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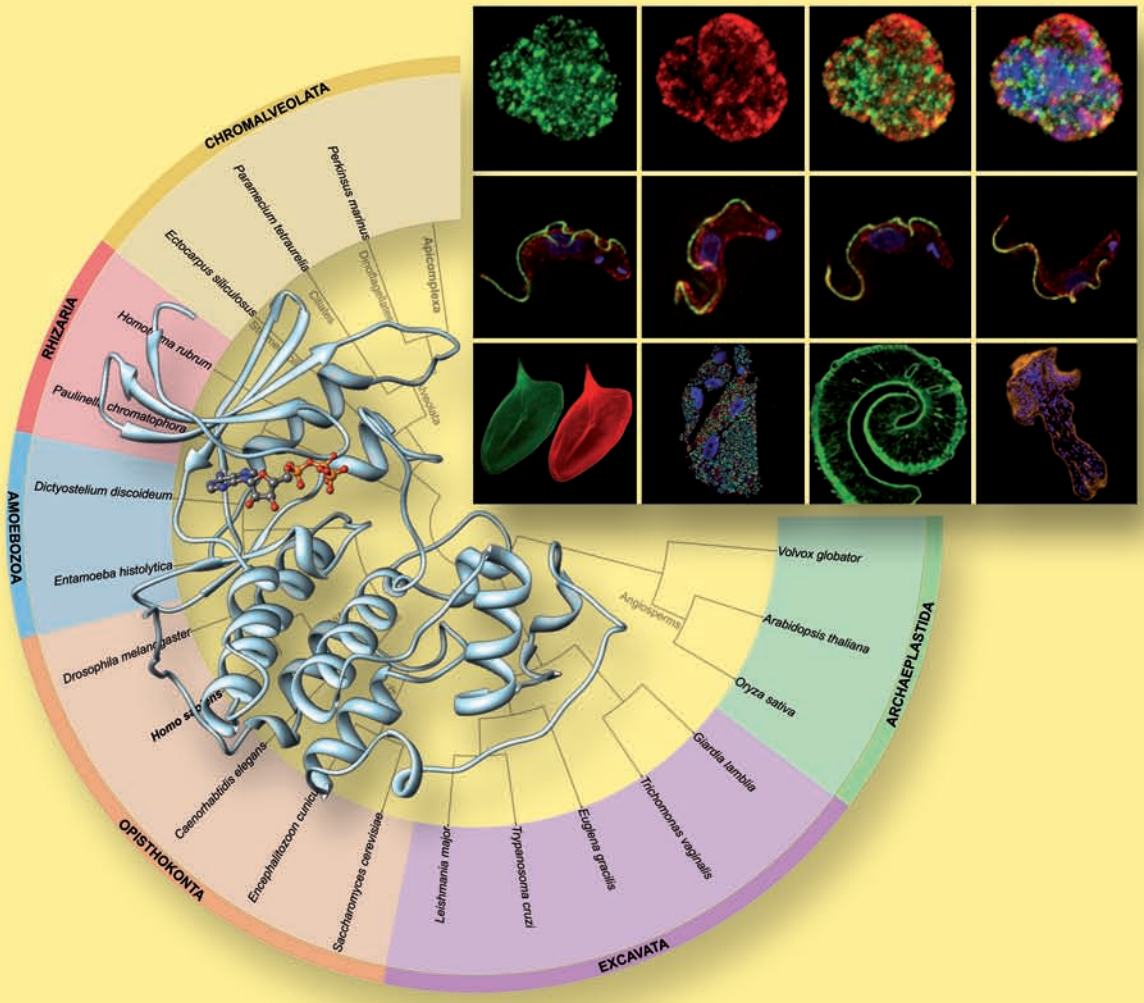
Protein Phosphorylation in Parasites

Novel Targets for Antiparasitic Intervention

Edited by Christian Doerig, Gerald Späth, and Martin Wiese



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Contents

Foreword: Protein Kinases in Parasites

Today, despite the fact that this is not obvious to the vast majority of the people leaving in industrialized countries, a large part of the world is still massively suffering and dying from parasitic diseases as a result of the lack of efficacious and/or affordable treatments. Each year 30,000 people pass away due to human African Trypanosomiasis (HAT or African sleeping sickness), a disease caused by the parasite *Trypanosoma brucei spp*. Available treatments for this disease are poor, with unacceptable efficacy and safety profiles, particularly in the late phase of the infection when the parasite has invaded the central nervous system. In South and Central America, *Trypanosoma cruzi* is the infectious agent of Chagas' disease (American Trypanosomiasis) which represents the most important parasitic infection in this part of the world. It is affecting more than 10 million people, with about 100 million people at risk. Leishmaniasis is due to the infection by protozoa of the genus *Leishmania* and is affecting more than 10 million people worldwide. These parasites live in the alimentary tract of blood-sucking sand flies, and as nonflagellate intracellular forms mostly within the macrophages of mammalian hosts. The severity of the disease is ranging from cutaneous and/or mucosal to visceral infection. Malaria occurs following infection by *Plasmodium spp*. and is the most prevalent parasitic disease, affecting more than 250 million of people per year and still responsible for almost a million deaths, the vast majority of which impacting children below 5 years. Not only unicellular parasites bear a huge impact on global public health: parasitic helminths (worms), such as *Schistosoma ssp*, also represent a serious public health problem, mostly in the developing world. In view of such a dramatic situation, more than ever, it is crucial that the entire scientific community in basic research and industries develops all possible strategies leading to an arsenal of therapeutic weapons that will efficiently treat patients and eradicate these diseases. Among possible drug targets, enzymes that modulate the level of phosphorylation of parasite and host proteins such as protein kinases (PKs) and protein phosphatases are interesting candidates.

First, the kinomes of parasites like kinetoplastids and apicomplexans could reveal promising taxon-specific drug targets. Indeed, signalling pathways are well known to allow any organism to adapt to its environment by coordinating intracellular processes. Bioinformatics approaches revealed a total of 176 PKs in *T. brucei*, 190 in

T. cruzi and 199 in *L. major*. Compared to trypanosomatids, the human kinome contains 3 times more protein kinases while the size of the *Plasmodium* kinome is only about half that of trypansomatids. Trypanosomatids and *Plasmodium* do not contain receptor-linked tyrosine kinases, but possess divergent kinases with no orthologues in the mammalian kinome (Ward P, Equinet L, Packer J, Doerig C. 2004. BMC Genomics; Parsons, M, Worthey E, Ward P, Mottram J. 2005. BMC Genomics). The fact that trypanosomatids exhibit a large set of PKs, covering approximately 2% of each genome, suggests that phosphorylation may play a key role in the biology of most parasites.

Despite differences in kinome sizes and composition from one parasite to another, major signalling pathways and functions are conserved. Motility, for instance, is an essential attribute that allows some parasites finding their target cells in human hosts and/or arthropod vectors. In apicomplexans, this key driving force depends on a unique component whereby adhesins contained in the micronemes are released onto the parasite apical extremity and translocated to the posterior end of the cell, thus propelling the parasite forward. In *Toxoplasma gondii*, Calcium-dependent protein kinase 1 (TgCDPK1) is an essential regulator of calcium-dependent exocytosis and this could well be the case in most of the opportunistic human parasites. Recently, the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) was described elevated in dormant forms of apicomplexan parasites such as *Plasmodium spp*. and *Toxoplasma gondii*. (Zhang M. *et al.*, Eukaryotic Cell, 2013).

Kinases have been shown to be essential for survival of parasites in their mammalian hosts. Nevertheless, a parasite kinase-specific small molecule inhibitor still awaits to be identified and the question whether drugging the parasite kinome is more a dream or a reality begs for an answer. In complex parasite life cycles such as that of *Plasmodium*, most of the key developmental forms of the parasite such as sexual and liver stages rely on protein kinase-mediated regulations as highlighted by C. Doerig (Nat. Chem. Biol.) commenting the work of Kato *et al*. who has demonstrated that *Pf*CDPK1 plays a key role in asexual blood stage egress. Considering the 3-dimensional structure of protein kinases, there are increasing evidences that the ATP-binding pocket represents a druggable site. Specific kinomes like the one of *Plasmodium* display sufficient specificity, compared to the human one, to represent a potentially fertile source of novel targets. Interestingly, the counteracting biochemical reactions driven by *Plasmodium* phosphatases are similarly specific enough to envisage drug discovery programs targeting molecular events that are modulated by these enzymes (Wilkes and Doerig BMC genomics 2008).

Since helminths are Metazoan and have therefore a kinome that is very similar to that of their hosts, it is unlikely that highly selective targets will be identified. However, the kinome still remains an attractive target in this case to: precisely because of similarities between the helminthic and human kinomes, "piggy-back" approaches exploiting the wealth of resources devoted to targeting human kinases in the context of diseases such as cancer and neurodegenerative diseases is an attractive option as a strategy to combat diseases caused by worms.

Last but not least, the host-parasite interface might well be a target of choice to avoid induction of drug resistance and spreading. *Plasmodium* infection of host cells takes advantage of the plasticity of this parasite and the different forms produced along its complex life cycle. For instance during the infection of human hepatocytes (liver stage of Plasmodium's life cycle), not only part of the parasite kinome is solicited but some human protein kinases in liver cells such as MET, PRKWNK1, SGK2, STK35 and PKCζ seem to be crucial to *Plasmodium* sporozoite invasion mechanism and differentiation/growth (Prudêncio M. *et al.*, 2008, PLoS Pathogens). Evidence is emerging that even in the erythrocyte, host signalling pathways are activated and required for parasite survival (Sicard *et al* 2011). Host protein kinases such as MEKs and downstream MAPKs may play a key role in the host immune response to *Plasmodium*. Indeed, these protein kinases have been shown to regulate the production of pro-inflammatory cytokines produced in response to specific markers of various infectious agents that may modulate the specificity and effectiveness of adaptive immunity. Thus, small molecules could be used as immunomodulatory tools to control pathogen infections and resulting diseases by regulating specific host protein kinases. (Zhu J. *et al.*, 2009, J. Biol. Chem.).

In the present book, the bioinformatics approach leading to the study of parasite kinomes and phosphatomes will be described and followed by chapters addressing the functional analysis of some of the key enzymes. The potential roles of host cell kinome and phosphatome will be discussed. Finally, opportunities for drug discovery programs targeting parasite protein kinases and phosphatases will be explored in protozoan and helminthic parasites alike. There is no doubt that the holistic view described in this book will contribute to the future success of new efficacious and affordable therapeutics to treat the world population severely impacted by parasitic diseases.

Geneva, July 2013 *Didier Leroy*

Preface

Diseases caused by eukaryotic pathogens have been a scourge of human populations ever since the emergence of our species. Many of the major lineages of eukaryotes, from Excavata (*Giardia*), through Discicristata (*Trypanosoma, Leishmania*), Amoebozoa (*Entamoeba*) and Alveolata (*Plasmodium, Toxoplasma, Eimeria*), to Opisthokonta (metazoans, fungi), include species that have adapted to a parasitic lifestyle and have co-evolved with their hosts in the lineage that led to *Homo sapiens*. The burden imposed by parasitic diseases is disproportionally large in the poorest nations. While there has been immense progress in controlling some of these diseases in the second half of the XXth century, notably through the use of specific drugs, the global picture remains very gloomy: first, pathogens have responded to novel treatments by developing resistance against the drugs; thus, for example, that wonder antimalarial drug, chloroquine, has now become ineffective in a majority of malaria-affected countries. Even the latest generation of antimalarial drugs, based on artemisinin, shows signs of losing efficacy in some parts of the world. Second, many of these diseases have remained grossly neglected in terms of investment in research and development of novel control agents, largely because of the poor marketing prospects such agents would offer. Clearly, a renewed effort is urgently needed to address this global issue. Fortunately, awareness has increased in the last decade, which has led to an increase of funding from public institutions such as the European Commission and the research councils and agencies of many governments, as well as private bodies such as the Bill & Melinda Gates foundation. Furthermore, new organisational tools now exist to fund such research; for example, the Medicines for Malaria Venture (MMV, www.mmv.org), a Public-Private Partnership based in Geneva, and the Drugs for Neglected Diseases initiative (DNDi, www.dndi.org), have already had a tangible impact in this area. To eventually bring parasitic diseases under effective control, it is crucial that existing funding for fundamental research on eukaryotic pathogens is maintained and expanded, so as to prime the drug development pipeline. A high priority on the agenda is to develop control agents with un-tapped mechanisms of action.

Protein phosphorylation is an enormously important phenomenon in the biology of eukaryotic cells, where it regulates essentially all complex processes. This fundamental role has singled out protein kinases as potential targets for anticancer

agents, and indeed, a number of protein kinase inhibitors have reached the market in this context.

Could protein kinases represent targets for the treatment of parasitic diseases as well? A group of about 25 researchers interested in this idea convened in Paris in 2001, at the first EU-COST-funded meeting on "Protein kinases of eukaryotic parasites". This forum has reconvened in Glasgow in 2005, and in Lausanne in 2010. By then, the attending community had grown to 80 people, and significant progress had been achieved in (i) our fundamental understanding of the complement of parasite protein kinases and protein phosphatases (kinome and phosphatome) and the function of these enzymes in the biology of the parasites, and (ii) the identification of specific kinase targets in many eukaryotic parasites, and, in a few cases, of parasite kinase inhibitors. It was also emerging that the phosphorylation machinery in the host was playing a crucial role in parasite survival and development, suggesting that kinase inhibitors developed against cancer might be repositioned for the treatment of parasitic diseases.

The present book is an outcome of the 2010 meeting in Lausanne, and offers a written and updated version of some of the highlights that were presented there. It covers bio-informatics analyses of the kinomes and phosphatomes of selected eukaryotic parasites, recent advances in our fundamental understanding of the biology of selected kinases and phosphatases (inclusive of host signalling elements), and finally the state-of-the-art with respect to anti-parasitic drug discovery efforts targeting protein kinases.

We consider protein kinases offer huge potential for the development of urgently needed control agents against devastating diseases caused by eukaryotic parasites. This will happen only if the research community embraces the idea and constitutes compelling supporting data, so that policymakers and industrial partners can be convinced that there would be a significant return on investment in terms of impact on global public health. The purpose of this book is therefore to stimulate interest of established researchers and students in this topic, which offers a combination of both fascinating biology and potential tangible impact.

The Editors are aware of the significant additional commitment that engaging into the writing of a chapter represents in the busy life of research scientists, and are therefore very grateful to all authors for their timely contributions. We are indebted to the series editor, Dr. Paul M. Selzer of MSD Animal Health Innovation GmbH, for his constant encouragements and active involvement in the preparation of this volume, and to Anne du Guerny, Project Editor at Wiley Blackwell, for her patience and excellent support throughout the publication process.

Melbourne, Paris, and Glasgow
September 2013

Christian Doerig, Gerald Späth, and Martin Wiese

Cover Legend

The cover is composed of several illustrations coming from or being related to the articles in this volume. The underlying phylogenetic tree illustrates the evolutionary relationships among eukaryotic species, including model organisms and protozoan pathogens, selected across all eukaryotic supergroups (courtesy of D. Miranda-Saavedra, see chapter 1 for details). The protein structure shows the homology model of EtCRK2 a CDK2-like protein of *Eimeria tenella* with ATP docked into the ATP binding pocket. The protein is shown as ribbons, while ATP is depicted in ball-and-stick representation with atoms colored according to the CPK model (courtesy of R. J. Marhöfer, see chapter 15 for details). The black matrix panel shows fluorescence microscopy images of different parasites. The top row of the panel shows an intra-erythrocytic *Plasmodium falciparum* schizont. The mitotic regulator Aurora kinase 3 is labeled in green, the *Plasmodium* homologue of centrosome protein Centrin-3 is labeled in red and the parasite DNA is stained in blue (courtesy of T. Carvalho, see chapter 1 and 13 for details). The middle row of the panel shows immunofluorescent staining of *Trypanosoma brucei* bloodstream forms. PKA-like kinase substrates are labeled in red, the paraflagellar rod protein in green as reference for the flagellum, and nuclear and kinetoplast DNA are stained blue with DAPI (courtesy of S. Bachmaier and M. Boshart, see chapter 5 for details).The lower row, from left to right, shows in the 1st image, eggs of *Schistosoma mansoni* purified form livers of infected hosts. Due to tyrosine-rich eggshell precursor proteins, which are fused via quinone tanning during eggshell synthesis, green and red auto-fluorescence is observed by fluorescence microscopy (courtesy of C. G. Grevelding, see chapter 16 for details). The 2nd image shows several human fibroblast cells with large blue nuclei massively infected with a transgenic strain of *T. gondii* tachyzoites visualized by small blue nuclei expressing GFP in its single mitochondrion. Cellular lipid bodies are stained red with Oil red O (courtesy of F. Seeber, Robert Koch Institute, Berlin, Germany). The 3rd image shows a section of a *Schistosoma mansoni* male worm labeled with anti-*S. mansoni* Insulin Receptor 1 antibodies. The antibody was localized at the basal membrane of the tegument, in muscles and in intestinal epithelium of worms (courtesy of C. Dissous, see chapter 16 for details). The 4th image shows an *Echinococcus multilocularis* protoscolex with DAPI/phalloidin staining (courtesy of K. Brehm, see chapter 17 for details).

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Part One
Bioinformatics

Protein Phosphorylation in Parasites: Novel Targets for Antiparasitic Intervention, First Edition.
Edited by Christian Doerig, Gerald Späth, and Martin Wiese.

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Computational Analysis of Apicomplexan Kinomes

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Abstract
Apicomplexan parasites are responsible for a large number of diseases affecting much of the World's population, and as a result place a tremendous burden on the economic development of many countries. Protein kinases, a large family of enzymes regulating almost every known cellular process, have emerged as potential key drug targets for antiparasitic therapies. In this chapter we review recent bioinformatic investigations aimed at identifying the most promising protein kinase drug targets. An overview of the resources available for the study of apicomplexan genomes is first provided, especially databases of protein kinases and custom methods for the sequence analysis of kinases, as well as some practical guidelines for the annotation of protozoan kinomes. Finally, recent findings on apicomplexan kinomes obtained from comparative studies of multiple species are summarized, and an explanation is provided as to how heterogeneous datasets (functional genetic, expression, phylogenetic and structural data) are integrated not only to identify the most important protein kinase drug targets but also to find their Achilles' heels in order to achieve their selective targeting.

Introduction

The Apicomplexa – derived from the Latin *-apex* (top) and *complexus* (composed of parts) – is a diverse phylum of unicellular parasitic protozoa named after a characteristic cellular structure (the apical complex) that is used to invade animal host cells. The apical complex is itself a set of unique organelles (the conoid, rhoptries, micronemes and polar or apical rings). Most apicomplexans also contain apicoplasts, a unique nonphotosynthetic plastid, which was acquired ancestrally through the secondary endosymbiosis of a red alga [1]. This event endowed some

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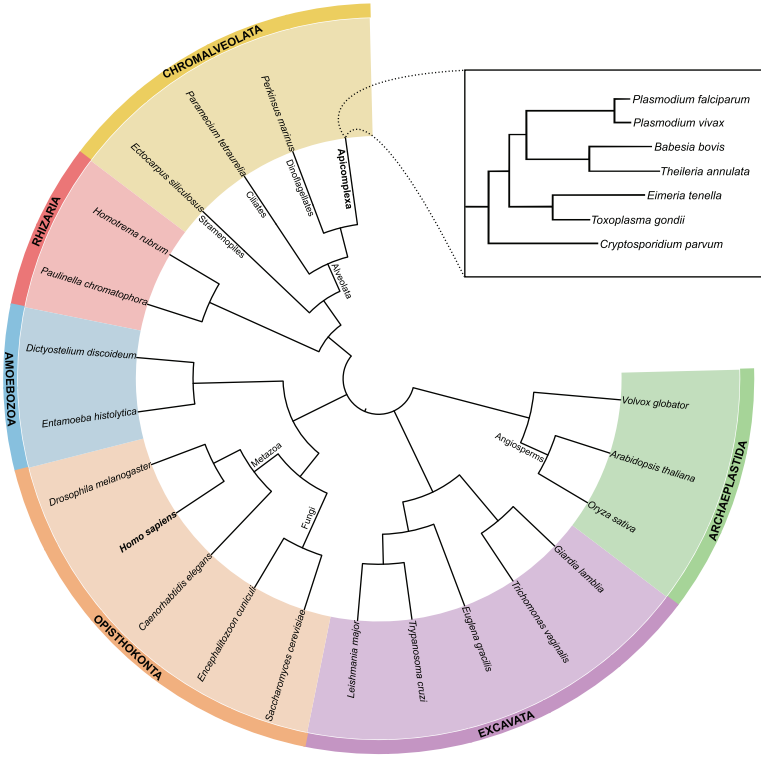

Figure 1.1 Eukaryotic and apicomplexan species relationships. Phylogenetic relationships between apicomplexan species, as per Kuo *et al*. [127], and between selected outgroup and model eukaryotes representing the major eukaryotic supergroups [4]. The tree image was rendered by the Interactive Tree of Life server (iTOL) [128] and edited in Inkscape (http://inkscape.org).

apicomplexans with plant-like characteristics such as plant-specific gene families, and also a vulnerability to some herbicides [2,3].

The Apicomplexa form part of the chromalveolate eukaryotic supergroup, which also includes dinoflagellates and ciliates [4] (Figure 1.1). The biodiversity and wide environmental distribution of apicomplexans are astounding, with an estimated 1.2 to 10 million species, of which only about 0.1% have been characterized to date [5]. The results of phylogenetic studies have suggested that the first apicomplexans originated nearly one billion years ago at the dawn of eukaryotic multicellularity, well before the Cambrian explosion and the emergence of land-dwelling animals [6]. Therefore, although the apicomplexans *Plasmodium falciparum* and *Toxoplasma gondii* are grouped together within the same phylum and are often considered in the same context, evolutionarily speaking they are about as distant as humans and mosquitoes, having diverged about 800 million years ago [7].

Like many parasites, apicomplexans exhibit complex life cycles involving one or two host species, and may pass through multiple stages in each host [8,9]. For

instance, the *P. falciparum* life cycle involves an initial sporozoite stage in the *Anopheles* mosquito vector, transmission by the mosquito to a human host, followed by a maturation stage in the liver and a blood stage in which parasites invade the host erythrocytes. Once inside the red blood cells, the parasites multiply synchronously, differentiate, and then burst from the cells, causing the characteristic episodes of fever. Reinvasion of the red blood cells occurs with a two-day periodic pattern. After invasion of the red blood cells, some parasites differentiate into nonproliferating male or female gametocytes, which may be taken up again by a mosquito to finally undergo gametogenesis and fertilization. Variations in the life cycle strategies of different apicomplexan species are well documented. For example, *Cryptosporidium parvum* has only a single host (human); *Theileria* spp. and *Babesia bovis* escape the parasitophorous vacuole (a special parasite-made membrane which surrounds the intracellular parasite and which is very different from endosomal membranes or the membrane of phagolysosomes) shortly after entering the host lymphocyte cell; and *T. gondii* is capable of infecting a wide variety of mammalian hosts and cell types, unlike most apicomplexans, which have restricted host species ranges. An understanding of the characteristics of each life cycle stage is essential for devising successful therapeutic intervention strategies, as distinct sets of genes are expressed (and pathways activated) in each of these stages through mechanisms that are poorly understood [10,11]. In fact, many promising treatments affect different aspects of the parasite's reproductive and transmission abilities by interfering with mechanisms specific to different stages of the cell cycle [9,12]. For instance, the erythrocyte invasion process and the intraerythrocytic stage constitute promising targets for the treatment of malaria, while vaccination strategies to inhibit the liver or vector stages are also under development [13].

As parasites of animals, apicomplexans have a tremendous impact on human health and economic development. The malaria parasite *P. falciparum* has traditionally been the focus of major research programs as it still causes 300–600 million clinical cases per year, and more than one million deaths (WHO World Malaria Report 2011; http://www.who.int/malaria/world_malaria_report_2011/en/). Malaria is currently endemic in more than 100 countries, and *P. falciparum* has probably killed more humans throughout history than any other single factor [13]. Other apicomplexan diseases include cryptosporidiosis (caused by *Cryptosporidium* species), which afflicts humans mainly in developing countries; in immunocompetent adults it causes acute gastroenteritis and diarrhea.

Toxoplasmosis (caused by *Toxoplasma gondii*) affects about 30% of the human population worldwide; in the vast majority of cases it exists as dormant cysts and does not cause physiological symptoms, but it can be a serious threat to young children and pregnant women. The presence of *T. gondii* cysts in the brain has also been linked to schizophrenia and paranoia [14]. The AIDS pandemic has created large immunocompromised populations in many of the same tropical areas where apicomplexan diseases are endemic, and infections by these opportunistic pathogens may be fatal.

Among the apicomplexans causing diseases of veterinary/agricultural importance we may cite *Babesia bovis* (hemolytic anemia or babesiosis in cattle); *Theileria*

annulata and *T. parva* (tropical theileriosis and East Coast fever, respectively, in cattle); *Eimeria tenella* (coccidiosis in chickens); *Sarcocystis neurona* (myeloencephalitis in horses); and *Neospora caninum* (neosporosis in cattle and neurological problems in dogs). Most of these diseases have poor or no treatments, and since parasites are known to develop resistance to therapies it is essential to characterize as many drug targets as possible for antiparasitic intervention.

One specific protein family that holds great promise for antiparasitic therapies are the protein kinases (PKs), which have been successfully targeted in a number of human conditions, with 16 distinct kinase-specific drugs currently available in the market and over 150 undergoing clinical trials [15]. The present understanding of the "druggability" principles of PKs, the existence of compound libraries and assays for high-throughput screening, and the identification of not only important differences between orthologous PKs in human and apicomplexans [16–20] but also essential Apicomplexa-specific PKs [21–23], have led PKs to become a very attractive set of potential drug targets in these parasites.

In this chapter, an overview is first provided of the public resources presently available for the study of apicomplexan genomes, and more specifically of those focused on genes encoding PKs. A set of practical guidelines is then introduced for the analysis and annotation of kinases in protozoan genomes. Finally, an overview is given of the apicomplexan kinomes that have been characterized to date, and an explanation provided of the integration of heterogeneous datasets (including genetic, expression, phylogenetic, amino acid sequence and structural data), not only to identify the most important kinase drug targets but also to find their Achilles' heel in order to achieve selective targeting.

Public Resources and Computational Methods for Annotating Apicomplexan Kinomes

Apicomplexan Resources

In 2002, research into malaria entered the post-genomic era when details of the genomes of *P. falciparum* [24], *Plasmodium yoelii* [25] and the mosquito vector *Anopheles gambiae* [26] were published. Ten years down the line, a total of 15 apicomplexan genomes – including representatives from all four clades (coccidians, gregarines, hemosporidians and piroplasmids) – have been sequenced and at least partially annotated [10,24,25,27–34] (Table 1.1). The analysis of this wealth of data has revolutionized the study of these parasites and provided fundamental insights into their biology.

Apicomplexan genomes are highly dynamic and characterized by rapid genetic recombination, large-scale genome rearrangements (making syntenic relationships difficult to detect across genera), a relatively small size (~3700–8000 genes), and frequent gene losses relative to the nearest free-living Eukaryotes [35–37]. Indeed, like other parasites, apicomplexans can withstand massive gene losses as long as the host can supply nutrients and a sheltered environment. It has been estimated that