 5.4.4). For example, a sample whose PMF spectra from MALDI-TOF found no match in the Genpept and pdbEST databases was further analysed by PSD. Two peaks (1192.6847 kDa and 1325.7778 kDa) were selected for fragmentation by PSD. The resulting peptide fragmentation spectra were used to search the Genpept protein database for all organisms. Both peptides were found to have homology to peptide sequences from Histone H4, from *Plasmodium falciparum* and from *Homo sapiens* (Figure 5.6a-c). Retrospective analysis of proteins that had been previously identified as *T. gondii* proteins, but without reference to the *T. gondii* database, revealed that some of these could have been correctly characterised by homology to other organisms. These included actin (*Plasmodium falciparum*, *Entamoeba histolytica*, *Caenorhabditis elegans*), HSP70 (*Theileria parva*, *Trypanosoma cruzi*, *Saccharomyces cerevisae*), ATPase (*S. cerevisiae*, *Mycoplasma*

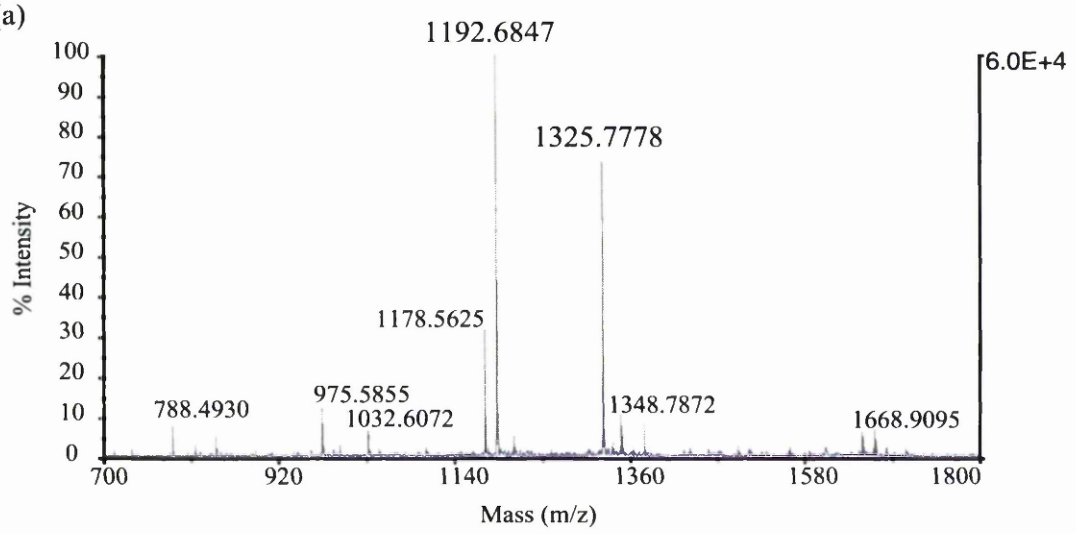
*genitalium*) and phospolipase-2C (*Neospora caninum*). However, others such as GRA1, lactate dehydrogenase and ROP1 could not be characterised by homology.

### 5.4.7 Identification of silver- and fluorescence-stained proteins

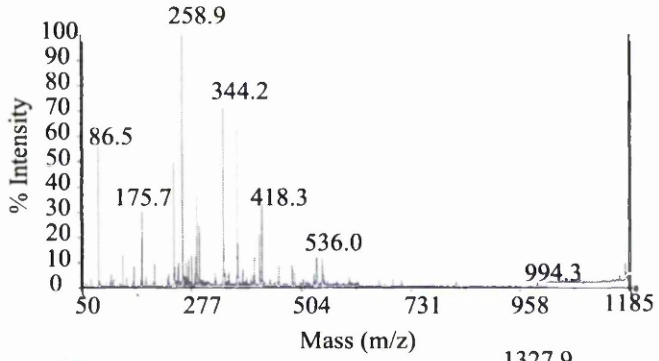
Having established a working method for the reliable identification of *T. gondii* proteins from protein and genome databases using peptide mass fingerprint and peptide fragmentation data, the sensitivity of detection of silver- and fluorescence-stained *T. gondii* proteins was investigated. A modified silver stain was used to visualise proteins. Several changes were made to the procedure, (i) gels were fixed twice in fixative (ii) glutaraldehyde was omitted from the sensitizer solution; and (iii) 100 µl formaldehyde was added to the developer solution (per 250 ml). Only a few samples were analysed and of these good spectra were obtained for the more abundant proteins, i.e. those that were previously identified on Coomassie blue stained gels. For example, actin (U10429) was readily identified from PMF data (Figure 5.7a) and confirmed by PSD analysis. A second sample could not be identified from PMF data alone but was subsequently assigned to a *T. gondii* consensus sequence, Ctoxqual2_349 by PSD analysis (Figure 5.7b).

Although silver staining detects proteins as low as 5 ng, incompatibility with MS poses problems, which may be overcome using the recently developed fluorescent dyes. Good MS spectra were obtained with Sypro red enabling the identification of several proteins. For example, actin (U10429) was easily identified using PMF data from Sypro red stained protein (Figure 5.8a). Although the spectra contained fewer peptides than the spectra obtained for the equivalent protein stained with silver, the peak intensities were 5 times greater. A second sample stained with Sypro red resulted in no

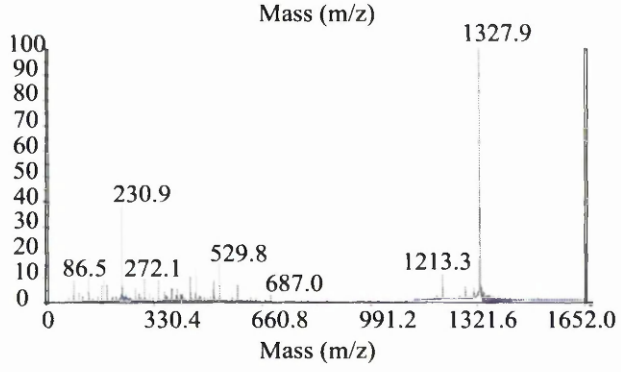
(a)




(b) i)




ii)

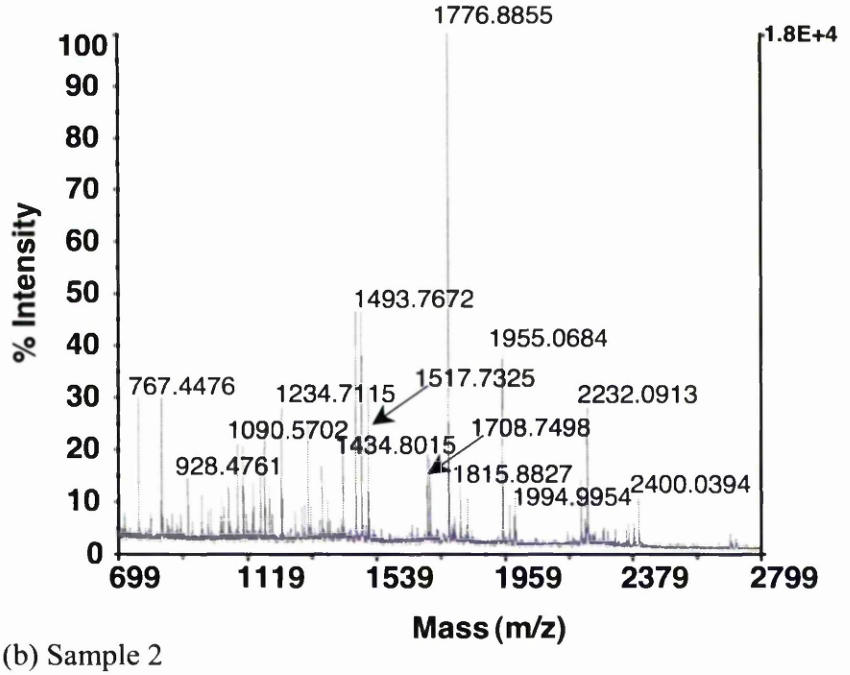



(c)

| Measured Peptide Mass (Da) | Matched ions | MH+ Error (Da) | Peptide Sequence | Protein molecular mass (kDa) / pI | Species | Genpept Accession # | Protein Name |
|---|---|---|---|---|---|---|---|
| 1192.6847 | ≥ 30 | 0.0269 | (R)ISGLIYEEIR(G) | 9.2 / 11.00 | *Plasmodium falciparum* | U65675 | Histone H4 |
| 1325.7778 | ≥ 27 | 0.0237 | (R)DNIQGITKPAIR(R) | 11.4 / 11.36 | *Homo sapiens* | X60482 | H4 Histone |

**Figure 5.6.** Identification of a putative *T. gondii* protein using MALDI-PSD. (a) Peptide mass fingerprint (PMF) of a protein spot excised from a 2-DE gel, digested with trypsin and analysed on a MALDI-TOF/PSD mass spectrometer. Searching protein and EST databases with PMF data alone resulted in no matches. (bi) Peptide 1192.6847 and (bii) peptide 1325.7778 were fragmented by MALDI-PSD mass spectrometry. Both peptides were found to have homology to peptide sequences from Histone H4, from *Plasmodium falciparum* and from *Homo sapiens* (c).

(a) Sample 1




(b) Sample 2

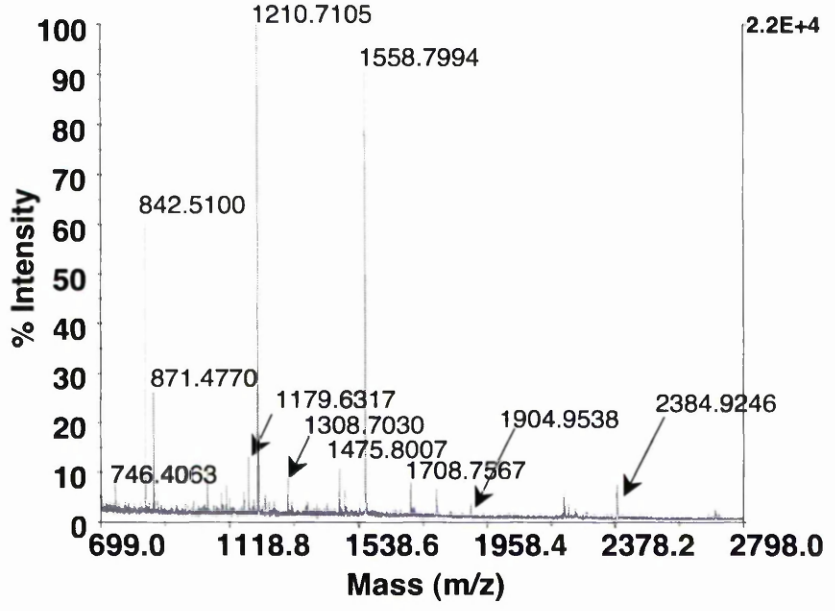
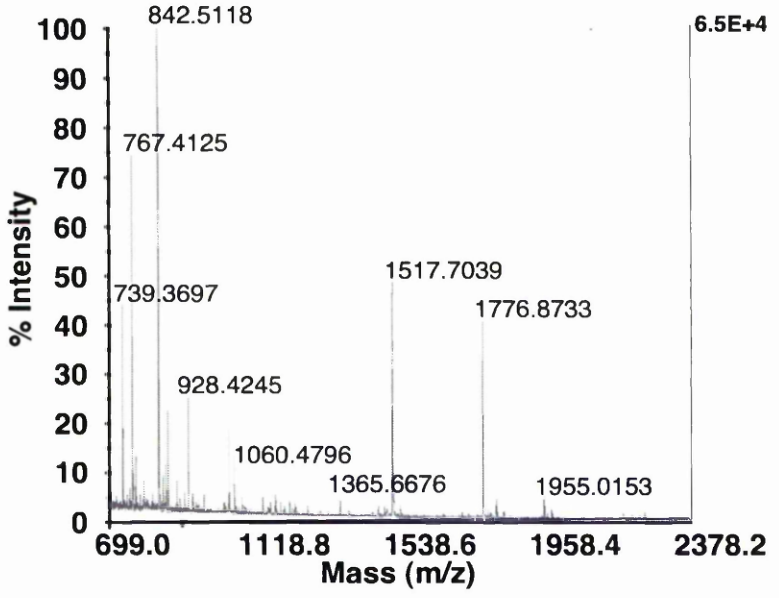



**Figure 5.7.** Analysis of silver-stained proteins by MALDI-TOF mass spectrometry. *T. gondii* proteins, separated by 2-DE and visualised using a modified silver stain, were analysed by MALDI-TOF/PSD MS. Sample 1 (Figure a) was identified as actin (U10429) by searching Genpept and confirmed by PSD analysis (not shown). PMF data for Sample 2 (Figure b) was insufficient to identify the protein; post source decay analysis of two peptides from this sample identified the *T. gondii* consensus sequence Ctoxqual2_349.

(a) Sample 1




(b) Sample 2

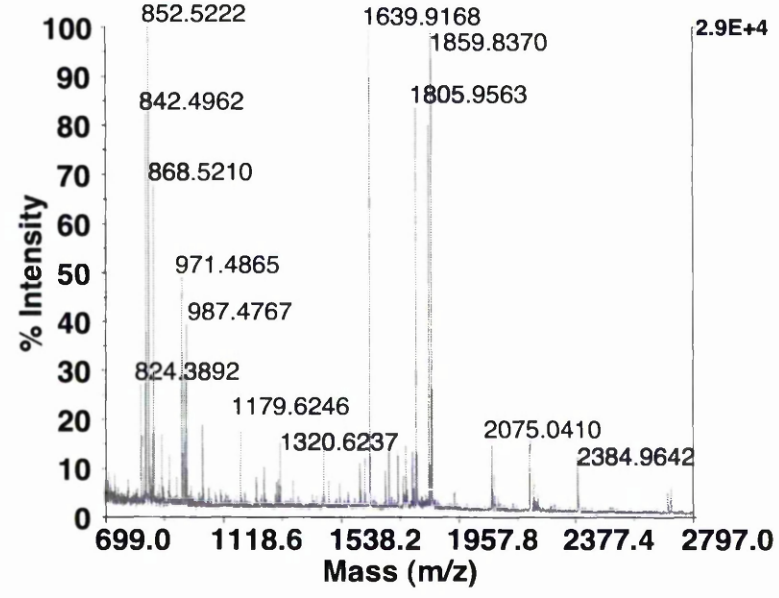
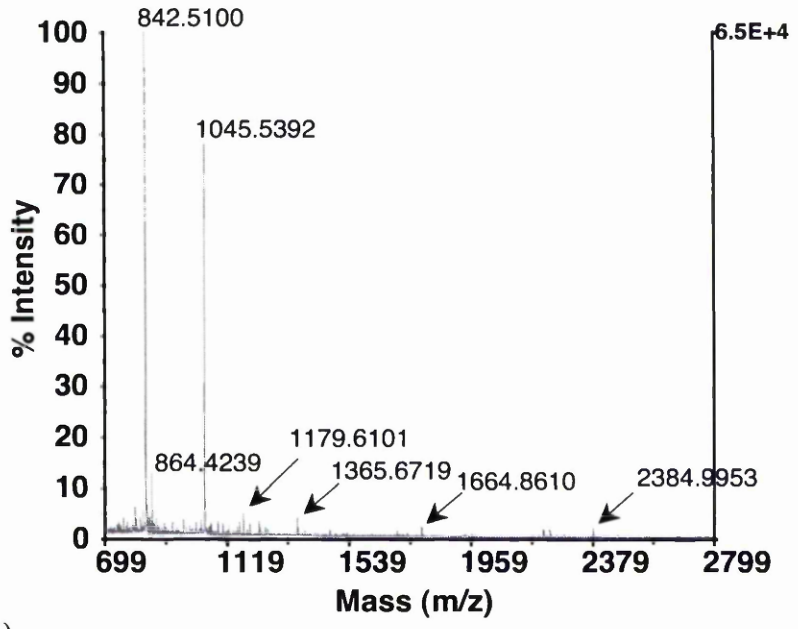



**Figure 5.8.** Analysis of Sypro Red-stained proteins by MALDI-TOF mass spectrometry. *T. gondii* proteins, separated by 2-DE and visualised using Sypro Red fluorescent stain, were analysed by MALDI-TOF/PSD MS. Sample 1 (Figure a) was identified as actin (U10429) by searching Genpept and confirmed by PSD analysis (not shown). PMF data for Sample 2 (Figure b) was insufficient to identify the protein; post source decay analysis identified the protein as having homology to enolase (X55981) from *Zea mays*.

(a)




(b)

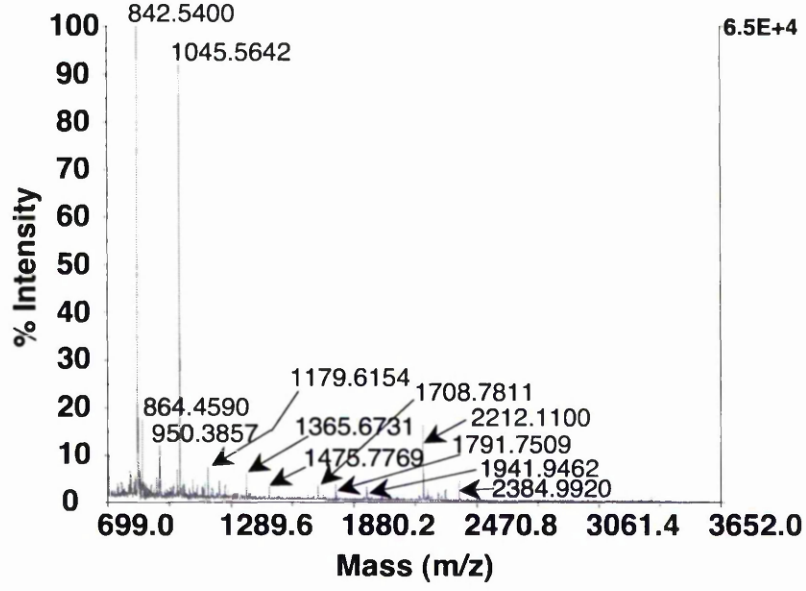



**Figure 5.9.** Analysis of a differentially expressed *T. gondii* protein by MALDI-TOF and MALDI-PSD mass spectrometry. The proteins of arprinocid-1-N-oxide-sensitive and -resistant *T. gondii* tachyzoites were compared using 2-DE. A differentially expressed protein present in the resistant line, but not in the sensitive line, was analysed by MALDI-TOF/PSD mass spectrometry. The protein was visualised using the modified silver stain but proved to be present in insufficient quantities to identify by MS (a) even when 4 spots were pooled together (b).

matches from PMF data but was identified as enolase (X55981) from *Zea mays* by PSD analysis (Figure 5.8b).

### 5.4.8 Attempts to identify a protein specific to drug-resistant parasites

The protein expression profiles of isogenic arprinocid-1-N-oxide-sensitive and -resistant *T. gondii* strains were analysed by 2-DE and silver staining. Protein spot differences (see Chapter 4) were analysed by ImageMaster 2D and one such difference excised, tryptically digested and analysed by MALDI-TOF/PSD MS. The protein spot was weak in intensity and as expected only a weak PMF spectra could be obtained (Figure 5.9a) even when 4 spots were pooled together (Figure 5.9b). Post source decay analysis was attempted and weak spectra obtained with ion peaks indistinguishable from background noise. Database searches of Genpept and pdbEST using both the PMF and PSD data yielded no matches.

## 5.5 Discussion

The introduction of delayed extraction techniques used in conjunction with MALDI-TOF MS has enabled the accuracy of peptide mass database searches to be as low as ± 10 ppm (Clauser *et al.*, 1999). Together with the ability to gain *de novo* sequence information by PSD analysis, these methods have significantly increased the likelihood of correct protein identifications from the rapidly growing size of protein and EST databases. Analogous to sample preparation determining the outcome of a 2-DE gel, the isolation procedures used for extracting digested proteins from 2-DE polypeptide spots and the preparation of samples for MS can have a significant impact on the sensitivity of detection of peptides. When processes are optimised, protein identifications may be obtained from low picomole to high femtomole quantities (Gevaert and Vandekerckhove, 2000).

The original protocol for tryptic digestion (Hellman *et al.*, 1995) suggested that after removing the supernatant from the overnight incubation in trypsin, two further extractions should be performed. In agreement with a detailed study of the recovery of peptides (Speicher *et al.*, 2000), these two additional extracts provided minimal benefit to the overall quality of the PMF data. Speicher *et al.*, (2000) demonstrated that over 75 % of peptides were recovered in the first extract and that subsequent extractions contribute no more than 10 % of total peptide recovery. Additional extracts and the subsequent concentration of extracts by vacuum centrifugation increase peptide losses due to adsorption to plastic surfaces and increase salt contamination.

The critical parameters in achieving optimal sample preparation for MS are: (i) achieving a high concentration of peptides relative to contaminants such as salt residue; and (ii) the formation of a homogeneous crystalline matrix. Desalting and concentration of peptide samples using ZipTips proved to be a fast (each sample was completed in approximately one minute) and reliable method of increasing the signal to noise ratio. The nitrocellulose solution phase method of preparing targets has been reported to be far more effective than the dried droplet method and a much-improved version of a 'fast evaporation' method which suffers technical difficulties due to the speed at which acetone evaporates (Landry *et al.*, 2000). In contrast, data presented in this thesis indicate that there was no significant difference between the dried droplet and solution phase nitrocellulose methods (the fast evaporation method was attempted unsuccessfully). The difference in findings may be a reflection of proteins analysed in this report being separated by 2-DE and that contaminating substances often present in 2-DE and not in simple SDS-PAGE techniques, e.g. CHAPS, may have reduced the effectiveness of the solution phase nitrocellulose method. Since neither method offered any overwhelming advantage over the other, the dried-droplet method of preparing samples was adopted because it involved less sample manipulation.

MALDI-TOF mass spectrometry is a fast and reliable method of protein identification which relies on comparing peptide mass fingerprints from analysis of enzymatically digested protein samples to computer generated theoretical fingerprint profiles from protein/EST databases. Protein matches are ranked according to algorithms that take into account protein properties, such as species of origin and expected mass range, in addition to the percentage sequence coverage and mass accuracy, to calculate the probability (and confidence level) of a correct identification. One example is the

modified MOWSE scoring algorithm, developed by Pappin *et al.,* (1993), and utilised by MS-FIT (ProteinProspector tools). Whilst strict rules can be applied to govern the likelihood of a correct identification, such as a minimum of 5 peptide matches, results are often open to user interpretation. For example, a large MW protein identified by 5 peptides may score lower than a small MW protein identified with a similar number of peptides because the percentage coverage has decreased. Recently a new search engine, ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound), employing a new type of scoring algorithm based on Bayesian probability theory, has been developed and is claimed to be superior to the ProteinProspector algorithms (Zhang and Chait, 2000). Indeed, identification of proteins from mass spectrometry data is relatively simple with these newer algorithms, but ultimately the protein must initially be present in the database. Designing algorithms that minimise user interpretation when searching EST databases is more difficult. For instance, an expected protein mass range cannot be applied to an EST sequence database search.

Proteome studies of fully sequenced organisms such as yeast have been able to identify the vast majority of proteins using mainly genome sequence information (Parker *et al.*, 1998; Shevchenko *et al.*, 1996a). In our study, 28 % of the samples analysed were identified by correlating the peptide mass fingerprint data with complete gene sequences, but as samples were analysed mainly from Coomassie blue stained gels there would have been an unavoidable bias towards more highly expressed proteins. For example, dense granule, rhoptry and structural proteins were readily identified in the analysis. Interestingly, these proteins which appeared to be highly expressed on the protein gel also show high representation in non-normalised EST databases suggesting that there is a good correlation between mRNA and protein expression for these

molecules. The relationship between the transcriptome and the proteome of *T. gondii* awaits future analysis, but it is perhaps unlikely that there will be a simple stoichiometry between the two. Further work on integrating transcriptome and proteome data will be essential for more meaningful interpretation of the emerging microarray data for *T. gondii* and other protozoa.

In order to increase the success of protein identification, PMF data were used to search the pdbEST database. PMF data obtained by MALDI-TOF are often unsuitable for EST database searches, since EST sequences are usually too short (approx. 200-400 bp) for reliable identification and there is the uncertainty of the correct reading frame (Boguski, 1995; Shevchenko *et al.*, 2000). However, since a highly developed consensus EST database is available for *T. gondii*, its suitability for searches on six-frame translations of these longer consensus sequences was tested. Matches to ESTs were found in a large number of samples, but only in a small number of samples were matches with PMF data considered reliable; these corresponded to large consensus EST sequences such as those for fructose bisphosphate aldolase (Ctoxqual2_2078, Table 5.4.3). Thus, whilst PMF data alone may be used to search EST databases, these matches must be treated with caution because EST sequences are not long enough to ensure confident matches. Perhaps this may be remedied by using multiple digestions of protein samples, each with enzymes of differing specificity (James, 1997). For example, PMF data from two separate digests of a sample using two different enzymes may be used to search Genpept (all organisms)/pdbEST. The top 50 hits on each results page from each digest may then be compared and cross checked for proteins that appear on both lists. In this event further analysis would still be required for increased confidence of a correct identification.

In contrast to PMF data, peptide fragmentation data obtained by MALDI-PSD gives information that can be used to search protein databases and six-frame translations of ESTs to yield primary sequence. This was demonstrated to be a highly successful strategy for assigning ESTs to polypeptide spots excised from the 2-DE gels based on matches to one or more peptides. However, although many matches to ESTs with PSD data were unambiguous (especially those with matches to two or more peptides), the exact nature and function of the proteins coded by the matching EST remains to be elucidated.

In some instances MS analysis of *T. gondii* proteins provides information that can help interpret the EST database. For instance, where two or more ESTs were demonstrated to be linked to the same gene by virtue of the fact that they were both identified from a single protein spot. Significantly, peptide fragmentation data could also be used to identify *T. gondii* proteins based on homology of peptides to known proteins from other organisms. This was found to work well with highly conserved proteins such as histone H4 or ATP synthetase β subunit, molecules which are likely to share peptide homology with *T. gondii*, but not as well for more species-specific proteins, such as dense granule proteins or rhoptry proteins. Although homology searching was demonstrated for *T. gondii* tachyzoites, this method also should be applicable more generally, thus allowing proteome analysis of other organisms whose genome is currently unsequenced, for example, *Neospora caninum* or *Eimeria* species. As protein/EST databases increase for other apicomplexan parasites the success of homology searching with peptide fragmentation data obtained either by post source decay analysis or tandem mass spectrometry will become an increasingly valuable approach for those apicomplexan parasites still lacking genomic analysis.

The initial aim of this work was to identify the differentially expressed protein found between the arprinocid-1-N-oxide-sensitive and -resistant *T. gondii* lines (pRH and $ARP^R$). Although an extensive *Toxoplasma* EST database permitted the putative identification of many proteins by MALDI-TOF/PSD MS, this differentially expressed protein remains unidentified. The relative incompatibility of silver-staining with mass spectrometry analyses was significant in the inability to obtain sequence information for this protein. However, recent advances in fluorescent staining, such as Sypro Ruby® (discussed below), and the introduction of automatic spot pickers, both of which were not available during this study, now enable mass spectrometry analysis of low abundance proteins. Further advances in the genome sequencing of *T. gondii* should increase the possibility of identification of proteins. The following strategies (1 - 5) offer conceivable solutions for the identification of the differentially expressed protein.

(1) The advantage of silver staining over Coomassie blue staining is the sensitivity of detection, between 1 and 10 ng of protein (Rabilloud, 1992). Although silver staining methods require several steps that are time consuming, some protocols can be completed in less than 2 h and, in conjunction with automatic gel stainers, with minimal manual input. However, the lack of linearity of staining and protein modifications induced by strong oxidising and sensitising agents, e.g. $Ag^+$, sodium thiosulphate and glutaraldehyde, often make mass spectrometry more difficult. This is in contrast to some published work that have identified low nanogram concentrations of known proteins stained by silver and observed no chemical modifications (Shevchenko *et al.*, 1996b). The modified silver stain used in this report worked well allowing the identification of abundant proteins. However, although the removal of glutaraldehyde and the reduction of formaldehyde served to reduce crosslinking effects that are

problematic for MS, the sensitivity of detection of proteins within the 2-DE gel was reduced. This simply may be overcome by increasing the amount of protein loaded onto the gel. Alternatively, the removal of silver ions prior to digestion using chemical reducers such as potassium ferricyanide and sodium thiosulphate has been demonstrated to enhance peptide detection from silver-stained spots (Gharahdaghi *et al.*, 1999). This technique used in conjunction with MALDI-TOF allowed the detection of 10 ng of protein.

(2) Alternative fluorescent stains such as Sypro Ruby® (Gorg *et al.*, 2000) are best placed to overcome the problem of silver-staining since they rival the sensitivity of silver and are more compatible with MS. Analysis of a small number of protein spots from this study that had been stained with Sypro red showed that good MS spectra could be obtained; Sypro Ruby® is the stain of choice for highest sensitivity in 2-DE analysis. This or other fluorescent dyes may therefore become the stain of choice in future proteomic studies where high sensitivity and compatibility with MS is required. Recently a new fluorescent metal chelate, ruthenium II tris was shown to have similar sensitivity to Sypro Ruby® and have even better sensitivity in mass spectrometry analysis (Rabilloud *et al.*, 2001).

(3) Manual interpretation of PSD spectra is often challenging due to the large numbers of peaks obtained representing sequence specific and internal fragment ions. Recent advances in chemistry have now produced methodologies for charge derivatisation of tryptic digests. One such protocol involves the attachment of a charged tag to the N-terminus of peptides which, when analysed by PSD, fragments along the CHR-C(O) backbone of the peptide, yielding predominantly a-type sequence ions

(Shen *et al.*, 1999). The resulting spectrum is easier to interpret manually, since the mass differences between consecutive pairs of a-type ions correspond to sequential amino acid residues.

(4) By increasing the sequence coverage of the parent protein, a higher level of confidence in identification of the protein is obtained. Despite the many advantages of MALDI-TOF MS, such as rapid detection of picomolar amounts of protein, it suffers the disadvantage of being biased towards the detection of ions that contain an arginine residue over those that contain lysine residues (Krause *et al.*, 1999). However, this may be overcome by increasing the basicity of lysine by chemically converting lysine to homoarginine with *O*-methylisourea (Hale *et al.*, 2000).

(5) Work involved in this chapter has centred on the use of a MALDI ionisation source, however, another common source is electrospray ionisation (ESI). In ESI the analyte is in solution and enters the mass spectrometer either by infusion or nanoelectrospray, or increasingly in combination with reversed phase liquid chromatography (LC-MS). The advantages of nanoelectrospray ionisation techniques are that very low flow rates, a few nanolitres per minute, may be achieved thus allowing complex mixtures to be analysed. LC-MS allows sample cleanup, separation and concentration all in one step reducing sample loss due to manipulation (Mann *et al.*, 2001). It also has the added advantage of allowing automated high-throughput screening. The most common ESI mass spectrometer is the triple-quadrupole that consists of three sections or quadrupoles (Q1 - Q3). Typically, quadrupole mass spectrometers are operated in MS-MS mode, whereby an ion having a specific mass:charge ratio is selected in Q1 and subjected to collision induced dissociation (CID) in Q2 with the resulting fragment ions determined in Q3. Recently, the Q3

quadrupole has been replaced with a TOF analyser (Q-TOF) resulting in higher accuracy and resolution (Borchers *et al.*, 2000). In addition, the ESI source may also be replaced with a MALDI source (MALDI-Q-TOF) enabling proteins from complex mixtures to be identified by searching protein and EST databases with increased accuracy (Shevchenko *et al.*, 2000). These improvements in mass spectrometric technologies will allow increased sample handling, analysis of low copy number proteins and, together with the development of more powerful database search algorithms, should allow the identification of previously unsequenced proteins by homology searching (Shevchenko *et al.*, 2001).

In conclusion, *T. gondii* tachyzoite proteins separated by 2-DE may be identified, despite limited genome information, by using a combination of MALDI-TOF and -PSD mass spectrometry data to search both protein and EST databases. Not all of proteins analysed were subsequently identified but other approaches exist to improve MS sensitivity and aid manual interpretation of spectra. Although proteomics will not tell us about protein function directly, it will prove to be an essential tool in pinpointing the proteins and genes for which future detailed functional analysis is required. For example, the identification and characterisation of proteins thought to be involved in acquisition of drug resistance. Proteomics will be a key technology in helping to interpret the complexity of the genome and will provide a better understanding of disease.

# CHAPTER 6

# Characterisation of Purine Transporters in *T. gondii*: Effects of Arprinocid and Arprinocid-1-N-oxide on Purine Salvage

## 6.1 Introduction

### 6.1.1 Purine transport in *T. gondii*

*Toxoplasma gondii* is incapable of purine *de novo* synthesis and must salvage essential purines from its host environment (Perrotto *et al.*, 1971). Consequently, purine transporters and salvage enzymes provide potential conduits and attractive targets, respectively, for the development of improved anti-*Toxoplasma* drugs. Although the purine salvage pathways in *T. gondii* are fairly well understood, little is known about the transporters in *T. gondii* and much controversy surrounds the theory that adenosine is the preferred salvage substrate.

Using mutant Chinese hamster ovary (CHO) cells with a defect in purine synthesis as host cells for *T. gondii*, neither radiolabelled formate nor glycine were incorporated into *T. gondii* purines and thus it was concluded that *T. gondii* is an auxotroph for purines (Schwartzman and Pfefferkorn, 1982). Similarly many other protozoa, such as *Eimeria spp* and *Plasmodium spp*, are incapable of *de novo* purine synthesis (Hassan and

Coombs, 1988). The purine salvage pathways present in *T. gondii* are shown in Figure 6.1.

With the exception of adenosine, *T. gondii* nucleosides are first hydrolysed to the free base before being incorporated. All four purine bases (hypoxanthine, xanthine, guanine and adenine) are incorporated into their respective nucleotides *via* phosphoribosyltransferase (PRTase) enzymes (Krug *et al.*, 1989). The enzyme responsible for phosphoribosylisation of 6-oxypurines, hypoxanthine-xanthine-guanine-phosphoribosyl-transferase (HXGPRT), has been cloned (Donald *et al.*, 1996). A separate enzyme, adenine phosphoribosyltransferase, for the incorporation of adenine, exhibits nearly 3-fold lower activity compared with HXGPRT (Krug *et al.*, 1989). Using adenosine deaminase deficient host cells, deaminase activity was detected for adenosine, adenine and guanine. The low specific activity of adenosine deaminase conflicts with data suggesting that intracellular *T. gondii* tachyzoites convert 15 % of adenosine to inosine (Krug *et al.*, 1989). The exact role of adenosine deaminase is unclear. Adenosine is incorporated directly *via* adenosine kinase and since this enzyme exhibits a ten-fold higher activity than the HXGPRT-mediated salvage of 6-oxypurine bases (Krug *et al.*, 1989), it is possible that adenosine is the preferred substrate for purine salvage. Consistent with this theory, an adenosine transporter (TgAT1) has been identified in the plasma membrane of *T. gondii* tachyzoites (Schwab *et al.*, 1995). However, the sub-micromolar concentration of free adenosine in the host cell cytoplasm is inconsistent with the low affinity of this transporter ($K_m$ = 120 μM). (Schwartzman and Pfefferkorn, 1982) showed that tachyzoites are capable of using adenine nucleotides as a purine source, after degradation to adenosine. However, tachyzoites lack an uptake system for adenine nucleotides (Ngo *et al.*, 2000). An NTPase capable of hydrolysing ATP to AMP has been identified in the parasitophorous vacuole

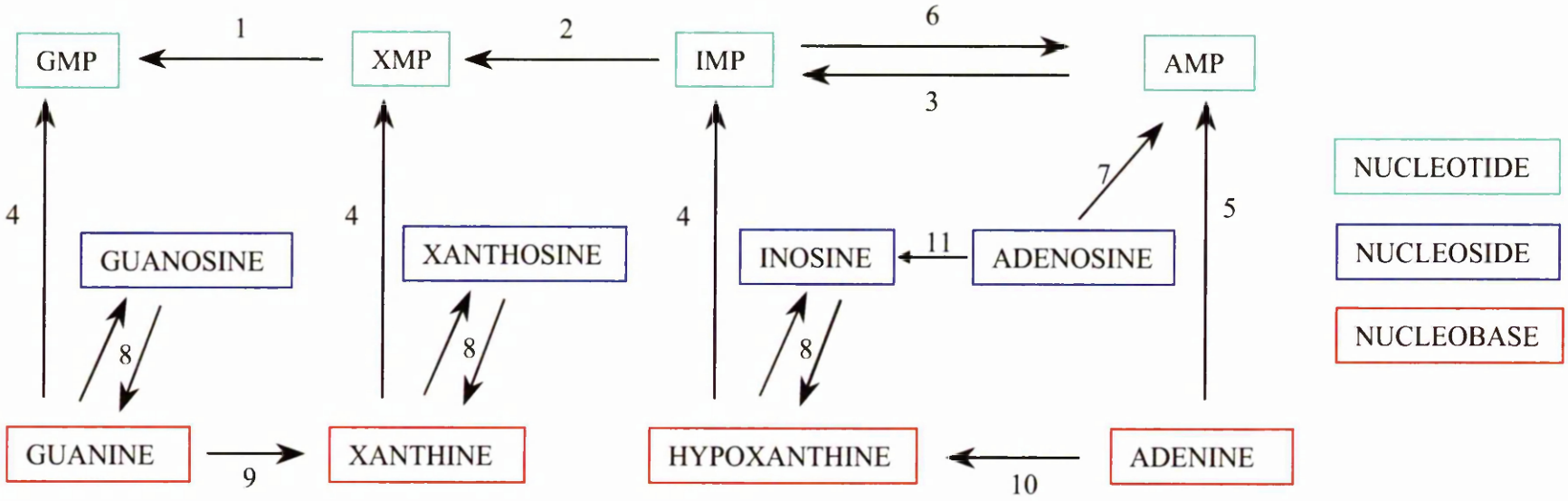



**Figure 6.1.** Purine salvage pathways in *T. gondii*. Enzyme key: 1. GMP synthetase; 2. IMP dehydrogenase; 3. AMP deaminase; 4. Hypoxanthine-xanthine-guanine phosphoribosyltransferase; 5 Adenine PRTase; 6. Adenylosuccinate synthetase/lyase; 7. Adenosine kinase; 8. Nucleoside phosphorylase; 9. Guanine deaminase; 10. Adenine deaminase; 11 Adenosine deaminase (Coombs *et al*, 1997; Hassan and Coombs, 1988; Schwartman and Pfefferkorn 1982).

(Asai *et al.*, 1983; Asai *et al.*, 1995), but the parasite appears to lack the ecto 5'-nucleotidase (5'NT) required for the final hydrolysis to adenosine (Ngo *et al.*, 2000). Consistent with the notion that *T. gondii* has multiple purine salvage pathways, hypoxanthine and guanine are rapidly incorporated into extracellular tachyzoites (Schwartzman and Pfefferkorn, 1982). Moreover, separate *T. gondii* mutants containing gene deletions of HXGPRT (Donald *et al.*, 1996), TgAT1 transporter (Chiang *et al.*, 1999) or adenosine kinase (Darling *et al.*, 1999) have been shown to be viable.

### 6.1.2 Mammalian nucleoside/nucleobase transport

In mammalian cells, nucleotides are synthesised either *de novo* or from exogenous nucleosides and bases *via* a salvage pathway. Purine salvage first requires transport across the plasma membrane. Nucleoside/base transporters are of particular interest for designing therapeutic agents since these transport routes are potential conduits for the entry of drug molecules into the host cell cytoplasm. Consequently, these transporters are possible entry routes for drugs against intracellular pathogens, for example *T. gondii*. Nucleoside transporters expressed by mammalian erythrocytes and the majority of cultured cell lines are non-concentrative equilibrative transporters with a broad specificity (Plagemann and Woffendin, 1988). Two forms are identifiable based on their inhibition by nitrobenzylthioinosine (NBTI), either to nanomolar amounts (NBTI-sensitive) or micromolar amounts (NBTI-resistant). Human erythrocytes express only the equilibrative NBTI-sensitive (*es*) transporter (Plagemann and Woffendin, 1988). Two distinct nucleobase transporters have been characterised, (i) sodium dependent nucleobase transport in epithelial cells, and (ii) facilitated diffusion in non-epithelial

cells. The latter has been characterised in human red blood cells and found to transport, with a high affinity, adenine and guanine (Domin *et al.*, 1988).

## 6.2 Aims

Since arprinocid and arprinocid-1-N-oxide are both purine analogues, it is feasible that they may penetrate *T. gondii via* a purine transporter and/or exert their effects by interfering with purine salvage pathways. Indeed, previous work has demonstrated that arprinocid and arprinocid-1-N-oxide may affect hypoxanthine transport (Chapter 1). Hence, the main objectives of this work were to

(i) Characterise purine nucleoside (adenosine and inosine) and nucleobase transport (adenine and hypoxanthine) in extracellular *T. gondii* tachyzoites and investigate whether any of these transporters are involved in the uptake of arprinocid or arprinocid-1-N-oxide.

(ii) Investigate whether arprinocid-1-N-oxide resistance in *T. gondii* is the result of altered purine transport kinetics. Resistance to chemotherapeutic agents is often associated with mutations in the transporter responsible for their selective accumulation. Examples include resistance to tubercidin (7-deazaadenosine) in *Leishmania donovani* (Vasudevan *et al.*, 2001) and to arsenicals in *Trypanosoma brucei* (Maser *et al.*, 1999).

(iii) Investigate whether arprinocid and arprinocid-1-N-oxide inhibit mammalian nucleoside/nucleobase transporters, specifically adenosine and adenine transport. These transporters have been characterised extensively in red blood cells and, although human erythrocytes do not provide a model of *T. gondii* infection, transport analysis in these cells will give further information on the activity of arprinocid and arprinocid-1-N-oxide against mammalian cells.

## 6.3 Methods

### 6.3.1 Preparation of *T. gondii* tachyzoites

Tachyzoites were separated from host cells by filtration as described (section 2.2.2) and washed twice in assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM $NaH_2PO_4$, 0.3 mM $MgCl_2$, 23 mM $NaHCO_3$ and 14 mM glucose, pH 7.3) by centrifugation at 1500 × g for 15 min at 4°C. Tachyzoites were counted and re-suspended in assay buffer at a density of between 1.5 - 2.5 x $10^8$/ml.

### 6.3.2 Transport studies using *T. gondii*

The analysis of transport of purine nucleosides (adenosine or inosine) and purine nucleobases (adenine or hypoxanthine) into extracellular *T. gondii* tachyzoites was performed using a modified oil-stop technique previously described for transport measurements in *Trypanosoma brucei brucei* (de Koning, 2001). Assay buffer (100 µl) containing radiolabelled nucleoside or radiolabelled nucleobase (permeant) and, where appropriate, various concentrations of test compound, was layered over 200 µl of a 7:1 mixture of di-*n*-butylphthalate (BDH, Poole, U.K.) and light mineral oil (Sigma). Arprinocid and arprinocid-1-N-oxide were diluted from stock concentrations of 50 mM and 3 mM, respectively, in 100 % DMSO. Final concentrations of DMSO were 1 % throughout, which had no noticeable effect on cells. Transport assays, performed at room temperature, were initiated by rapid pipetting of tachyzoites (100 µl) to the permeant. Assays were stopped by the addition of 1 ml ice-cold 4 mM (hypoxanthine or adenine) or 10 mM (inosine or adenosine) ice-cold unlabelled permeant. Parasites were centrifuged through the oil layer at 16,000 x g for 1 min using a Heraeus Biofuge

centrifuge (Jencons PLS, Bedfordshire, UK). Subsequently, each tube was flash-frozen in liquid nitrogen and the bottom of the tube, containing the cell pellet, cut into 6 ml scintillation vials. Tachyzoite pellets were lysed in 250 µl 5 % (w/v) TCA for 90 min before being mixed with 3 ml Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA). Radioactivity was determined by using a 1219 RackBeta Spectral scintillation counter (LKB Wallac, Finland). All assay data points were performed in triplicate.

### 6.3.3 *T. gondii* growth measurements

The *in vitro* growth of *T. gondii* tachyzoites was measured by quantification of the incorporation of $^{3}$H-uracil, as described in Chapter 3.

### 6.3.4 Transport studies using human red blood cells (hRBCs)

Human blood cells from healthy volunteers were obtained from the Glasgow and West of Scotland Blood Transfusion Service. The blood was preserved in citrate-phosphate-dextrose adenine (16 mmol/l citric acid, 89 mmol/l sodium citrate, 16 mmol/l sodium dihydrogen phosphate, 161 mmol/l glucose and 2 mmol/l adenine) and stored for less than 1 week. Blood cells, in a total volume of approximately 10 ml, were washed three times by centrifugation at 1800 x g for 10 min at 4°C in 50 ml erythrocyte medium (EM, 140 mM NaCl, 5 mM KCl, 20 mM Tris/HCl pH 7.4, 2 mM $MgCl_2$ and 0.1 mM EDTA). After each wash, white blood cells were removed using a pasteur pipette. Washed hRBCs were resuspended in three times their volume of EM (25 % haematocrit) at a density of 2 - 2.5 x $10^9$ cells/ml. Transport assays were performed as described above except that radiolabelled permeant and inhibitor was layered over 250

μl di-*n*-butylphthalate (BDH). Assays were initiated by rapid pipetting of the cell suspension (100 μl) into permeant and were stopped by the addition of 1 ml ice-cold stop medium containing 19 mM papaverine in 165 mM NaCl for adenine uptake assays or 20 μM dilazep in EM for adenosine uptake assays. Cells were then pelleted by centrifugation at 16,000 x g for 1 min, as described above. The aqueous and oil layers were removed using a suction pump and remaining radioactivity above the pellet wiped away using cotton buds. RBC protein was solubilised in 0.25 ml 1 % triton X-100 for 20 min, precipitated by the addition of 0.25 ml 5 % (w/v) TCA and centrifuged at 16,000 x g for 10 min. Supernatant was transferred to scintillation vials and radio-activity measured, as described, after the addition of 3.5 ml scintillation fluid. All assay data points were performed in triplicate.

### 6.3.5 Calculation of transport kinetics

Kinetic parameters were calculated using the FigP graphical package (Biosoft, Ferguson, MO) and are presented as a mean ± S.E. of three independent experiments. $K_i$ values were calculated using the equation, $K_i = IC_{50}/[1 + (L/K_m)]$ (Cheng and Prusoff, 1973). Consistent with monophasic competitive inhibition, Hill coefficients were found to be consistently close to -1. Experimental data and kinetic values from separate experiments are shown in the text as averages ± standard errors (n = 3); representative data from an experiment is presented in figures and corresponding legends. Transport of permeant refers to mediated transport minus radioactivity associated with the cells due to diffusion or binding. Counts per minute resulting from diffusion and binding were taken to be those associated with the cell pellet in parallel incubations but in the presence of saturating concentrations of unlabelled permeant.

## 6.4 Results

### 6.4.1 Purine nucleoside transport

Uptake of [$^3$H]adenosine at 1.25 μM and 2.5 mM by pRH *T. gondii* tachyzoites was linear for at least 90 s (Figure 6.2a). Uptake of [$^3$H]adenosine was saturable, with 2.5 mM unlabelled adenosine greatly reducing uptake rates (Figure 6.2a). The remainder of uptake is assumed to be as a result of diffusion since 1 mM adenosine saturated uptake (Figure 6.2b inset). Therefore, in subsequent experiments uptake at 2.5 mM adenosine was subtracted from every datapoint to yield mediated uptake. [$^3$H]Adenosine transport (1.25 μM), in the presence of unlabelled substrate (0 - 1000 μM) conformed to simple Michaelis-Menten kinetics (Figure 6.2b inset) having $K_m$ and $V_{max}$ values of 108 ± 20 μM (n = 3) and 0.066 ± 0.002 pmol/$10^7$ parasites/s (n = 3), respectively. Adenosine transport was found to be inhibited by both arprinocid ($K_i$ = 3.3 ± 1.1 μM, n = 3) and arprinocid-1-N-oxide ($K_i$ = 10.4 ± 3.4 μM, n = 3) with Hill slopes of approximately -1, consistent with competitive inhibition (Figure 6.2b). At the highest concentrations tested, arprinocid (50 μM) and arprinocid-1-N-oxide (60 μM) greatly inhibited adenosine transport, compared with similar assays in the absence of drug, by 82.9 ± 4.4 % (n = 3) and 98.9 ± 4.1 % (n = 3), respectively ($P < 0.001$). These results are in agreement with arprinocid and arprinocid-1-N-oxide being taken up by the *T. gondii* adenosine transporter.

The kinetic constants, $K_m$ and $V_{max}$, for [$^3$H]adenosine (1.25 μM) transport in an arprinocid-1-N-oxide-resistant strain of *T. gondii* ($ARP^R$) were not significantly different ($P > 0.05$) from those for adenosine transport in the isogenic parental line

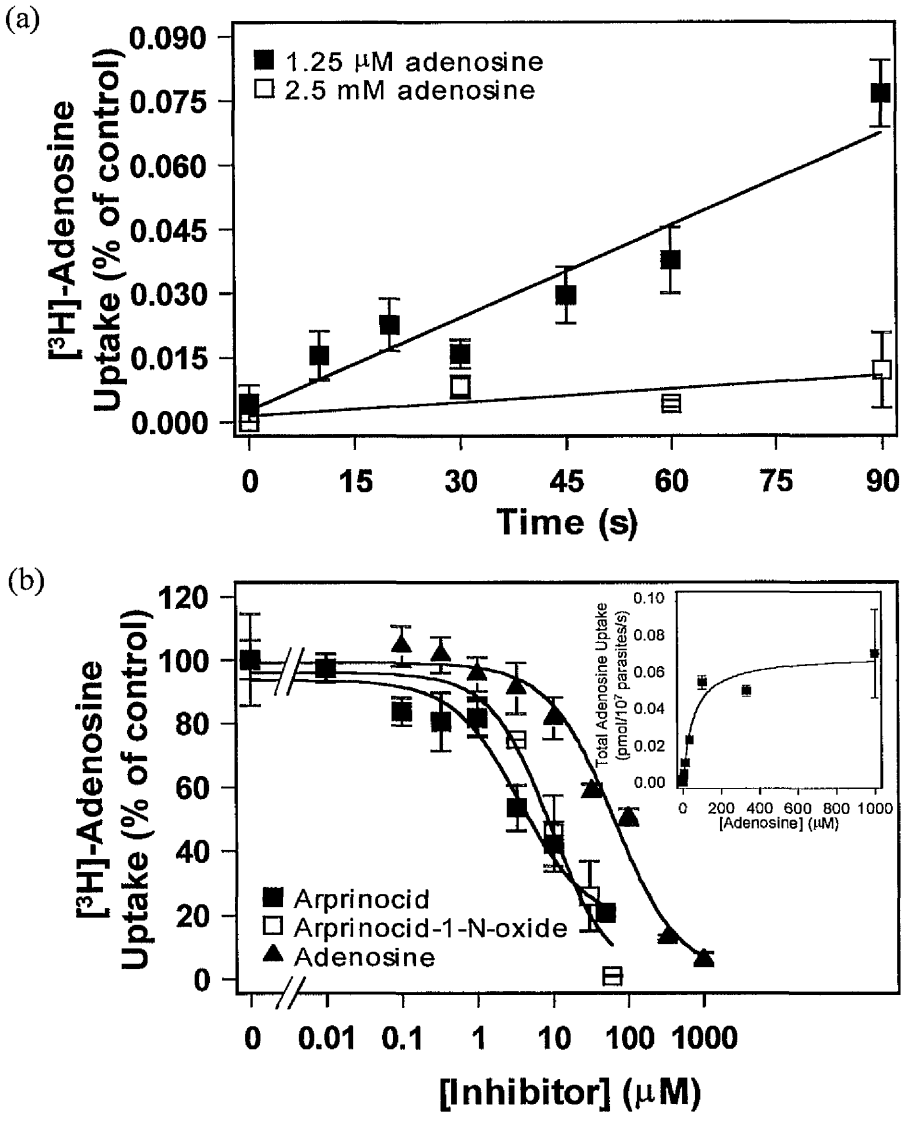



**Figure 6.2.** Adenosine transport in *T. gondii* tachyzoites. (a) Uptake of 1.25 μM [$^3$H]adenosine (7.21 ± 1.04 x $10^{-4}$ pmol/$10^7$ parasites/s) and 2.5 mM [$^3$H]adenosine (1.07 ± 0.56 x $10^{-4}$ pmol/$10^7$ parasites/s) was measured over various intervals between 0 and 90 s. (b) Uptake of [$^3$H]adenosine (1.25 μM) over 45 s by RH *T. gondii* in the presence of unlabelled adenosine ($IC_{50}$ = 63.5 ± 17.0 μM), arprinocid ($IC_{50}$ = 3.5 ± 1.4 μM) and arprinocid-1-N-oxide ($IC_{50}$ = 9.3 ± 4.7 μM). Michaelis-Menten graph, inset, shows calculated $K_m$ and $V_{max}$ values, for this experiment, for adenosine transport of 63.5 ± 17.0 μM and 0.069 ± 0.004 pmol/$10^7$ parasites/s, respectively. 100 % control rate was 1.1 ± 0.2 x $10^{-3}$ pmol/$10^7$ parasites/s.

(pRH), and similarly conformed to Michaelis-Menten kinetics (Figure 6.3 inset). The kinetic constants, $K_m$ and $V_{max}$, were determined to be 70 ± 31 μM (n = 3) and 0.050 ± 0.027 pmol/$10^7$ parasites/s (n = 3), respectively. The adenosine transporter in the resistant strain was equally sensitive to arprinocid-1-N-oxide ($K_i$ = 11.3 ± 2.8, n = 3) as the parental strain (Figure 6.3).

Transport of [$^3$H]inosine at 0.1 μM, 1 μM and 10 μM was linear for up to 180 s (Figure 6.4a). Uptake of inosine (1 μM), measured at 120 s, was saturable by unlabelled inosine over the range 0 - 10 mM and exhibited Michaelis-Menten kinetics (Figure 6.4b and inset). $K_m$ and $V_{max}$ values for this transporter were calculated as 656 ± 259 μM (n = 3) and 0.28 ± 0.09 pmol/$10^7$ parasites/s (n = 3). In three independent experiments, arprinocid (50 μM) and arprinocid-1-N-oxide (50 μM) had no significant effect on the transport of [$^3$H]inosine.

### 6.4.2 Inhibition of TgAT1 with dipyridamole

Data obtained so far were consistent with arprinocid and arprinocid-1-N-oxide being transported by TgAT1 and therefore, to ascertain whether either compound was transported *via* TgAT1, a $^3$H-uracil assay to measure parasite growth (Section 3.3.1) was conducted using dipyridamole to block adenosine transport. Dipyridamole (10 μM) has been shown to block adenosine transport (Schwab *et al.*, 1995). Therefore a change in the efficacy of arprinocid or arprinocid-1-N-oxide, measured in the presence of dipyridamole, would verify whether or not the *T. gondii* adenosine transporter (TgAT1) was involved in the mechanism of action of arprinocid or arprinocid-1-N-oxide. Control experiments in the presence of only dipyridamole died, indicating that dipyridamole,

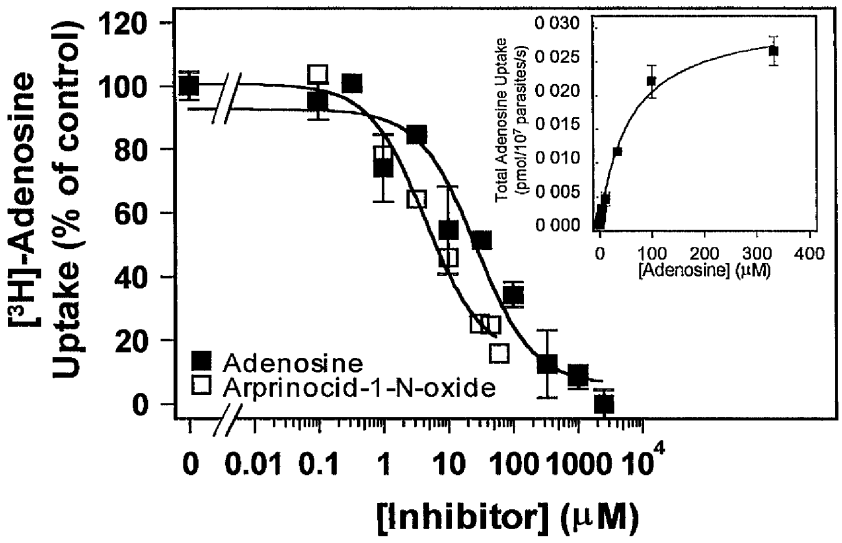
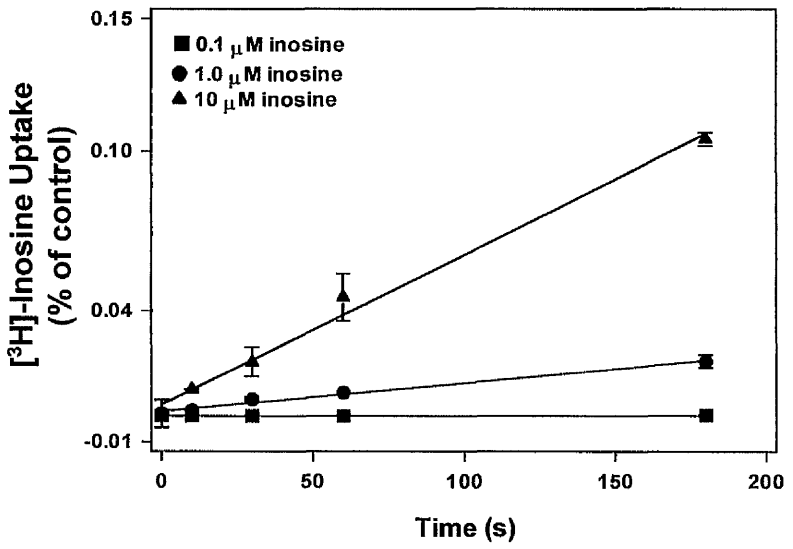



**Figure 6.3.** Adenosine transport in arprinocid-1-N-oxide-resistant *T. gondii* tachyzoites ($ARP^R$). Uptake of [$^3$H]adenosine (1.25 μM) over 45 s by ARPr *T. gondii* in the presence of unlabelled adenosine ($IC_{50}$ = 54.0 ± 18.5 μM) and arprinocid-1-N-oxide ($IC_{50}$ = 4.5 ± 1.1 μM). Michaelis-Menten graph, inset, shows calculated $K_m$ and $V_{max}$ values, for this experiment, for adenosine transport of 59.5 ± 7.4 μM and 0.031 ± 0.001 pmol/$10^7$ parasites/s, respectively. 100 % control rate was 1.1 ± 0.2 x $10^{-3}$ pmol/$10^7$ parasites/s.

(a)




(b)

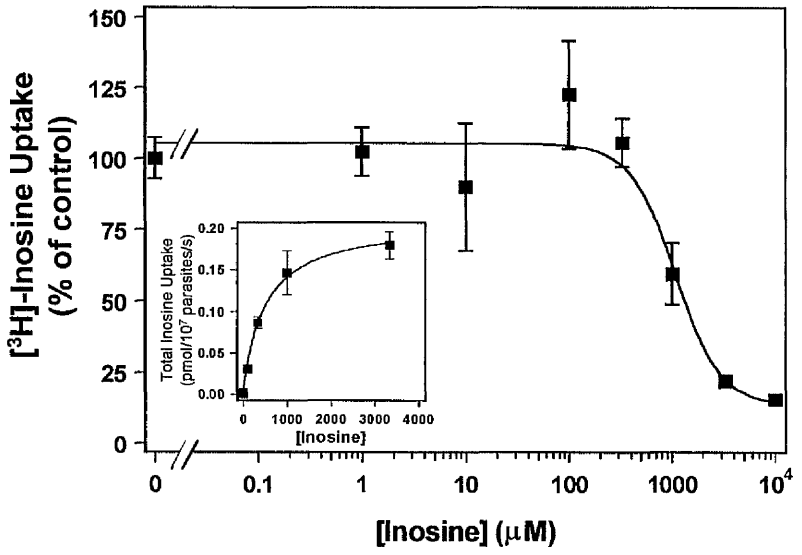


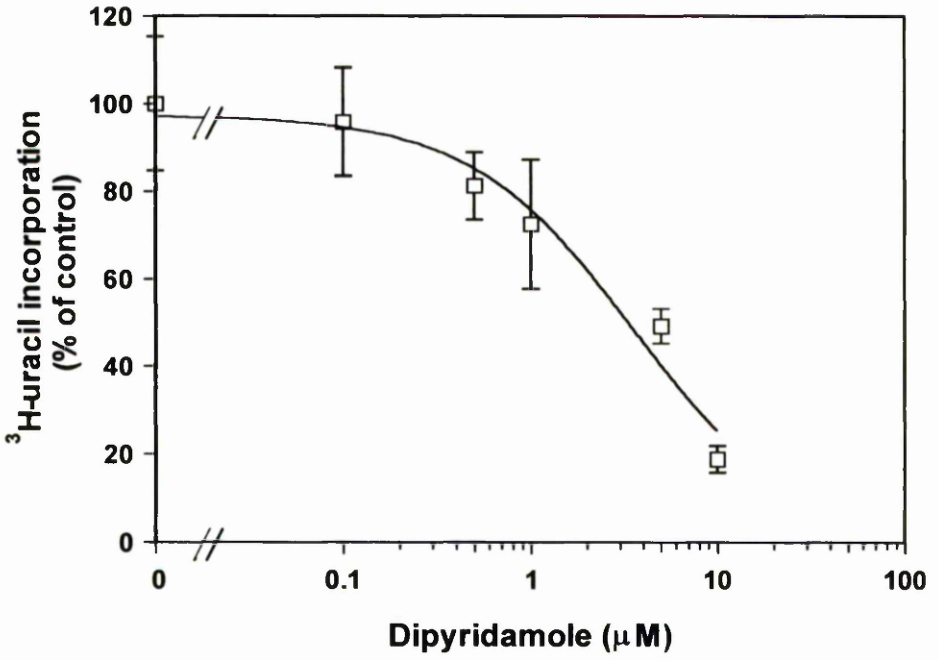

**Figure 6.4.** Inosine transport in pRH *T. gondii* tachyzoites. (a) Uptake of 0.1 μM [$^3$H]inosine (1.60 ± 0.21 x $10^{-6}$ pmol/$10^7$ parasites/s), 1 μM [$^3$H]inosine (1.08 ± 0.076 x $10^{-4}$ pmol/$10^7$ parasites/s) and 10 μM [$^3$H]inosine (5.67 ± 0.31 x $10^{-4}$ pmol/$10^7$ parasites/s) measured over various intervals between 0 and 180 s. (b) Uptake of 1 μM [$^3$H]inosine by RH *T. gondii* in the presence of unlabelled permeant ($IC_{50}$ = 1074 ± 313 μM). Michaelis-Menten graph, inset, shows calculated $K_m$ and $V_{max}$ values for this experiment of 473 ± 39 μM and 0.208 ± 0.005 pmol/$10^7$ parasites/s, respectively. 100 % control rate was 2.5 ± 0.18 x $10^{-4}$ pmol/$10^7$ parasites/s.

itself, had anti-*Toxoplasma* activity. Further investigations revealed that dipyridamole could indeed inhibit *T. gondii* growth with an $IC_{50}$ = 3.5 ± 0.75 μM (Figure 6.5a). Manual counting of concurrent dipyridamole-treated-infected cultures also indicated that dipyridamole inhibited the growth of *T. gondii* (Figure 6.5b). Consequently, measurement of arprinocid- and arprinocid-1-N-oxide-uptake by the adenosine transporter could not be achieved.

### 6.4.3 Effect of arprinocid and arprinocid-1-N-oxide on adenosine and adenine transport in human erythrocytes

To investigate the effect of arprinocid and arprinocid-1-N-oxide on facilitated transport in human erythrocytes, a modified oil-stop technique was performed. Papaverine and dilazep have been shown to be efficient inhibitors of nucleobase and nucleoside transport, respectively, and therefore act as effective stop solutions for the reliable measurement of initial rates of transport (Domin *et al.*, 1988; Plagemann and Woffendin, 1988). Transport of 1 μM [$^3$H]adenine measured using seven datapoints was linear up to 15 s with a rate of 0.059 ± 0.005 pmol/$10^7$ cells/s ($r^2$ = 0.96) (unpublished observation, H. de Koning, 2001) and similarly uptake of 1 μM [$^3$H]adenosine was linear using 10 datapoints over 20 s with a rate of 0.035 ± 0.001 pmol/$10^7$ cells/s (Figure 6.6). All transport assays were performed at 5 s, well within the linear phase. Neither arprinocid (1 - 50 μM) nor arprinocid-1-N-oxide (1 - 60 μM) had any significant effect on the transport of adenine or adenosine in red blood cells ($P > 0.05$) compared with equivalent assays in the absence of drug. Controls with 1 mM unlabelled permeant completely inhibited uptake (n = 3; data not shown).

(a)




(b)

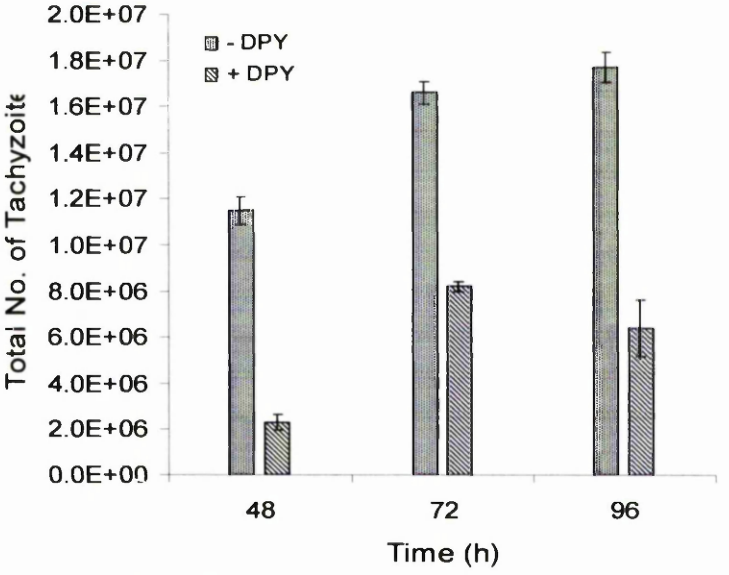



**Figure 6.5.** Inhibition of *T. gondii* growth by dipyridamole. (a) Growth of *T. gondii* tachyzoites was quantified by $^3$H-uracil incorporation, as described in Chapter 3, in the presence of dipyridamole (0 - 10 μM). The $IC_{50}$ of dipyridamole was calculated as 3.5 ± 0.75 μM. Data points are the mean ± SE (n = 3). (b) Parasites grown using exactly the same parasite:cell ration in concurrent infected culture flasks were counted at 48, 72 and 96 h post-infection. Uninfected drug-treated Vero cells showed no obvious signs of distress compared to untreated uninfected control cells.

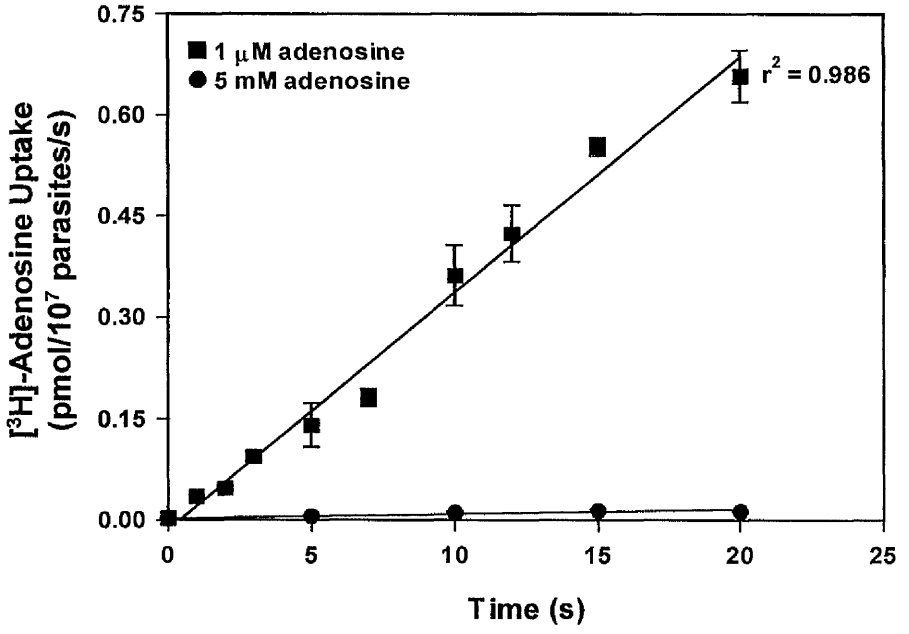


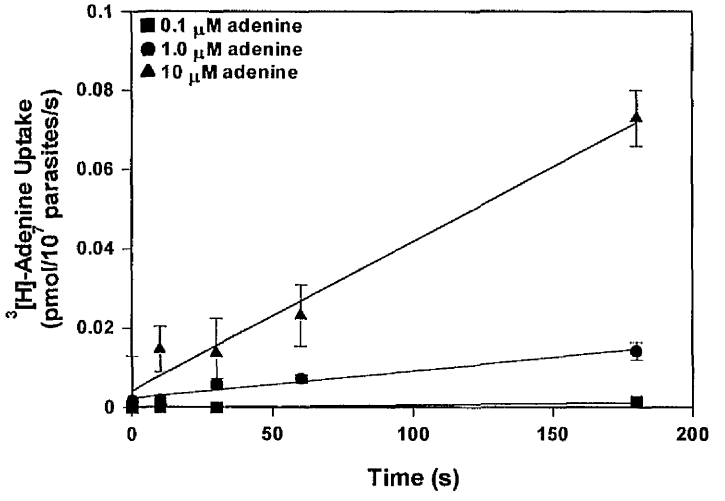

**Figure 6.6.** Adenosine transport in human erythrocytes. Uptake of 1 μM [$^3$H]adenosine (0.035 ± 0.001 pmol/$10^7$ cells/s ) and 5 mM [$^3$H]adenosine (6.6 ± 1.4 x $10^{-4}$ pmol/$10^7$ cells/s) over various time intervals between 0 and 20 s.

### 6.4.4 Purine nucleobase transport

Uptake of $^{3}$[H]-adenine at 0.1 μM, 1 μM and 10 μM by extracellular *T. gondii* tachyzoites (pRH) was comparatively slow and linear for up to 180 s, with rates of 8.2 ± 2.0 x $10^{-6}$ pmol/$10^{7}$ parasites/s, 6.8 ± 0.89 x $10^{-5}$ pmol/$10^{7}$ parasites/s and 3.9 ± 0.7 x $10^{-4}$ pmol/$10^{7}$ parasites/s, respectively (Figure 6.7a). At 1 μM, [$^{3}$H]-adenine uptake was not saturable by unlabelled adenine concentrations up to 1 mM (Figure 6.7b).

Uptake of [$^{3}$H]-hypoxanthine at 0.1 μM, 1 μM and 10 μM by *T. gondii* tachyzoites was rapid and linear for up to 180 s (Figure 6.8a). [$^{3}$H]-Hypoxanthine transport (0.3 μM) measured at 120 s over a range 0 - 1000 μM unlabelled permeant was saturable and complied with simple Michaelis-Menten kinetics, with a $K_m$ value of 0.91 ± 0.19 μM and a $V_{max}$ value of 0.0045 ± 0.0014 pmol/$10^{7}$ parasites/s (n = 3) (Figure 6.8b and inset). The inhibitory effects of potential substrates for this hypoxanthine transporter, designated TgHT1, were tested by measuring initial uptake rates at 0.3 μM [$^{3}$H]-hypoxanthine, in the presence of varying concentrations of test compounds (Figure 6.9). The 6-keto nucleobases, guanine (6.9 μM) and xanthine (134 μM) inhibited hypoxanthine transport by 50.2 ± 2.9 % ($P < 0.005$) and 61.4 ± 2.1 % ($P < 0.005$), respectively, whereas the aminopurine adenine and the nucleosides, adenosine and inosine, were less potent inhibitors (21 ± 3.8 %, 50.5 ± 2.0 % and 47.6 ± 5.7 %, respectively, at 1 mM). The hypoxanthine analogue allopurinol also weakly inhibited hypoxanthine transport, by 29.7 ± 8.4 % at 1 mM ($P < 0.05$). Arprinocid (50 μM) had no significant effect on the transport of hypoxanthine whilst, arprinocid-1-N-oxide (30 μM) reduced uptake by 24.2 ± 8.7 % ($P < 0.05$).

(a)




(b)

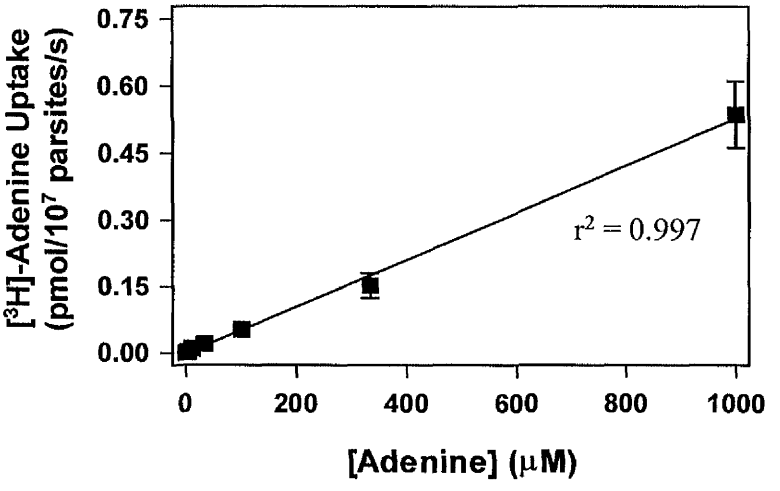



**Figure 6.7.** Uptake of adenine by extracellular pRH *T. gondii* tachyzoites. (a) Uptake of $^3$[H]-adenine at 0.1 μM, 1 μM and 10 μM by *T. gondii* tachyzoites was linear for up to 180 s (rates are shown in text). (b) Uptake of [$^3$H]adenine (1 μM) by *T. gondii* tachyzoites was determined at 120 s in the presence of unlabelled substrate (0 - 1000 μM). No carrier-mediated transport of adenine was observed as indicated by the non-saturable linear uptake of adenine (5.31 ± 0.12 x $10^{-4}$ pmol/$10^7$ parasites/s).

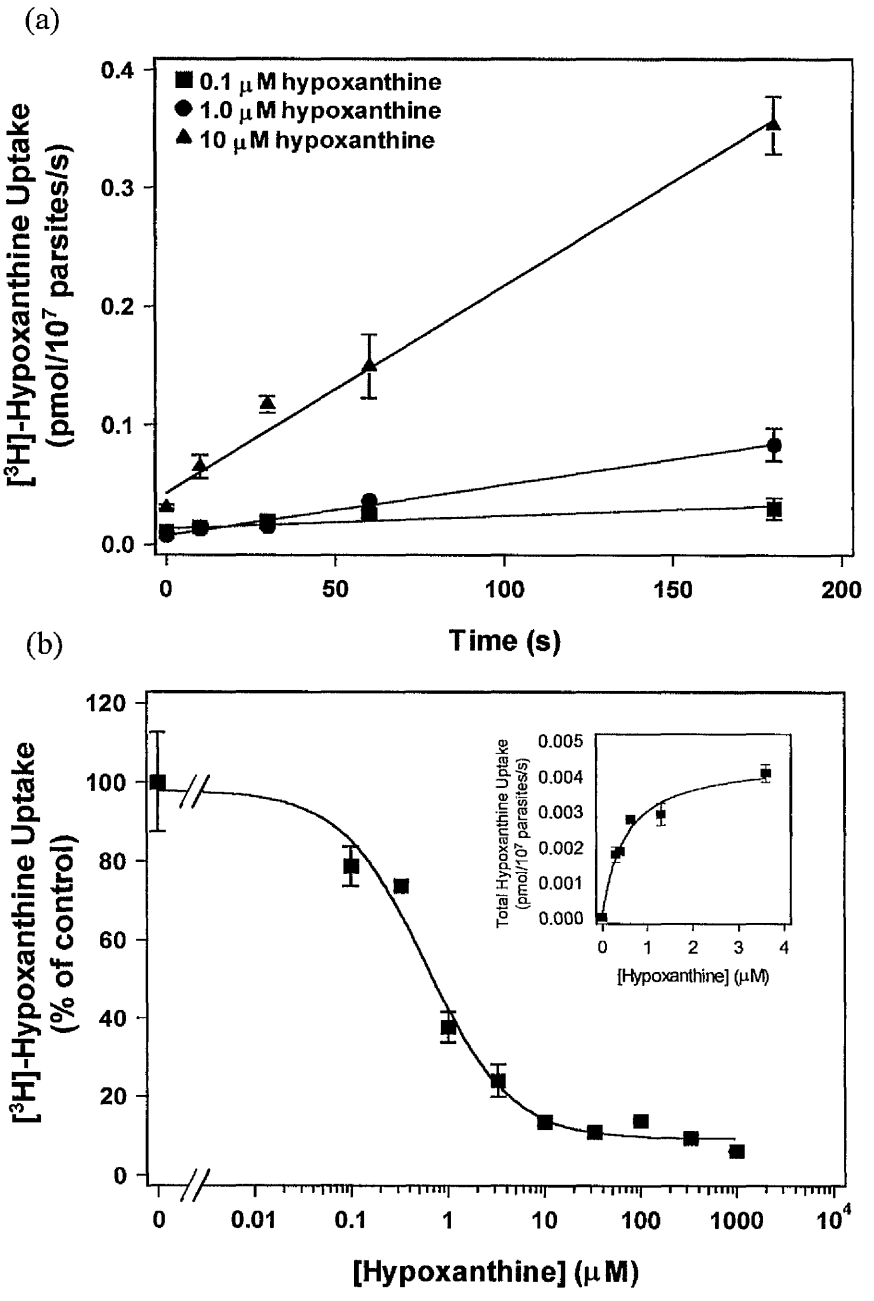



**Figure 6.8.** Hypoxanthine transport in RH *T. gondii* tachyzoites. (a) Uptake of 0.1 μM [$^3$H]hypoxanthine (1.04 ± 0.27 x $10^{-4}$ pmol/$10^7$ parasites/s), 1 μM [$^3$H]hypoxanthine (4.26 ± 0.22 x $10^{-4}$ pmol/$10^7$ parasites/s) and 10 μM [$^3$H]hypoxanthine (1.75 ± 0.092 x $10^{-3}$ pmol/$10^7$ parasites/s) measured over various intervals between 0 and 180 s. (b) Uptake of [$^3$H]hypoxanthine (0.3 μM) over 120 s by *T. gondii* in the presence of unlabelled hypoxanthine ($IC_{50}$ = 0.59 ± 0.12 μM). Michaelis-Menten graph, inset, shows calculated $K_m$ and $V_{max}$ values for this experiment of 0.49 ± 0.11 μM and 0.0045 ± 3.3 x $10^{-4}$ pmol/$10^7$ parasites/s, respectively. 100 % control rate was 0.002 ± 2.2 x $10^{-4}$ pmol/$10^7$ parasites/s.

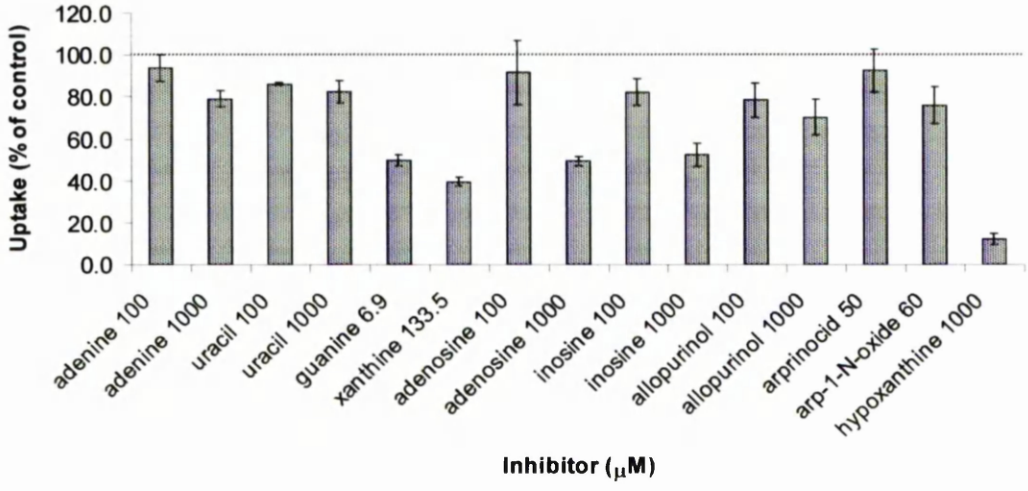



**Figure 6.9.** Inhibition profile of the *T. gondii* hypoxanthine transporter, TgHT1. Uptake of [$^3$H]hypoxanthine (0.3 μM) was measured in the presence of various inhibitors. Guanine (6.9 μM; $P < 0.005$), xanthine (133.5 μM; $P < 0.005$), adenosine (1 mM; $P < 0.005$) and inosine (1 mM; $P < 0.05$) significantly reduced the transport of hypoxanthine. Data shown are the average of three independent experiments and S.E.

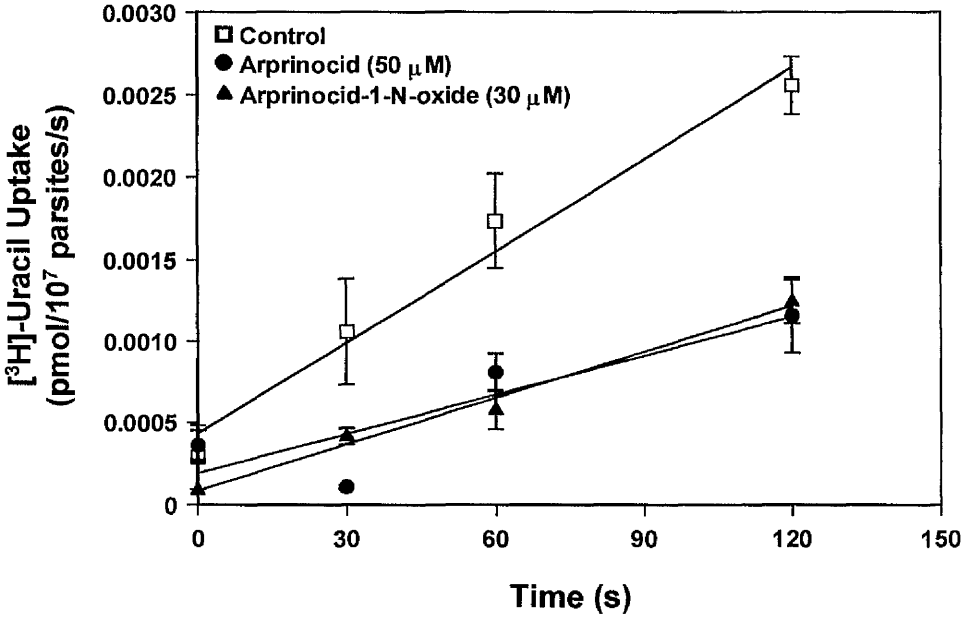



**Figure 6.10.** Uracil transport by *T. gondii*. Uptake of 1 μM [$^3$H]-uracil (1.8 ± 0.2 x $10^{-5}$ pmol/$10^7$ cells/s), and in the presence of 50 μM arprinocid (8.0 ± 3.0 x $10^{-6}$ pmol/$10^7$ cells/s) or in the presence of 60 μM arprinocid-1-N-oxide (9.4 ± 0.7 x $10^{-7}$ pmol/$10^7$ cells/s) over various time intervals between 0 and 180 s (n = 2).

### 6.4.5 Effect of arprinocid and arprinocid-1-N-oxide on $^{3}$H-uracil transport in *T. gondii*

Uptake of 1 μM $^{3}$H-uracil by *T. gondii* tachyzoites was measured over various time points between 0 and 120 s (Figure 6.10). The rate of uptake of $^{3}$H-uracil was reduced approximately two-fold by the presence of 50 μM arprinocid and by the presence of 30 μM arprinocid-1-N-oxide (Figure 6.10).

## 6.5 Discussion

Purine salvage by *T. gondii* from the host cell requires translocation of the purine across the parasitophorous vacuole membrane (PVM) and subsequently across the parasite cell membrane. Studies of the PVM, poses technical difficulties and consequently little is known about its exact function. However, the PVM is permeable to small solutes up to 1300 - 1900 Da, allowing free exchange of small molecules between the host cell cytoplasm and vacuolar spaces and so is unlikely to provide any barrier to purine bases and nucleosides (Schwab *et al.*, 1994). In contrast, the tachyzoite cell membrane will form a barrier amenable to the study of transport across this membrane, after tachyzoites have been separated from the host cell.

Results from investigations of the purine transporters of *T. gondii* tachyzoites using the oil-stop technique provide evidence for the presence of facilitated transport routes for adenosine (TgAT1), inosine (designated TgIT1) and hypoxanthine (designated TgHT1). The calculated value of $K_m$ for TgAT1 ($K_m$ = 105 ± 22 μM) is in agreement with that from previously published work (Schwab *et al.*, 1995) and is also in agreement with the $K_m$ calculated for TgAT1 expressed in oocytes (Chiang *et al.*, 1999).

The published $K_m$ for inosine transport is 81 μM (Schwab *et al.*, 1995) whilst data presented in this report suggest a $K_m$ value 8-fold higher. The reason for this discrepancy is unclear. Furthermore, in this study no evidence of two inosine transporters (Schwab *et al.*, 1995) was found and careful evaluation of Schwab and co-workers' data suggest that inosine was transported only by the dipyridamole-insensitive transporter (TgIT1). Data obtained for the uptake of inosine by *T. gondii* tachyzoites

exhibited a monophasic inhibition profile, consistent with a single transporter for inosine. Whilst TgAT1 has been reported to be inhibited by inosine (Chiang *et al.*, 1999; Schwab *et al.*, 1995) no evidence has been presented that prove inosine is actually transported by this transporter. Clearly more work needs to be done to establish the exact substrate specificities of these transporters, but it is certain that purine nucleosides are taken up with only low affinity. In contrast, TgHT1 has a high affinity for hypoxanthine, and is likely to also transport guanine and perhaps xanthine into *T. gondii*.

Schwab *et al.,* (1995) proposed that TgAT1 also transported hypoxanthine and adenine, on the basis of the ability of these nucleobases to inhibit adenosine uptake at 1.6 mM. Similarly, Chiang *et al.,* (1999) showed that hypoxanthine and guanine, but not adenine and xanthine, inhibit approximately 35 % of adenosine uptake at 0.5 mM. However, results obtained in this study suggested uptake of adenine was due to diffusion. This is consistent with the observation that the incorporation of [$^3$H]adenine is negligible in extracellular *T. gondii* tachyzoites (Schwartzman and Pfefferkorn, 1982). While TgAT1 may have some very low affinity for hypoxanthine, it is unlikely to contribute significantly to the overall hypoxanthine flux.

The previously reported low affinity of TgAT1 for adenosine (Schwab *et al.*, 1995) seems to contradict the hypothesis that adenosine is the preferred substrate for purine salvage by *T. gondii*. Currently, the most convincing evidence for this theory is (i) the high activity of adenosine kinase (Krug *et al.*, 1989) and (ii) the presence of an adenosine transporter, TgAT1. However, it is highly unlikely that adenosine is accumulated in the parasitophorous vacuole at a much higher concentration (closer to the $K_m$ of TgAT1) than is present in the host cytoplasm because the PVM is effectively

porous to small molecules such as adenosine. Therefore, adenosine will be in equilibrium between the parasitophorous vacuole and the host cell cytoplasm at a concentration far lower than the $K_m$ of TgAT1.

Whilst the hypothesis of preferential uptake of adenosine by *T. gondii* cannot be discounted, all in all it is likely that the TgHT1 transporter described here makes a very significant contribution to purine salvage. This is consistent with the observation that both TgAT1 and adenosine kinase null mutants are viable (Chiang *et al.*, 1999; Darling *et al.*, 1999). One theory could be that adenosine deaminase present within host cells may convert adenosine to inosine (Krug *et al.*, 1989) and subsequent metabolism of inosine to hypoxanthine by mammalian inosine phosphorylase would allow transport of hypoxanthine into *T. gondii via* TgHT1 and the HXGPRT-salvage pathway.

In *P. falciparum* two nucleoside/nucleobase transporters PfNT1 and PfENT1, differing only by one amino acid, have been identified by two independent research groups (Carter *et al.*, 2000; Parker *et al.*, 2000). PfENT1 has a low affinity for adenosine ($K_m$ = 320 ± 50 μM) (Parker *et al.*, 2000) and similarly TgAT1 also has a low affinity for adenosine. In contrast, PfNT1 has been reported to have a much higher affinity for adenosine ($K_m$ = 13.2 ± 4.3 μM) (Carter *et al.*, 2000). Further work is necessary to address this discrepancy, to elucidate whether or not PfENT1 and PfNT1 are distinct transporter proteins, and whether or not *P. falciparum* has similar transport proteins to *T. gondii*. Interestingly, *T. gondii* has a high affinity hypoxanthine transporter (TgHT1) and *P. falciparum* PfENT1 has a low affinity for hypoxanthine ($K_m$ = 420 ± 10 μM) (Parker *et al.*, 2000) suggesting that they both have distinct purine salvage requirements.

Several protozoan purine transporters have been implicated in the uptake of and resistance to various chemotherapeutic agents. Examples include the *T. cruzi* adenosine transporter and tubercidin resistance (Finley *et al.*, 1988; Nozaki and Dvorak, 1993), the *Leishmania donovani* LdNT2 transporter and resistance to formycin B (Aronow *et al.*, 1987), and the involvement of the *T. b. brucei* P2 transporter in uptake of diamidines and melaminophenylarsenicals (Maser *et al.*, 1999). Data presented in this thesis indicate that resistance to arprinocid-1-N-oxide is unlikely to be due to a modified adenosine transporter (TgAT1) since inhibition of adenosine transport by arprinocid-1-N-oxide in the drug-resistant line was similar to that observed in the drug-sensitive line. In this study, it was investigated whether arprinocid and its 1-N-oxide metabolite enter host cells by the broad-specificity equilibrative nucleobase and *es* nucleoside transporters present in human red blood cells and many other tissues (de Koning and Diallinas, 2000; Plagemann *et al.*, 1988). The lack of inhibition of these transporters by arprinocid and its metabolite suggests that these drugs may enter the host cells by diffusion. The diffusion gradient, however, would only lead to effective accumulation if the compound were subsequently salvaged from the host cell cytosol by a high affinity *T. gondii* transporter, after diffusion into the parasitophorous vacuole. Alternatively, arprinocid may accumulate within host cells by binding to intracellular organelles or membranes. It should also be recognised that perhaps arprinocid and arprinocid-1-N-oxide do not enter hRBC. In addition, erythrocytes are not good models of *T. gondii* host cells and further work using other cells, perhaps macrophages, is necessary. Both arprinocid and arprinocid-1-N-oxide inhibited TgAT1, but not TgIT1 or TgHT1, with much higher affinity than its natural substrate, adenosine, suggesting these drugs may be substrates for the transporter – leading to selective accumulation within the parasite.

Conclusive evidence that arprinocid or arprinocid-1-N-oxide enters *T. gondii via* TgAT1 could not be obtained using dipyridamole (an inhibitor of adenosine transport) since this compound also inhibits the *in vitro* growth of the tachyzoites. The reason for this inhibition is not clear but is likely to involve inhibition of phosphodiesterase (PDE). The regulation of cAMP by adenylate cyclase and phosphodiesterase (PDE) is important in controlling the proliferation of *T. gondii*. PDE activators, for example imidazole, lower cAMP levels and inhibit growth of *Toxoplasma*, whereas PDE inhibitors, for example 3-isobutyl-1-methylxantine (IBMX), raise cAMP levels and promote growth of *Toxoplasma* (Choi *et al.*, 1990). Two classes of PDE enzymes have been identified in *T. brucei* (i) TbPDE1 and (ii) TbPDE2. In contrast to the observations made by Choi *et al.*, (1990), a mutant *T. brucei* isolate lacking the gene encoding for PDE1 exhibited no phenotypic difference except for a marginal increase in growth rate. Whilst IBMX is a broad spectrum PDE inhibitor, dipyridamole is selective for type-2 PDE enzymes (Seebeck *et al.*, 2001). Inhibition of *T. gondii* growth by dipyridamole would suggest that the parasite contains type-2 PDE or related enzymes. *Trypanosoma brucei* cyclic nucleotide-specific phosphodiesterase PDE2A (Accession number AF263280) sequence data was used in a BLASTn search of the *T. gondii* EST database and resulted in a match to the *T. gondii* consensus sequence Ctoxqual_4522 with 62 % homology (P = 1.5e-10). This indicats that *T. gondii* tachyzoites may contain a PDE2-related enzyme. Dipyridamole has been demonstrated to inhibit the *in vitro* growth of bloodstream *T. brucei* with an $IC_{50}$ of 5 - 10 μM (Seebeck *et al.*, 2001), a similar concentration to that shown to inhibit the proliferation of *T. gondii* tachyzoites. Currently, little is known about the role of PDE in *T. gondii* but it is evident that these enzymes are essential for normal metabolism and could be explored as potential chemotherapeutic targets.

Results from chapter 3 (Section 3.4.5) led to the conclusion that both arprinocid and arprinocid-1-N-oxide act within a short period of time. In this chapter, it was demonstrated that arprinocid (50 μM) and arprinocid-1-N-oxide (30 μM) reduce the rate of incorporation of $^{3}$H-uracil over a period of 120 s. This, perhaps, could explain the rapid action of the compounds. However, although there is a direct correlation between $^{3}$H-uracil uptake and parasite growth (Chapter 3), the significance of a two-fold reduction in the rate of $^{3}$H-uracil uptake measured over a period of 120 s, considering that the tachyzoite replication cycle lasts approximately 4-6 h, is not known. In addition, the concentration of arprinocid-1-N-oxide used in the transport experiment is 490-fold greater than its $IC_{50}$ value, and hence the decreased rate of $^{3}$H-uracil uptake for arprinocid-1-N-oxide may not be significant.

# CHAPTER 7

## Final Discussion and Conclusions

The central theme of this study was to investigate the mode of action of two purine analogues, arprinocid and arprinocid-1-N-oxide, by comparing arprinocid-1-N-oxide-sensitive and -resistant *T. gondii* lines. Two main approaches were taken:

- a global approach using two-dimensional electrophoresis and mass spectrometry (proteomics).

- a directed approach to test the hypothesis that arprinocid and arprinocid-1-N-oxide may exert their action by interfering with purine salvage and/or purine transport.

These approaches are discussed below in relation to their applicability to the drug discovery process and the results obtained in this work.

Initial work focussed on determining the efficacies of the compounds by using incorporation of $^{3}$H-uracil into tachyzoites as a measure of parasite abundance and viability. Although these investigations gave limited information on how the compounds work, the assays provided some details on basic properties of the compounds, such as the rapidity of their action, and in eliminating the hypothesis that hypoxanthine transport was involved in their mechanism of action. Further investigations of the rapidity of action of arprinocid and arprinocid-1-N-oxide showed

that they caused a two-fold reduction in the rate of uptake of $^{3}$H-uracil, but this was not thought to be significant. The adenosine transporter was found to be a possible mode of entry of the compounds into the parasites.

A major emphasis of this project was to apply techniques of proteomics to *Toxoplasma*. The ability to separate proteins in two dimensions has been in existence for over 25 years. However, the recent improvements in both protein separation and mass spectrometry have enabled proteomics to become an important technology. Proteomics offers the distinct advantage over mRNA based techniques in that information regarding post-translational modifications or protein expression levels may be obtained. The study of genes alone cannot give this information, and given that there are over 400 possible chemical modifications that a protein may undergo (Pennington and Dunn, 2001), all with consequences to its function/activity, analysis of expressed gene products is vital. Changes in the structure and abundance of proteins are causes of disease and resistance to drugs. Ultimately, most therapeutic drugs are targeted against proteins and the design of new improved compounds requires increased knowledge of protein expression.

The use of proteomics in parasitology is in its infancy but the technique is applicable to the analysis of virulence factors, the analysis of protein changes as a consequence of genetic manipulation, and the analysis of protein expression changes between drug-sensitive and -resistant lines. These have been demonstrated in this work. Clearly, proteomics is also applicable to many other aspects of parasitology research, such as characterising the proteins specifically expressed in different *T. gondii* life stages. This could aid in understanding the mechanisms by which the parasite has evolved to be a successful intracellular pathogen.

On a wider perspective, proteomics may be used in the drug discovery process, itself (Page *et al.*, 1999). The drug discovery process has largely depended upon the screening of vast compound libraries but in recent years has frequently been initiated by the identification of a drug target by molecular, cellular or genomic approaches with experiments based on pre-determined hypotheses. For example, arprinocid being a purine analogue may inhibit purine salvage/transport. Working on this hypothesis a high affinity hypoxanthine transporter, TgHT1, was discovered, which itself may be a potential drug transporter. The next step would be to clone the gene, express it and target or characterise the transporter. In contrast to this approach, proteomics is "hypothesis-generating" and starts from the detection of a protein differentially expressed between diseased and healthy tissues/cells. The rapid identification of disease-specific proteins (or modifications of proteins) can be applied in the investigation of pathogenesis. Recently, comparative analysis of proteins from the brains of *p*53-deficient mice (*p*53 is a tumour suppressor gene and mutations in this gene are known to account for more than 50 % of human cancers) and control mice revealed 7 proteins out of the 886 analysed that were specific to the *p*53 null mice (Araki *et al.*, 2000).

The detection of differentially expressed proteins is insufficient to enable the start of the drug discovery process. The identification of these proteins by mass spectrometry and subsequent validation of their importance in the onset of disease is vital. For example, if the gene encoding the protein was deleted (by molecular methods) and resulted in a reversal of the disease phenotype, then this would validate the protein a drug-target. This is why proteomics is described as "hypothesis-generating". Proteomics can also be of great advantage in assessing changes in metabolic pathways as a consequence of a

drug directed against a particular protein and therefore provide information on potential toxic side-effects of this therapy.

Elucidating the mechanism of action of a compound is the next crucial part of the drug discovery process. This may be undertaken by analysing particular possible targets. In this study, *T. gondii* purine transporters were analysed using the oil-stop technique and this showed that the adenosine transporter might play a pivotal role in the mechanism of action of arprinocid and arprinocid-1-N-oxide. This possibility requires further investigation, perhaps by using an adenosine transporter knockout line (Chiang *et al.*, 1999). Proteomics also may be applied to mode-of-action studies, for example, in determining the up- or down-regulation of proteins in response to drug treatment. Treatment of hepatoma cells (Huh7) with $IC_{50}$ doses of 5-fluorouracil identified 19 proteins that were up-regulated by 5-fold or more (Page *et al.*, 1999). Several of these proteins were related to pyrimidine pathways and cell cycle/growth pathways, thus giving information on the mechanism of action of 5-fluorouracil. This approach could be an important method to determining (or comparing) the mechanisms of action of arprinocid and arprinocid-1-N-oxide, and potential toxicity pathways. In addition, it is conceivable that a protein involved in resistance may be minimally expressed under normal passage conditions, but up-regulated under drug pressure.

Although the reliable identification of proteins using mass spectrometry and EST databases was demonstrated, this failed to identify the protein that was differentially expressed between the arprinocid-1-N-oxide-sensitive and -resistant lines. The success of identification of proteins was, arguably, related to the fact that these samples were Coomassie-stained and that the failure to identify the differentially expressed protein may have been because it was silver-stained, which is much less compatible with mass

spectrometry analysis. Since Sypro Ruby fluorescent staining is equal in sensitivity to silver-staining but more compatible with mass spectrometry analysis, this is, perhaps, the best strategy for future attempts to identify this and other proteins of interest. In addition Sypro Ruby staining allows more accurate assessment of subtle protein expression changes. However, the disadvantage of Sypro-staining is that an automated spot-picker is required for excising the protein from the gel. This equipment was not available during this study, but although expensive, is now commercially available.

It could be that even when using mass spectrometry-compatible protein staining methods together with the current status of the EST database, or even when full gene sequence information is available for *T. gondii*, that the differentially expressed protein would still remain unidentified. Certainly, not all the spots visualised with Coomassie-blue staining were identified. Thus the question needs to be asked, is proteomics a viable technique for identification of protein expression changes in organisms with limited genome information? The data presented suggest the answer is yes, and if further increases in the size of genome and protein databases are made, the possibility of proteins remaining unidentified should be minimised. Moreover, work in this thesis has demonstrated that proteins may be reliably identified from known proteins of other organisms (homology searching). These reasons for optimism are reinforced by the report that a proteomics approach was used successfully for the investigation of erythromycin-resistance in *Streptococcus pneumoniae* (Cash *et al.*, 1999).

The techniques comprising proteomics are advancing very rapidly. In addition to the advances in 2-DE separation technology, improvements in fluorescent protein labelling (i.e. Cy™ dyes) and analysis software (Beaumont *et al.*, 2001) are further enhancing our ability to reproducibly and quantitatively analyse protein expression changes between

two systems, with less manual input. The success of this "difference gel" electrophoresis (DIGE) technology is yet to be confirmed but the preliminary literature suggests that the techniques are very promising. One alternative to this technology is post-gel labelling, known as isotope-coded affinity tagging (ICAT). In this approach, proteins from two different cell states (A and B) are separated by 2-DE and protein spots of interest are excised. Proteins from A and B are digested and peptides labelled at the *N*-terminus with H4NicNHS (light isotope containing hydrogen) and D4NicNHS (heavy isotope containing deuterium), respectively. Mass spectrometric analysis of the combined digests then provides information on the expression levels of those proteins by comparison of H4/D4 ratios of individual peptides (Quadroni and James, 2001). All of the techniques mentioned above currently require 2-DE to separate proteins. However, recent improvements in HPLC analysis have meant the technique can be miniaturised to using capillary columns (Manabe, 1999; Jensen *et al.*, 2000). The advantage of this technique is that it is readily automatable and can be linked directly to a mass spectrometer (LC-MS). The ICAT technique described above, also may be performed using LC-MS without the need for 2-DE gel separation (Gygi *et al.*, 1999a). More recently, protein separation and digestion techniques have been miniaturised onto 'chips' containing fluid channels, measuring 50 μm (depth) x 30 μm (width) x 16 cm (length), and reaction chambers (Figeys and Pinto, 2001). Thus, high-throughput proteomics is under development and will be available in the near future.

Microarray technology can rapidly identify disease-specific mRNAs but the lack of correlation between mRNA and protein expression limits its use for drug discovery. Microarray technology has been used in the identification of genes that are up- or down-regulated during the first 24 h of invasion of a cell by *T. gondii* (Blader *et al.*, 2001).

Similar types of experiments should soon be possible using protein-array chips. The technique requires an interaction between a molecule and a protein moiety, and a method of detection of this interaction. Clearly molecular recognition of proteins in a similar fashion to that used for cDNA arrays has inherent problems. For example, fluorescently labelled proteins (for detection purposes) may alter the function of the protein or its active site and differences in the efficiency of labelling the same protein with different fluorophores may undermine quantitative analyses (Jenkins and Pennington, 2001). Thus problems remain to be overcome, but the potential advances are clear.

In conclusion, proteomics is at an early development 'proof of principle' stage and is currently limited by technology (Lee, 2001). However, with continued investment proteomics will become an important collection of tools for the investigation and characterisation of proteins in biological systems, including parasites and their interactions with host cells. It is certainly evident that proteomics is unrivalled in its ability to analyse simultaneously many thousands of proteins from complex mixtures. For the full potential of proteomics in parasitology to be realised, continued investment in biochemical and molecular techniques, and in genome-sequencing projects, are essential.

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