# Drugs against parasitic diseases: R&D methodologies and issues

## Discoveries and drug development

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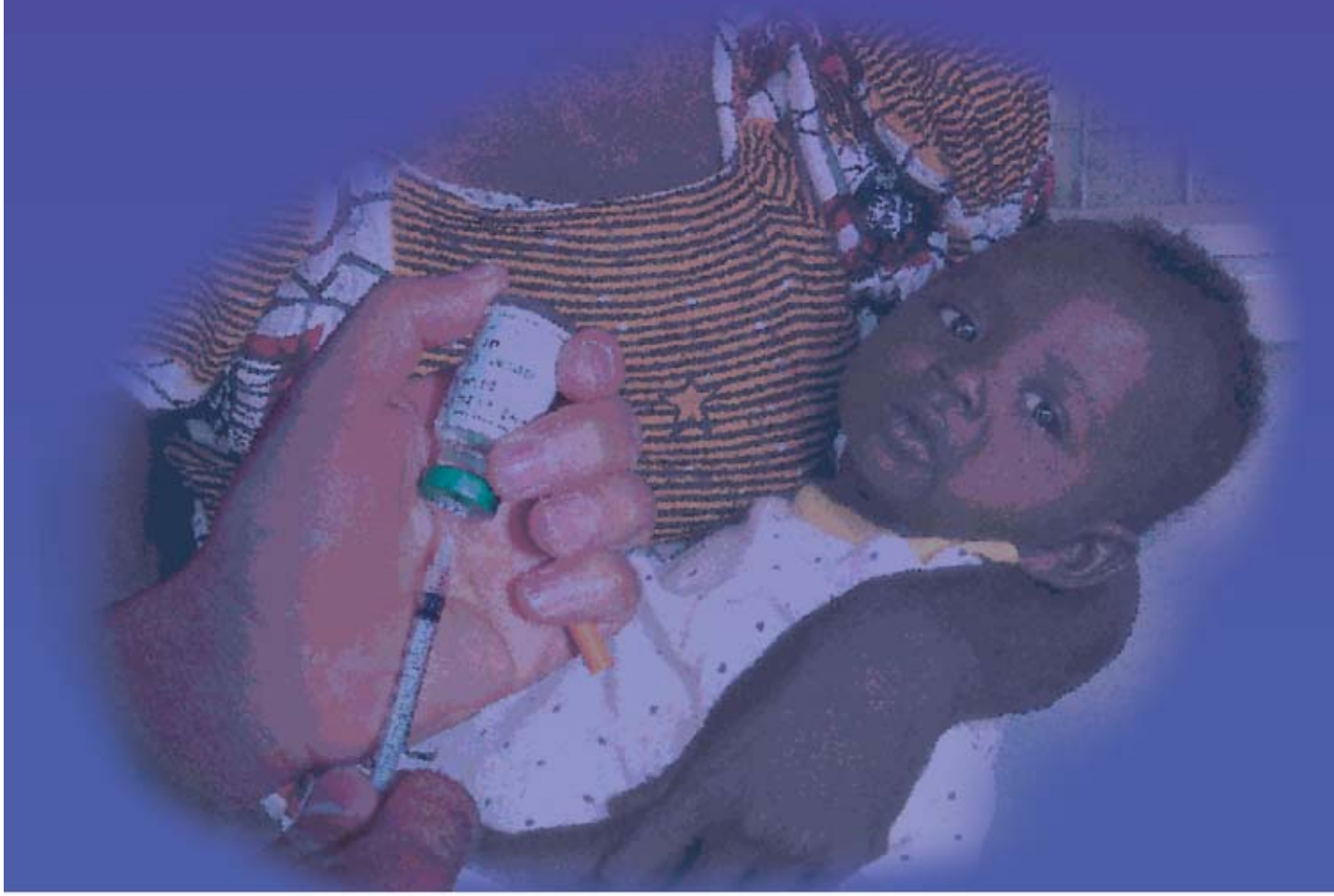

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Discoveries and drug development

UNDP / World Bank / WHO
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C O N T E N T S

# Discovery and drug development for tropical diseases: new opportunities, new trends

Viewed globally, parasitic diseases pose an increasing threat to human health and welfare. Leishmaniasis and trypanosomiasis continue as a cause of suffering for many millions of people in both tropical and subtropical zones of the world and in the last 25 years, malaria has made a comeback and remains one of the greatest threats to the health and economic prosperity of mankind.

However, the available therapeutic tools for the treatment of most parasitic diseases are extremely limited. Many of them were developed in the first part of this century and are not without risk. The development of parasites resistant to many of the available drugs is also responsible for the depressing picture of disease persistence and death. Drug resistance is spreading faster than ever, new drugs are not being developed quickly enough and potential vaccines have so far not fulfilled expectations in field trials. Although alternative antiparasitic drugs are urgently needed, the response to this crisis is inadequate. The field suffers not so much from a lack of promising scientific approaches, but a lack of funding and commitment from both public sector agencies and the pharmaceutical industry to convert these approaches into new drugs.

The past 20 years has witnessed a huge increase in our understanding of the biochemistry, molecular and cell biology of the pathogens that cause these diseases, with attention focused on the characterisation of metabolic pathways and cell structures that are different between parasite and host. The advent of genomics has increased this opportunity and offers a new challenge. Studies on pathogenic bacteria and fungi have already shown the impact that genomic information can have on our level of understanding of such organisms. For example, microarrays describing of the transcription response profile of *Mycobacterium tuberculosis* have helped to characterise the metabolic pathways of this organism. Coordinated research on the model yeast, *Saccharomyces cerevisiae*, particularly the methodical knocking out of each gene and characterisation of resultant phenotypes, has shown that it is possible to characterise the role of each gene.

Within the next five years, the genome sequences of the parasites that cause malaria, leishmaniasis and trypanosomiasis will be completed and this new data will significantly alter the directions of laboratory research. This expansion of molecular and biochemical knowledge will reveal many of the essen-

tial differences between parasite and host and will open the door to identify metabolic differences that could result in defining potential drug targets. One of the aims of the post-genomic agenda for research on parasites is to provide the tools necessary to use the massive amount of information contained in the genomic sequence and to address key biological questions concerning pathogens. Particular foci of interest will include identification of parasite-specific genes essential for infection and development of pathogenesis, characterisation of molecular structures and metabolic pathways, elucidation of unique parasite-specific mechanisms of gene regulation and RNA processing, and analysis of the protein profile of the parasite to identify functionally important genes (proteomics). In addition improved technology for the transfection of these parasites and for complementation will help to determine the function of the various steps of a number of essential metabolic pathways. These techniques will validate new drug targets, lead to new pharmacological paradigms and hopefully be a source of innovative chemotherapy.

This new environment made the symposium on "Drugs against Parasitic Diseases" that was held in Montpellier from 24-26 May 1999 particularly timely. The goal of the meeting was the development of a framework for research into antiparasitic drugs into the 21st century. Parasitic diseases are diverse and complex, and multiple approaches often have to be used for their control. Even when multiple approaches are taken they rarely result in complete success. One of the major goals of parasitology is to explain, in molecular terms, the identity of the parasites, the processes that allow their structural and functional stability and their interactions with their host. The increasing knowledge of parasite genomes is revolutionising research in this field, but there is a need to focus on what genomics can deliver and what its limitations are. Successful utilisation for drug discovery and development of all the data evolving from the knowledge of the genome and post-genomics, requires the interaction of many disciplines, among them bioinformatics, biochemistry, cellular and molecular parasitology, chemistry, protein crystallography and structural biology, pharmacology, toxicology, as well as competence in the field of drug testing, and human clinical expertise. Research on drugs against parasitic diseases is now being a truly multidisciplinary activity.

The Montpellier meeting addressed the complex questions posed for antiparasitic drug discovery and development and its need to incorporate the industrial stages of development if it is ever to be significantly successful. The objective of the symposium "Drugs against Parasitic Diseases" was to provide a guideline around the novel strategies being developed to fight this crucial health issue for humankind. There was a focus on parasitic genomes, their promise and limitations. Priority topics also included issues relating to the discovery of new pharmacological targets, the criteria needed to validate a

target, and the key decision-making factors and sequential and iterative steps needed when taking molecules from the bench through to clinical development and use. The meeting also provided research managers with a forum in which to discuss advances and update their knowledge concerning drug discovery in the area of parasitic diseases so that they might better facilitate an environment in which drug development in this crucial field can be accelerated and more effectively contribute to improved control strategies.

We hope that the publications of the proceedings, with a selection of manuscripts on top priorities for immediate research and development will be helpful, and contribute to helping set priorities for creating the facilities and mechanisms that are desperately needed to develop novel antiparasitic drugs. We also hope it will strengthen the collaboration between scientists, experts, and professionals both in the North and the South, promoting excellence not only in research but also in its practical implementation.

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## SECTION I: Global picture on antiparasitic drugs and the need for control strategies. Economic and patent issues

The burden of parasitic diseases in terms of Disability Adjusted Life Years (DALY) has a renewed and intriguing position at the dawn of the 21st century. Of the 1399 new chemical entities registered and marketed between 1975-1999, only 13 specifically concerned tropical diseases. Despite the fact that the demand for novel antiparasitic drugs is extraordinarily high, the pharmaceutical industry is not very enthusiastic about supporting development. Industrial partners are often only interested in compounds close to or already undergoing Phase-I trials. However, drug development costs are enormous, and focus more on the know-how of an industrial partner than on that of the scientist who developed the pharmacological model. Besides, socio-economic conditions in endemic countries are often insufficient to allow the emergence of new weapons against parasitic diseases, and alternative strategies therefore need to be considered. Finally, to make explicit such outcome, it is commonly said that the R&D process is a costly, risky and lengthy business, particularly since intellectual property rights are not universally respected. Are these arguments relevant, if so what are the possible solutions?

## SECTION II: How to discover novel targets for pharmacological intervention

Within the next few years, the genomes of a several medically important parasites will be completely sequenced and assembled, presenting unparalleled opportunities for both basic and applied research. How best to capitalise on this information in the post-genomic era? Which of the biochemical pathways and biological processes of these organisms can be used for future drug development? How to identify molecular targets critical for proliferation of a pathogen or for progression of a disease and how to determine whether the target molecules are crucial for the pathogenic agent or the pathological process?

## SECTION III: Compound acquisition and rationale for drug development

Selective toxicity is key to all chemotherapy. This involves identification of biochemical or biological processes that can be selectively disrupted in a parasite without harm to the host. Differences between proteins that are common to host and parasite can be exploited for drug design. Unfortunately, most amino-acid residues implicated in substrate and coenzyme binding and catalysis are often highly conserved. How to identify specific regions in a protein that can be selectively attacked? Is it essential to fully characterise the molecular structure of a target to obtain lead compounds that disrupt target function? With an experimental or hypothetical structure in hand, how best to generate novel structures with high affinity and selectivity that can serve as leads in drug development?

## SECTION IV: Methods for drug screening and evaluation of pharmacological activity

Throughout the development of a pharmacological model, lead compounds have to be tested for their effects on parasite survival or pathogenicity, while their toxic effects against the host organism also have to be evaluated. These tests have to be done in a sequential order, from the in vitro to the in vivo condition, and meet specific criteria concerning the potential of a lead. Such experiments are expensive and facilities for testing in primate models are rare.

Other specific problems include monitoring of resistance under controlled conditions, and the discovery of other pharmacological properties (especially broad spectrum antimicrobial activity) to increase economic interest in further development.

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SECTION I

# Global picture of antiparasitic drugs and need for control strategies. Economic and patent issues

# The need for new approaches to tropical disease: Drug discovery and development for improved control strategies

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## 1. Introduction

It is now well recognised that there is an increasing medical need for new drugs to cover a range of "neglected" infectious diseases. A retrospective analysis of new drugs introduced over the past 24 years clearly illustrates this need. Between 1975 and 1999, of 1393 new chemical entities (NCEs) introduced on to the global market, only 13 were specifically for neglected infectious diseases and many of these came out of primary research and development for other disease indications such as veterinary medicine and cancer[1] (see also P. Trouiller this issue). This paper summarises the medical need for a number of tropical diseases in terms of their global disease burden and lists the existing treatments available and their limitations, highlighting desirable product profiles for current and future drug discovery and development. It further investigates the reasons for the lack of industrial activity in these diseases and identifies the key elements of any new mechanisms initiated to ensure the sustainable enhancement of neglected disease drug R&D in the future.

## 2. Disease burden

The Disability Adjusted Life Year (DALY) provides a means to measure disease burden that is more informative than crude measures of mortality and prevalence and is fast becoming the standard by which different disease burdens are compared. The concept is well described in reference 2. Table 1 provides disease burden in terms of DALYs from the 1999 World Health Report.[3] HIV/AIDS clearly dominates the global picture with an alarming increase in disease burden to 89.9 million DALYs. However, this disease does receive a

huge amount of R&D funds both from national science agencies such as the NIH and from industry itself. The level of activity in this area is apparent by the fact that although only one anti-HIV medicine existed in 1987, 15 anti-retrovirals are now registered and listed for use in the USA.[4] The contrasting information for the other diseases listed in Table 1 makes sorry reading. Although equivalent in disease burden, malaria has only had 4 NCE's registered over the years 1975 to 1999 (artemether, atovaquone, halofantrine, mefloquine).[1] Although equivalent to leukaemia in the scale of their global impact, there have only been one NCE for African Trypanosomiasis (eflornithine), two for Chagas' Disease (benznidazole and nifurtimox, the latter no longer being available), two for schistosomiasis (oxamniquine and praziquantel) and two for the filariases (albendazole and ivermectin).[1] Another point deserves to be remembered. The DALY figures in Table 1 represent a global disease burden. If one were to take into account the high concentration of these diseases in specific regions of the world, especially Africa, then the levels become truly horrendous. Malaria alone may account for about 10% of total disease burden in many sub-Saharan African countries and may account for over 30% of hospital admissions.[2]

Table 1 also demonstrates that it is not too fine a point to describe the impact of these diseases as being equivalent to war and conflict in terms of their impact on human health and development. If one compares the funds spent annually on weaponry with that spent on neglected disease R&D, there is a clear indication that our global sense of priorities is in need of some realignment.

***Table 1.*** **Disease burden in terms of Disability Adjusted Life Years**

| Disease | Disease Burden DALY's[3] (1999 figures)[3] |
|---|---|
| Malaria | 39.3 million |
| Filariases (lymphatic filariasis and onchocerciasis) | 5.8 million |
| Leishmaniasis | 1.7 million |
| Schistosomiasis | 1.7 million |
| African trypanosomiasis | 1.2 million |
| Chagas disease | 0.6 million |
| War | 20.0 million |
| HIV | 89.8 million |
| Leukaemia | 4.6 million |

## 3. Liabilities of existing drugs to treat neglected diseases

It was mentioned above that there are several drugs available to treat the diseases listed in Table 1. However, these drugs, combined, fail to meet existing medical need and the adverse side-effects of some of the drugs in use for tropical diseases would probably not be tolerated if the diseases were prevalent in North America, Europe or Japan. In this section we briefly review the drugs available to treat tropical diseases and the problems associated with their use and effectiveness.

### *3.1. Malaria*

For an excellent account of malaria drug treatment and use please refer to a recent WHO report on the use of antimalarial drugs.[5] The mainstay of malaria treatment remains chloroquine, though development of resistance over the last two decades has rendered this drug ineffective in many regions. The frequently used next drug of choice is sulfadoxine–pyrimethamine, often referred to by the trade name Fansidar® or by the abbreviation SP. This also suffers from extensive drug resistance and is ineffective in many parts of the world. Quinine is frequently used, often as an injection, but has some side-effects, notably tinnitus. It also has to be administered over five to seven days, which often results in poor compliance. In addition, a course of treatment costs several dollars, a high burden in sub-Saharan Africa. It is often used in combination with tetracycline or doxycycline, adding to cost and compliance issues.

Among the newer drugs, the artemisinin derivatives such as artemether and artesunate are increasingly finding use in malaria treatment. They are fast acting and efficacious. However, these drugs also suffer from short half-lives and a need for treatments of five to seven days. It is generally agreed that the identification of appropriate combination partners that can reduce the treatment course of these drugs would be valuable, but to what extent this can be achieved in the setting of sub-Saharan Africa remains an open question.[6] Two drugs related to quinine, namely mefloquine and halofantrine, were registered in the mid-1980s. It has been found that resistance develops relatively rapidly to both these compounds and both suffer from potential side-effects. Because of neuropsychiatric effects in some people, mefloquine has a liability in certain situations. Halofantrine is contra-indicated for people with heart conditions. A combination of atovaquone-proguanil (trade name Malarone®) has recently been registered. Its broad utility is hindered by its high price (over $40 per treatment) and it is being used primarily as a prophylactic agent for travellers. Resistance already exists, however, to its individual components and it is possible that if it became more widely used resistance would develop similarly to sulfadoxine-pyrimethamine. Another new combination drug, lumefantrine-artemether (trade name Coartem®) has also been registered. It is used twice daily for three days and may prove to be a valuable addition to the antimalarial armamentarium. From a critical perspective, lumefantrine is related to quinine, mefloquine and halofantrine and potential cross-resistance with these drugs may be a liability. Another issue that has been raised is variable adsorption and bioavailability in malaria patients.

There is an urgent need for affordable, orally active, new chemical entities to replace chloroquine and sulfadoxine–pyrimethamine for uncomplicated 'drug-resistant' malaria. Ideally these should be active over a 3-day treatment course, have negligible side-effects, including negligible side-effects in pregnant women and children, and development of resistance should be slow. It is becoming increasingly likely that any such new chemical entity will be combined with a partner drug to optimise the chances of delaying drug resistance. In addition to this major need, improved treatments for severe malaria and radical cure of vivax malaria are also required.

*3.2. Leishmaniasis*

Leishmaniasis has several manifestations: cutaneous, mucocutaneous and visceral and there are several causative Leishmania species.

The major liability of all three major anti-leishmanial drugs, namely the antimonials, pentamidine and amphotericin B, relate to their safety and the fact that they have to be administered by injection. They also need to be given over a 21 to 28 day period. There is no orally active anti-leishmanial drug yet available and drug resistance has been reported for both the antimonials and pentamidine. Some of the safety issues with amphotericin B have been ameliorated by the development of a liposomal formulation. However, the cost of this treatment takes it out of the reach of poor populations.

There is an urgent need to develop an oral drug or a short-course injectable treatment for treatment of visceral leishmaniasis, the most lethal form of the disease. Similarly there is a need for an oral or topical treatment for cutaneous and mucocutaneous leishmaniasis. The compounds need to be active against all species of leishmania and be affordable in the context of developing country healthcare systems.

*3.3. African Trypanosomiasis*

Sleeping sickness afflicts many parts of sub-Saharan Africa and many of the drugs currently in use date back to before the Second World War. There are two clinical forms of African trypanosomiasis, the acute form in which the parasite is restricted to the bloodstream and the chronic form in which the parasite has crossed the blood brain barrier. There are also two species of trypanosome largely responsible for the disease *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*.

Suramin and pentamidine can be used to treat the early stage, acute, infection if given over a 14-day period. Both are injectable drugs and have to be given under close medical supervision. There is a real dilemma when it comes to treating the chronic late stage disease. The only drug that treats both *T. b. rhodesiense* and *T. b. gambiense* is melarsoprol, an extremely toxic arsenical drug. It is estimated that in a significant number of cases, death may actually result from drug use and not disease. A drug exists that can treat the chronic form of *T. b. rhodesiense*, namely eflornithine. Its major liability is its cost, but current agreements between WHO, Medecins Sans Frontieres and the manufacturer, Aventis, will hopefully guarantee supply of this drug for the coming decade and studies are under way to establish whether 7-day dosing can be as effective as the standard 14-day dosing.

There is an urgent need for new chemical entities that can serve as affordable drugs against both *T. b. rhodesiense* and *T. b. gambiense*. They need to be made available, both as an oral formulation for treatment of the early-stage disease, and as a parenteral formulation for treatment of the late-stage disease. Such compounds must be capable of crossing the bloodbrain barrier.

*3.4. Chagas disease*

Chagas disease, causative agent *Trypanosoma cruzi*, differs in many respects from its fellow kinetoplastid species of the Leishmania and *Trypanosoma brucei* families, but is another debilitating disease that has both an acute and chronic form. The acute blood stage form is becoming less prevalent due to vector control measures and the availability of the drug benznidazole. However, after the acute phase the parasite may remain dormant for many years before recrudescing. It may lodge in the heart muscle and upon reactivation lead to a severely degenerative heart disease. A large number of people in South America suffer from the chronic form of the disease for which no treatment is currently available. There is an urgent need to identify such a treatment.

*3.5. Filariases*

The filariases, lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia malayi*, and onchocerciasis caused by *Onchocerca volvulus*, each cause significant pathologies. Lymphatic filariasis manifests itself as 'elephantiasis' and onchocerciasis primarily as 'river blindness' and as a debilitating itchy and disfiguring skin rash.

Lymphatic filariasis can be treated with DEC (diethylcarbamazine), with potential immunopathological side-effects due to the Mazzotti reaction. Currently an attempt is being made to develop and implement a combined treatment of albendazole and ivermectin to treat the disease. However, it is still unclear whether this combination will kill the adult worms (macrofilaria) or only target the microfilarial offspring.

Onchocerciasis control is dependent entirely on the use of ivermectin, which kills the microfilariae and can prevent the pathology associated with river blindness. Currently, annual treatment with ivermectin is required to keep the pathology of the disease in check, but there is no cure as the adult worms remain in the human host and are not killed by ivermectin.

An agent for either filarial disease that would kill the macrofilarial adult worms would be a significant advance. In addition there is no back-up treatment available for onchocerciasis should resistance develop to ivermectin. Back-up treatments for both indications would be valuable.

*3.6. Schistosomiasis*

Schistosomiasis, or Bilharzia, is a disease that afflicts many hundreds of millions of people. The major treatment available at the moment is praziquantel, which itself is only fully effective in about 60% of cases. There are anecdotal reports of resistance to the drug and a substitute agent is desperately needed.

## 4. Obstacles to obtaining new drugs for neglected diseases

The previous sections have identified both a healthcare need for drugs in the area of neglected diseases and have demonstrated that many of the current treatments are deficient. What are the obstacles to achieving new drugs? An objective assessment of this issue would need to look at a number of issues:

- Scientific feasibility of the objectives
- Level of scientific knowledge
- Mechanisms in place to carry out industry-like Research and Development work

Of the indications outlined in the previous section it would be fair to acknowledge that obtaining macrofilaricides to treat lymphatic filariasis and onchocerciasis is by no means a trivial objective. Likewise, an agent to address the chronic form of Chagas disease is a major scientific challenge. In each of these cases one would be trying to obtain a treatment that has not before been managed. However, these challenges are certainly not more daunting than many existing industry and public sector endeavours in areas such as Alzheimer's disease or many cancers.

For the other diseases mentioned, namely malaria, leishmaniasis, African trypanosomiasis and schistosomiasis, there are a number of scientific opportunities, combined with the availability of a significant body of genomic information, that could lead to new drugs. It is also worth noting that as drugs for these diseases have been identified in the past using minimal resources, an input of appropriate resources, combined with improved scientific knowledge, should be able to deliver significant improvements on existing therapies.

For all of these diseases the major problem in the past has been the lack of an appropriate financial and managerial mechanism to deliver new chemical entities. Industry is traditionally the bridge between research ideas and their reduction to new products, but the high cost of drug R&D and the lack of any significant commercial return from the neglected diseases has resulted in a general withdrawal of the pharmaceutical industry from R&D for tropical diseases. By way of example it is estimated that the antibacterial market, which generates approximately two NCEs per year, is about $16 billion. Malaria, for which about two new drugs per decade have been generated over the last 20 years, has a global market of between $200 million and $300 million. Obviously, if this level of 'market failure' exists for a major disease like malaria, the problem is multiplied many fold for the other neglected diseases such as leishmaniasis and African trypanosomiasis.

Many people point to the fact that there is much publicly funded scientific research in the area of neglected diseases, such as that funded by the NIH and the Wellcome Trust. However, this research is no substitute for targeted and focused product R&D of the type found in the R&D based pharmaceutical industry. In fact most public sector research funding stops before major expenditures on areas such as toxicology and process manufacture are encountered. In addition, this research is often focused on a narrow area of

drug discovery research and the groups funded do not have access to the necessary capacities or partners to reach the end goal of identifying a drug development candidate. This situation is illustrated in Figure 1 (see also reference [7]).

***Figure 1.*** **Traditional public sector funding not designed for drug R&D**

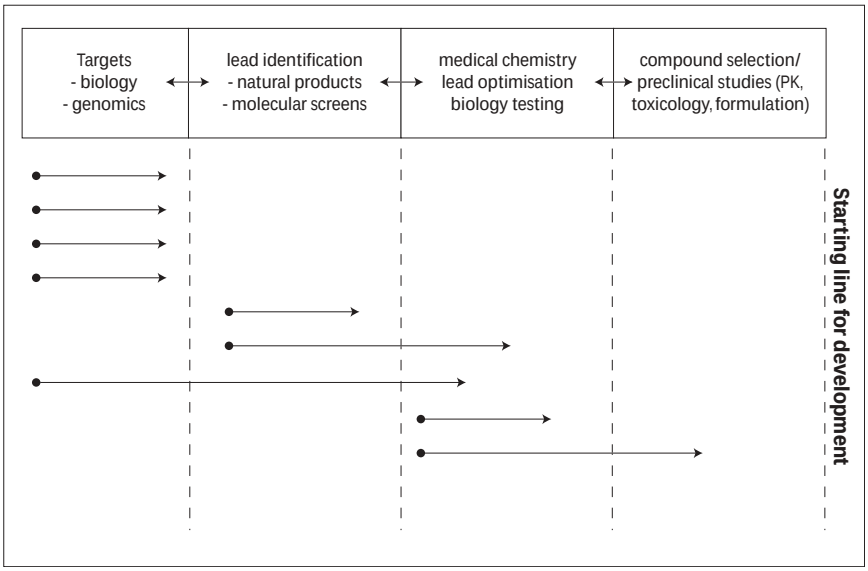


**Diagram shows the stages necessary to discover and identify a drug worthy of preclinical and clinical development. Arrows represent public sector funded research projects. Projects normally limited to specific sections of work that do not link to other necessary activities required to deliver a drug candidate. In particular, public sector funding of the final two stages of the drug discovery process, the stages involving medicinal chemistry and pharmacokinetics, metabolism and toxicology, are very weak.**

The scale of the effort required to discover a drug, let alone develop it through appropriate non-clinical and clinical studies to GLP and GCP, in a manner that meets regulatory requirements, is further illustrated in Table 2. This table lists the resources required to deliver a drug development candidate. The large manpower requirements and technological requirements begin to explain the high costs associated with this process, especially when one considers that most projects, even of the size outlined, will probably fail to deliver a registered drug. However, an equally critical element to the whole process that is often overlooked is the strategic and management resources and expertise that are needed to direct projects appropriately. Many of the skills, technologies and, particularly important, the mindset required for drug discovery and development are to be found in industry.

***Table 2.*** **Resources needed for full discovery project**

- **Manpower** (would benefit from access to industry expertise)
  - chemistry team
  - biochemistry team
  - microbiology team
  - preclinical expertise
- **Technology**
  - molecular high-throughput screening technology (industry)
  - modelling, databases, etc.
- **Strategy**
  - direction and management
  - experience (industry)
  - goal-oriented team leadership

Through 1997 and 1998 these issues and related ideas led to the establishment of a group of individuals representing several important organisations associated with both the public sector and industry to address the need for appropriate drug R&D in these areas. They defined the need for a new paradigm for the sustainable introduction of new drugs for neglected diseases. These included:

- adequate funding and management of a portfolio of discovery projects
- adequate funding and management of a portfolio of development projects
- an industry-like approach
- support of the pharmaceutical sector
- concerted efforts to secure commercialisation of products that are accessible by low income populations

This team ultimately focused on new drugs for malaria as meeting the most urgent public health need. It formed a Strategic Planning Group that, with the support and assistance of WHO/TDR, ultimately led in November 1999 to the foundation of the Medicines for Malaria Venture (MMV, **www.mmv.org**.[8]

This organisation seeks to obtain funding of $30 million per year to develop a portfolio of projects that can deliver one new antimalarial drug every five years. The key elements to its operation and potential future success are that it is:

- a public-private partnership capitalising on public sector scientific knowledge and clinical expertise and access to industry tools, expertise and know-how through industry partnering of specific projects
- a not-for-profit business utilising paradigms of industry, not those of a public sector science funding agency; progress towards a new drug rather than scientific publications drives continued funding
- a bridge between research, control and development through facilitating manufacture and commercialisation of products.

The current status of MMV is outlined in its 2000 annual report (**www.mmv.org**). It has developed a healthy portfolio that has approximate-

ly 10 major projects, nearly all with an active industrial partner. The target date for the first new antimalarial products, if all goes well, is 2004.

Subsequent to the formation of MMV, a major effort led by the Rockefeller Foundation resulted in the founding of a similar public private partnership for the discovery and development of tuberculosis drugs (**www.tballiance.org**). Further analyses of these and other public private partnerships can be found in a special issue of the WHO Bulletin (Vol 79, August issue) with several articles of particular significance.[9,10]

There is currently much discussion both on the part of WHO and the IFPMA and on the part of Médecins sans Frontières to develop a third public private partnership for other neglected diseases.

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# Drug development and registration for parasitic diseases: what are the barriers?

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***Abstract :*** *Therapeutic options for treating life-threatening parasitic diseases are extremely limited. The pharmaceutical industry has been prolific but has ignored diseases that kill the majority of people in poor countries. Of the 1 223 new chemical entities that were registred by Western health authorities between 1975-1996, only 11 were specifically indicated for tropical diseases.*
*The pharmaceutical industry is abandoning tropical diseases for various reasons: investment decisions are based on return on investment and developing countries markets are not profitable ; the research and development process is a costly and risky business; the current system of patents is a disincentive favouring counterfeiting; the level of regulatory requirements favours wealthy markets.*
*Pharmaceutical firms operate like all other private industry. Without any specific social welfare mission, they have no interest in finding treatments for tropical diseases. When the free-market economy does not provide such treatments it is the role of society to take appropriate steps.*

## 1. Introduction

Essential drugs are the foundation for nearly every public health programme. Provided they are available, affordable and properly used, they offer a simple, cost-effective solution to many health problems. During the past few decades, significant progress has been made in biomedicine and pharmaceutical research. However parasitic diseases by their nature and prevalence, are low priority for private pharmaceutical industry.[1]
Of the 1 223 new chemical entities (NCE) marketed worldwide between 1975-1996, 379 were considered as real therapeutic innovations but only

11 were specifically indicated for tropical diseases. This number was supplemented by a few reformulations of existing chemical entities. Only a minority may be claimed by Western pharmaceutical companies as genuine products of their research. The majority was either incidental discoveries recovered from veterinary medicine or entities discovered by governmental or academic institutions and only later acquired, registered and marketed by the Western industry (Table 1).[2]

Although drug discovery is conducted as a more or less collaborative effort between academia and pharmaceutical industry, generally the research-based pharmaceutical industry has been responsible for the development of over 90% of the new drugs produced worldwide.

**Table 1.** ***Tropical disease drug development output, 1975-1997***

1.1. MARKETING APPROVAL OF NEW MOLECULAR ENTITIES DURING 1975-1997

| Indication | Molecular entities | Year first marketed or approved | Pharmaceutical development context | Public-private Marketing strategy |
|---|---|---|---|---|
| Malaria | artemether * *(intramuscular)* | 1997 | Chinese academy discovery. Public-private collaboration (WHO-TDR/ Rhône-Poulenc-Rorer Co.). RPR/Kunming Co. (China) agreement | Preferential price for public sector. Artemether from other manufacturers cheaper |
| | atovaquone - proguanil | 1997 | Wellcome antimalarial research (now GlaxoWellcome Co.) atovaquone first approved for *Pneumocystis carinii* infection in HIV/ AIDS | Very expensive (high cost of goods). Currently partial drug donation programme |
| | halofantrine | 1988 | US DoD discovery (WRAIR). Public-private collaboration (WHO/WRAIR/SmithKline Beecham Co.). US Orphan Drug status | Expensive: producer price |
| | mefloquine | 1984 | US DoD discovery (WRAIR). Public-private collaboration (WHO/WRAIR/Hoffman LaRoche). US Orphan Drug status | Expensive but cheaper generic products exist |
| Human african trypanosomiasis (HAT) | eflornithine (DFMO) | 1990 | Marion Merrell Dow (now Hoechst Marion Roussel Co.). US orphan product designation and approval for the treatment of HAT (*T. b. gambiense*) | Very expensive. Product originally abandoned by HMR. WHO efforts restart and reduce price considered |
| Chagas disease | benznidazole | 1974 | Veterinary originally (Roche Co.) | Producer price |
| | nifurtimox | 1984 | Veterinary R&D originally (Bayer Co.) | Producer price |
| Schistosomiasis | oxamniquine | 1981 | Veterinary R&D originally (Pfizer Co.) | Producer price |
| | praziquantel | 1980 | Veterinary R&D originally (Bayer Co.). Public-private collaboration (WHO/Bayer) | Producer price and generic products |
| Helmintic infections | albendazole | 1987 | Veterinary R&D originally (SmithKline Beecham Co.) | Drug donation under consideration |
| Onchocerciasis | ivermectin | 1987 | Veterinary R&D originally (Merck Co.). Public-private collaboration (WHO/Merck) | Mectizan donation programme |

## 1.2. NEW APPROVALS FOR ALREADY MARKETED DRUG PRODUCT IN A NEW USE AND/OR A RE FORMULATION

| Indication | Molecular entities | Year first marketed or approved | Pharmaceutical development context | Public-private Marketing strategy |
|---|---|---|---|---|
| Human african trypanosomiasis | *pentamidine isethionate* | 1950/1984 | Rhône-Poulenc Co. : galenic re-formulation (mesylate to isethionate).US Orphan Drug status and new approval only for *P. carinii* infection | Drug donation for HAT (through WHO) |
| Leishmaniasis | amphotericin B lipid complex | 1962/1996 | Vestar (now NeXstar Co.): galenic re-formulation of Amphotericin B in liposomes. US Orphan Drug status and approval for treatment of invasive fungal infections | Extremely expensive. Efforts to cut price inconclusive as yet |

## 1.3. POSSIBLE FUTURE ADDITIONS

| Indication | Molecular entities | Year first marketed or approved | Pharmaceutical development context | Public-private Marketing strategy |
|---|---|---|---|---|
| Malaria | artemether * benflumetol | 1999 ? | Ciba Geigy (now Novartis Co.) | Probably expensive. Strategy unknown |
| | pyronaridine | 2000 ? | Chinese academy discovery, marketed only in China. International development by WHO/TDR Currently no industrial partner | Unknown |
| | artesunate rectal | 1999 ? | Chinese academy discovery. International development for limited indication by WHO/TDR and Mepha Co. | |
| | etaquine | ? | US DoD discovery (WRAIR). Public-private collaboration (WRAIR/SmithKline Beecham Co.) | |
| Leishmaniasis | paromomycin (aminosidine) | 1999? | Re-discovery of old aminoglycoside by Farmitalia-Carlo Erba (now Pharmacia-Upjohn Co.) developed by WHO/TDR, currently no industrial partner US Orphan Drug designation (1994) for the treatment of visceral leishmaniasis | |
| | WR6026 | ? | US DoD discovery (WRAIR). Public-private collaboration (WRAIR/SmithKline Beecham Co.) | |
| | miltefosine | ? | Product under development as anticancer agent. Public-private collaboration (WHO/TDR/Asta Medica Co.) | |

*Source : Trouiller P. & Olliaro P. (International Journal of Infectious Diseases, 1999)*

## 2. Past strategies used to encourage drug development

In accordance with free-market economy rules, the goal of marketing is to sell a product, maximize profit and maintain an edge in competition with other products. Different approaches have therefore been undertaken to encourage efforts within the private industry in order to address the unmet and uneconomic needs of poor countries. Strategies, used jointly either by international institutions or pharmaceutical industry, can be divided into two global categories : enabling conditions which are intended for creating a positive environment for drug development and/or a overcome hindrances ; and the discrete mechanisms which can be defined as strategies that can be planned and that have predictable outcomes.[3]

*2.1. Enabling conditions*

Public-private collaboration that includes scientific and/or financial support from both sectors, has been mainly used for :

(i) the different phase of the clinical development (e.g., ivermectin and praziquantel, both originated from veterinary medicine ; and pyronaridine),
(ii) strengthening clinical trials (e.g., development of artemisinin derivatives from Chinese pharmacopoeia),
(iii) manufacturing (e.g., upgrading good manufacturing practices for artemisinin derivatives).

Occasionally, interest in enhancing the public image of industry was decisive, meaning that a pharmaceutical company had an explicit interest in promoting itself as a socially responsible industry (e.g., atovaquone-proguanil, albendazole, ivermectin). Advocacy efforts, coming from civil society, facilitating development and/or distribution were conclusive for some compounds abandoned by industry as insufficiently profitable (e.g., pentamidine). Lastly, regulatory issues (e.g., expiry or absence of patent, patent approaching expiry, fast-track approval) could also provide a programme leverage (e.g., eflornithine, paromomycin).

*2.2. Discrete mechanisms*

These mechanisms pursued by international institutions programmes correspond to support strategies. Technical assistance strategy was the most productive one (e.g., artemisinin derivatives) while donation programmes were commonly used by pharmaceutical industry (e.g., albendazole, atovaquone, azithromycin, ivermectin, pentamidine). Apart from artemisinin derivatives (e.g., artemether), developing country production to lower costs and facilitate a transfer of technology has not been particularly successful (e.g., eflornithine, paromomycin, pyronaridine).

Developing drugs for communicable diseases prevalent in developing countries are activities of primary importance. No global strategy can be drawn from past successes and failures. The dominant approach has been to work on a case-by-case approach with an opportunistic mixing of the above mech-

anisms. With the exception of ivermectin and praziquantel, all examples of successful drug development have been for malaria (e.g., artemisinin derivatives, atovaquone, halofantrine, mefloquine). African and South American trypanosomiasis appear to be marginalized diseases with the likely cessation of production of melarsoprol, nifurtimox and eflornithine. Generally speaking, donation programmes are favoured by industrialists as an easy way to offset market failures, and to compensate for the lack of tropical research and development (R&D) policy.

## 3. Barriers and disincentives limiting drug development

The R&D process is becoming more and more complex and the imbalance observed in tropical pharmacy activities appears to be essentially structural. Four main reasons prevent drug companies from conducting clinical development for tropical diseases: financial costs of R&D, the commercial context, regulatory requirements and patent issues (Table 2).

***Table 2.*** **Conceptual Framework for drug development**

| | **Financial** | **Commercial** | **Regulatory** |
|---|---|---|---|
| Primary disincentive | Costs of R&D | Insufficient market size<br>Patients' inability to pay<br>Pricing pressures | High standards of regulatory requirements<br>Unenforcement of patents |
| Secondary disincentives | Shareholders value | Pressure from generics<br>Lack of secure patents | Lack of local clinical test sites |
| Underlying causes | Pharmaceutical R&D is a business | Political and social instability<br>Global competitiveness | Need for "zero risk" therapies<br>Weakness of pharmaceutical government policy<br>Weakness of local drug regulatory authorities |
| Possible solutions | • Faster, cheaper new discovery technologies<br>• Public-private collaboration<br>• Technical assistance<br>• International cooperation structuration<br>• Drug financing alternatives (insurance schemes)<br>• Creation of R&D innovation center outside the private sector | • Assessment and protection of markets.<br>• Pricing, supply and distribution strategies.<br>• Technology transfer (local development and production)<br>• Rational use of essential drugs | • Patents enforcement (TRIPs agreement)<br>• Compulsory licensing<br>• Market exclusivity<br>• Parallel imports<br>• Accelerated approval process |

*3.1. Financial costs of Research and Development*

The global cost of R&D has been estimated between US$ 224-300 million per successful product (figures include the cost of failures and the cost of capital, adjusted to 1995 dollars), shared between drug industry (mainly involved in clinical trials) and other resources (academia, international organizations for the discovery and research phase).[4,5] However these data are subject to controversy and private industry has much interest in keeping the perceived costs as high as possible.[6] Cost studies are retrospective and can change quickly as underlying scientific, technical or regulatory conditions change. It therefore means little to estimate future R&D costs from past experiences. In making R&D decisions, investors try to predict the possible future outcomes. R&D managers look ahead and do not invest simply because they have the cash on hand, they invest when prospects for future returns look promising, which contradicts the industry's contention that today's profits are needed to fund today's R&D.[7]

Traditionally R&D is considered as a costly, risky and time-consuming business, but it also remains a profitable one: global annual growth has been superior to 6% for many years and economic returns to the pharmaceutical industry exceed returns of other industries by about 2-3 percentage points.[8]

*3.2. Commercial context*

It is estimated that the minimal annual turnover must range between US$ 200-300 million per drug and more than US$ 300 million 3-4 years after introduction of a new drug with a margin superior to 30%. According to industrialists, if a new drug does not have that potential, it will not survive. Apart from malaria and tuberculosis, in all probability the market size of tropical diseases is insufficient on the basis of the current economic rules. Inadequate financing in most developing countries (public financing usually lower than 20%, health insurance coverage lower than 10% in Asia and 8% in Africa, and an out-of-pocket spending of households of 50-90%), the pricing pressure and competitive pressure from generic drugs (a sales decline of over 50% is expected within the first few months of generic entry),[9] are disincentives for any sustainable commitment from pharmaceutical industry.

The current global competitiveness of the industry environment can only amplify such a trend. Western drug companies growing bigger and bigger through repeated cycle of mergers, the target in terms of sales for a candidate product for development keeps climbing higher and higher, industry shifting to the most profitable segments of the market and leaving tropical medicine largely out of the equation.

*3.3. Regulatory requirements*

Regulatory requirements (information required by regulators when evaluating a product for marketing approval) can also be considered as disincentives and vary considerably from country to country. Whether the drug market requires normative measures, according to its content, a norm can favour evolution of one kind of market.[10] The current process of globalization and harmonization of documents (strengthened through the International Conference on Harmonization, ICH), tending to a high level of regulatory standards (Food

and Drug Administration-like system for drug safety and efficacy regulations) can act as a legal barrier and hinder the clinical development in tropical medicine, the case of sleeping sickness treatment being illustrative.[11] Clinical trial size increases from year to year in Western countries while clinical test sites decreases in developing ones. Paradoxically, increasingly demanding standards favour the larger and wealthier companies that are the least interested in tropical diseases.

Nevertheless, dossiers do not necessarily undergo the same level of review the world over, sometimes because of bare bones health budgets, and sometimes owing to a misconception of the regulatory process. If the core mission for all drug regulatory authorities is to promote public health by ensuring the quality, safety and efficacy of pharmaceuticals, the net result is that fewer drugs adapted to the needs of the poor can be anticipated.

*3.4. Patent issues*

Patents and protection of intellectual property rights (IPR) are important issues. The effective patent life (time between approval for marketing and expiration of the last patent) does not seem to still be a disincentive: after declining steadily throughout the 1970s and 1980s, effective patent life is rebounding. On the other hand, the present low level of patent protection in some countries may constitute a dissuasive effect for Western pharmaceutical companies, if resulting innovative products can be freely copied in other markets.[12] The history of development, registration and distribution of praziquantel illustrates the conflicts of interest between public health needs and free-market economy rules.[13] Nevertheless, according to the World Trade Organization agreement on Trade-Related Aspects on Intellectual Property Rights (TRIPs agreement, 1994), IPR must be enforced in all countries by the year 2005.

This enforcement raises certain concerns. Directors of pharmaceutical companies have stated repeatedly that the reason for not conducting development on tropical diseases is the lack of protection for innovation. Logically, drug development for tropical diseases should start again after the enforcement of the TRIPs agreement. However, it is unlikely that Western manufacturers will devote much of their effort to nonsolvent populations, with or without patents.

## 4. Conclusion

Pharmaceutical firms operate like any private industry, they have no specific social welfare mission and respond to economic rather than social or human imperatives. All things considered, drug development for parasitic diseases may not have a promising future in the current context, the profit-driven system beeing unable to keep pace with current and evolving needs in tropical medicine. No international pharmaceutical companies will develop new drugs against parasitic diseases of their own volition and this is not likely to be changed by technical breakthroughs such as the massive accumulation of knowledge in biology and/or the increasing role of instruments and computers in drug research.

When the market does not spontaneously provide the needed treatments, it is the role of society (developing and Western countries governments, international institutions) to take appropriate steps. There is much work to be done in lowering some of the market barriers (i.e., purchase funds, alternative routes of marketing and distribution, pricing strategies, essential package of health services definition), in creating mechanisms to incite industry interest (i.e., orphan drug schemes for rare diseases).[14] There is clearly room for new approaches and what is not appealing to the Western drug industry may well be suited to small to medium sized start-up companies, particularly in advanced developing countries.

For the most part, answers and solutions to these issues of drug development belong to developing countries provided that the rules and means are well and fairly shared.

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# Role of endemic countries in research and development

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***Abstract :*** *In the context of parasitic diseases, the ultimate aim of research and development is to reduce suffering and death from the diseases. For many years to come, for most parasitic diseases, this process will continue to rely heavily on drugs. The occurrence of resistance of the parasite is a constant threat. There is therefore the need for both a rational use of available drugs and the development of new compounds.*

*Endemic countries are facing the challenges to establish a regulatory system for good pharmacy practice; to define effective quality control mechanisms and to define mechanisms for surveillance of drug efficacy and resistance. But above all in the short to medium term, regional and international concerted actions are needed to take most of the process of R&D of anti-parasitic drugs to endemic countries. This is the prerequisite for the availability of good quality and effective drugs at affordable price at the most peripheral level of the health care system.*

## 1. The burden of parasitic diseases

The ultimate aim of Research and Development on parasitic diseases is to reduce suffering and death. The current morbidity and mortality figures are unfortunately not bright. It is estimated that about 2 million people are dying each year from malaria, 150 000 from African trypanosomiasis and 80 000 from leishmaniasis; while 200 million are infected with schistosomiasis and 120 million with lymphatic filariasis.[1, 2] No vaccines are available so far for any of these diseases; for many years to come, control strategies will continue to rely heavily on chemotherapy. Unfortunately currently available drugs are far from offering a definite solution to the problem. They all suf-

fer from either an unacceptable toxicity, poor efficacy or from an increasing drug resistance. Chloroquine is so far the most cost-effective drug against malaria; but there is clear evidence that the spread of resistance is causing an increasing malaria morbidity and mortality in Central and West Africa.[3] Praziquantel is still highly effective against all species of schistosomes, but it has been the only drug used during the last 15 years to control schistosomiasis in 40 out of the 46 endemic countries in Africa; and there is no new drug in the pipeline.[4] For onchocercasis and lymphatic filariasis there is no macrofilararicide available. Ivermectin is a good microfilaricide, but for more than 10 years it has been the only drug used in large-scale programmes by the Onchocercasis Control Programme in West Africa (OCP) and is now the only drug used by the African Programme for Onchocercasis Control in Central and East Africa (APOC).[5] Fortunately another microfilaricide, moxidectin, is under serious consideration and offers a hope of a back-up. Praziquantel and ivermectin are still considered as newly discovered drugs even though they are nearly 20 years old and the occurrence of resistance is a constant threat. Most of the other antiparasitic drugs, antimonials for leishmaniasis, suramin and pentamidine for African trypanosomiasis were discovered more than 50 years ago and suffer from toxicity, poor efficacy, high cost or the inconvenience of their administration by injection. This low speed of the research and development process is mainly due to the reluctance of industries in investing in the development of the so-called orphan drugs, simply because there is no prospect of a good financial return.

## 2. The socio-economic situation in endemic countries

Most of the endemic countries with high incidence of parasitic diseases are the highly indebted ones with obviously very limited purchasing power; 60-70% of the people live below the poverty line.[6] Clearly only those drugs that can be made available on the market at an affordable price will be useful to those who are most in need. Therefore, the real value of any research and development that would lead to the production of a very effective and safe drug will still depend on its affordability in endemic countries.

In many developing countries, health-seeking behaviour is related to socio-economic conditions. The usual first contact of patients is with traditional medicine; 54% of the people in a peri-urban area of Mali will first use self-medication, either in the form of a family remedy or modern drugs purchased from a local dealer.[7] Most of the patients will go to see health personnel only at an advanced stage of a disease. This situation is well known to health authorities of many counties; this is the main reason for the current initiative in improving traditional medicine and integrating traditional healers in the health system. In countries like China, India, Côte d'Ivoire and Mali, to name a few, a great emphasis has been put on the identification of natural sources of compounds through screening of medicinal plants. This is clearly a phase of the R&D process where scientists in endemic countries have been playing a significant role. Several initiatives have been developed and have led to good partnerships with northern scientists. The Department of

Traditional Medicine in Mali and that of Côte d'Ivoire have been developing strong links with Italian and German institutions on the development of anti-malarial drugs from medicinal plants.

These partnerships, however, have not helped in building the capacities of the developing country's institutions to enable them to go beyond the identification of the natural source of compounds; instead, there is a confinement in the production of so-called "improved traditional drugs".

In the R&D process, partnership has also been developed in the improvement of the role of several non-human primate centres in endemic countries. Under the INCO Programme of the European Commission, a concerted action has been initiated between the Biomedical Primate Research Centre of Rijswijk in the Netherlands, the Primate Centre of the Cali Immunology Institute in Colombia, the Oswaldo Cruz Institute in Brazil, the Institute of Primate Research National Museums of Kenya, The Guangdong Shunde Institute of Laboratory Animals in China and the International Centre for Medical Research in Gabon. This initiative has helped in harmonising the involvement of the primate centres in malaria and schistosomiasis drug and vaccine research. Based in endemic countries they could play a significant role in R&D; but they would need a lot of support and a lot more resources to build national capacities in order to be sustainable.

Scientists in endemic countries have a major role to play at the clinical phase of the R&D. Undoubtedly, their prime role would be the selection and preparation of suitable sites for drug or vaccine testing, taking into account the social and cultural perception of the population. Unfortunately, the capability of Developing country's scientists in setting and monitoring Good Clinical Practice for drug or vaccine trials needs further strengthening; and, together with their northern partners, they also need to be able to harmonise ethical issues in developing countries with Good Clinical Practice requirements. The Good Laboratory Practice and Good Clinical Practice training programme initiated by WHO/TDR hopefully will fill this gap. This programme should strengthen the capability of the institutions in the concept of product discovery and development, design and conduct of clinical trials.

When a drug is ready for the market, endemic countries have a very important role to play. This is the time when policy decisions should be made and their implementation carefully followed up. It is the responsibility of endemic countries to establish a regulatory system for Good Pharmacy Practice, with a clear definition of drug policy, including, the role of the private and public sectors, the rational prescription and use of drugs and a well-controlled self-medication. A very important role they would also have to play is the definition of mechanisms for quality control of the drugs used by the population; this includes the process of ensuring good storage and packaging conditions and the identification of defective drugs and counterfeit products. Last but not least, it is the role of endemic countries to define guidelines for the surveillance of drug efficacy and the occurrence of eventual resistance of the parasite. This could be specific to country or to region, as it obviously will depend on the organisation of the health care system.

## 3. The challenges

A commitment for long-term investment is needed to bring any new compound from the bench to the market. It is commonly estimated that this process takes about 10 years to be completed and could cost more than US$150 million (8). This is clearly out of the reach of endemic countries, even the most advanced ones. In the R&D process of anti-parasitic drugs there are many challenges.

In the short to medium term the main challenge is to get these so-called orphan drugs produced by industries in endemic countries. This would mean the development of a real partnership on the one hand between research institutions, and on the other between industries. This partnership should aim at strengthening the capability of endemic country's research institutions and at going beyond the identification of natural sources of compounds towards the transfer of the technology to qualified personnel and industries for an irreproachably high-quality science. A great emphasis will have to be put on the training of developing country's scientists to a high level, on all the processes of drug discovery.

However, what is urgently needed is to ensure:

- The establishment of a regulatory system for rational use of existing drugs. This would mean the definition a clear drug policy with Good Pharmacy Practice, the role of the private and public sectors, the rational prescription of drugs and a well-controlled self-medication.
- The development of proper storage and packaging facilities and an effective quality control mechanism for the detection of counterfeit and defective drugs.
- The establishment of an effective mechanism for surveillance of drug efficacy and resistance. This would mean strengthening the capability of research institutions to undertake this task.

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# Patent policy and stategies for tropical diseases

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***Abstract :*** *Patents are often a necessary prerequisite for development of new medicaments and their commercialisation. A patent policy needs to be part of an overall project strategy. The decision for filing a patent application may follow considerations on novelty, inventive step and utility of the invention, evaluation of economical potential, product lifetime, investments needed, project risks and third party patent rights. A number of technical patent issues have to be considered and assistance by patent experts are often necessary. Patenting of potential drugs for tropical diseases are furthermore complicated by the fact that patients are often from low-income countries making traditional income estimates less useful. International agencies may play a key role in formulating strategies to overcome this barrier ensuring effective drugs for all in future.*

## 1. Introduction

Patent protection is often a necessary prerequisite for the development and commercialisation of a new medication. A patent is a country-specific and time-limited right to prevent other parties in exercising an invention. This monopoly may ensure a return of an investment for a company developing and commercialising a product.

From the moment a new invention is made, a patent strategy needs to be considered. The inventor needs to decide whether the invention could have a specified usage e.g. lead to new therapeutics or diagnostics and whether it should be commercialised. Alternatively, the inventor may decide not to proceed with commercialisation of the invention and publish the invention. Patents may ensure the development of new medicines, and can be used as a strategic tool for revenue generation by out-licensing or sale of patent rights. If an inventor prefers commercialisation of an invention, then the

inventor needs to consider the expected lifetime of the invention, the economic potential of the invention and, if possible, the size of the necessary investment in order to evaluate whether there is basis for a patent application. The decision should be carefully considered as the patent filling procedure is both costly and time consuming. These considerations may include:

- evaluating novelty, inventive step and utility,
- evaluating economical potential,
- evaluating product/technology lifetime,
- evaluating investment needs and risks, and
- evaluating state of the art for third party patent rights.

## 2. Technical aspects of a patent

A patent application needs to be filed before any publications, presentations etc. The text of a paper intended for a journal can often be a good starting point for writing a patent specification but the usual structure of a scientific paper is markedly different from the typical structure of a patent application.[1]

A patent application contains among other things a description of the invention (specifications and drawings) and a number of claims which defines patent rights. The description should enable third party persons to perform the described experiments and describe potential applications of the technology. The claims are the enforceable components of a patent application and should, therefore, be as broad as possible to guarantee an extensive application of the technology. There are a number of prerequisites for getting patent protection of an invention including novelty (the invention should be new and different), utility and evidence of a sufficiently inventive step (i.e. non-obviousness and not previously predicted).

There are international conventions ensuring that the inventor may get priority to the invention. The Paris Convention laid the foundation for national treatment of patent applications, right of priority and some common rules. The Patent Co-operation Treaty (PCT) ensures a centralised patent filing process and evaluation prior to the filing of national applications. A considerable part of the patent costs are thereby being delayed giving the applicants more time to consider their position. The Trade-Related Aspects of Intellectual Property Rights (TRIPS) provides a minimum standards agreement in national legislation including a minimum of 20 years duration of patent protection from filing. Despite a TRIPS patent, legislation varies between countries and consultation with patent experts may be necessary to ensure an optimal patent strategy.

Pharmaceutical patents may include product, process, composition and use claims. Equipment and devices may also be patented. A patent inventor must have made a substantial contribution to the conception of the invention and to the practical application of the conception.

## 3. Patent filing strategy

A patent portfolio would normally be developed by filing first in the inventors home country, then filing a PCT application within 12 months retaining the earlier filing data, and then ultimately splitting the PCT application out at 30 months into an EPO patent application and national applications in the US, Japan and as many countries as possible justified by the potential importance (see Table 1). US patent law varies from the law of other countries. Patent rights are awarded according to the "first to invent" principle in the US, while patent rights are awarded according to the "first to file" principle in other countries. In the US, an applicant can file a patent application up to one year after the invention was made public (publication, etc.) while this grace period does not exist in many other countries. Professional assistance in filing patent applications is strongly recommended in order to assure optimal patent protection. Patent filing strategies have been discussed in the literature.[2,3]

***Table 1.*** **Example of a simplified patent life cycle**

| Time horizon | Patent action |
|---|---|
| | Filing of patent application in home country |
| 12 months | Filing of PCT patent application |
| 18 months | Publication of PCT patent application |
| 30 months | Filing of national patent applications |
| 2-5 years | Award of national patents |
| 20 (21) years | National patents expire |

The total period of exclusivity may be prolonged by various means including building a patent portfolio where product patents may be followed by later filed process patents and line extensions (formulation, device). Supplementary protection certificates adding a few years of exclusivity may be sought for pharmaceutical patents.

A number of technical questions need to be addressed if the inventor decides to proceed with a patent application, including the scope and type of claims and in which countries to seek patent protection. A publication strategy (when, what and why) besides the patent strategy is often appropriate and third party patent right needs to be monitored. Target countries for patent filing must have a large enough market for the invention to generate a profit (now or in the future) and a legal system which is strong enough to support the patent (now or in the future). A minimum filing in European countries, USA, Japan, Canada and Australia can be recommended. The inventor may have some difficult considerations for filing a drug patent in countries where a parasitic disease is endemic.

For a PCT filing, the cost typically amounts to £2 500-3000.[4] Costs for patent fees in the US, Europe and Japan may exceed 50 000 US$ for 10 years. In addition, there are expenses for translations and patent attorneys. These fees may easily double the official fee costs.[4] The cost will depend on complexity of the application and the number of designated countries. Finally, the process is often time and resource consuming for the inventor. Many inven-

tions are conceived in a collaboration involving different institutions. Proper and consistent ownership should be ensured from the start. Transfer, license and litigation rights should be regulated in agreements. Patent protection in collaborative research has been discussed in the literature.[5]

## 4. Licensing of patent rights

A sound patent policy can only be formulated if a complete project policy is formulated including a strategy for commercialisation and financing of the project. Patenting is a dynamic process with a continued resource demand, and patent-related work also continues after filing of the first patent application in order to secure a proper management of the patent portfolio. Thus, making a patent policy will be a major challenge for most scientists, yet it is a prerequisite for development of new therapeutics against tropical diseases. Specific questions need to be considered in the policy drafting when the work is related to tropical diseases where most patients will suffer from poverty as well.

One of the main obstacles for attracting company interest in developing new antimalarial medicines is the low economy valuation of the market. An international effort to make a more precise market estimate would benefit all parties. Such a market report should be credible and made freely available to all interested parties. People from economically poor countries need specific support to have access to new drugs. International agencies may play a key role in this respect.

## 5. Conclusion

Licensing of patent rights to companies or attracting venture capital may ensure development of the invention and will be crucial to most public inventors to cover patent expenses which may be substantial. Timing of licensing activities is important, price and risk consideration will depend on the stage of development of the invention. A number of topics will have to be negotiated when licensing patent rights, including: scope (patent portfolio or specified product or indication); territory; exclusivity; development and marketing obligations; party involvement; rights to new related inventions; economic conditions; duration; and legal aspects.

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SECTION II

# How to discover novel targets for pharmacological intervention

# Entering the post-genomic era of malaria research*

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***Abstract :*** *The sequencing of the genome of* Plasmodium falciparum *promises to revolutionise the way in which malaria research will be carried out in the future. Beyond simple gene discovery, the genome sequence will facilitate recently developed global analyses that will comprehensively determine parasite gene expression as a result of development, pathology and in response to the environmental variables such as drug treatment and host genetic background.*

*This paper reviews the current status of the* P. falciparum *genome sequencing project and the unique insights it has generated. Furthermore, the application of bioinformatics and analytical tools that have been developed for functional genomics is summarised. The aim of these activities is the rational, information-based identification of both new therapeutic strategies and targets based on a thorough insight into the biology of* Plasmodium spp.

## 1. Introduction

The development of genome sequencing technologies over the last five years has resulted in a wealth of sequence information, culminating in the recent announcement of a working draft of the human genome. Pathogen genomes, through their smaller size, have been even more tractable to these methodologies and are now well represented in genome science. Although not always

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suited as 'model' organisms, their importance in medicine and agriculture has made the exploitation of the sequence databases a high priority. But what does the production of long strings of A's, C's, G's and T's actually mean in terms of the alleviation of the burden of disease, particularly in developing countries. Genome sequencing has largely been the province of the developed world, where the resources and science infrastructure have allowed the formation of high-throughput sequencing centres. However, the use of sequencing information need not be restricted in this way, provided that resources for training can be met.

Probably the most important aspect of the post-genomic era (in other words, after the sequencing has been done) is the analysis of the primary sequence data. This has been called Bioinformatics and embraces a range of theoretical analyses aimed at converting the DNA sequence into biological information. A major part of this discipline involves the identification of genes through a number of processes from identifying similarities with previously identified genes from other organisms, to the use of computer-derived models based on existing data. Beyond this come predictions of biological function and molecular shape (structural genomics), both of which have scope for development.

Having the whole sequence of a pathogen genome also allows researchers to investigate the behaviour of organisms on a much broader basis than has previously been possible. Now, instead of studying the effect of drug treatment or differentiation on one or two genes, it is possible to study variation in all the genes at the same time using global transcriptional analysis. Equally, protein patterns may also be examined or the organism genetically modified (transfection), providing a direct link between these biological effectors and the gross phenotype. The technology for these experiments has been developed as a direct consequence of the desire to exploit the genome sequence data.

It is not hard to envisage that the ability to identify and characterise the genetic blueprint of pathogens will help us to recognise critical elements in the development and pathogenesis of disease-causing organisms and use this information to target our research efforts in the production of new therapies. For *Plasmodium falciparum*, genes and proteins acting at specific stages in the life cycle can be identified, their roles tested by genetic modification and used as vaccine candidates. Parasite metabolic pathways not present in the host can be used to design potent inhibitors that are non-toxic to humans or parasite drug-resistance mechanisms can be targeted, giving existing drugs a longer effective lifetime. The sequence of the *Plasmodium falciparum* genome will provide many opportunities for research into malaria, but this is only a beginning, with the challenge being to turn those opportunities into effective treatments in the field.

## 2. The *Plasmodium falciparum* genome

The genome of *P. falciparum* consists of three discrete components; a linear repeat of a 6kb element located within mitochondria, a 35kb circle within a

plastid-like structure (the apicoplast) and 25-30Mb of nuclear DNA (genomic DNA). Nuclear DNA is organised into 14 chromosomes, between 0.75 and 3.5Mb, as determined by pulse field gel electrophoresis (PGFE) and electron microscopic counts of kinetochore structures. Indirect evidence for the number of chromosomes was determined when genetic markers were organised into 14 linkage groups.

The nuclear genome is organised in a manner typical of eukaryotes, with linear chromosomes being bounded at either end with telomeric sequences. Genome plasticity seen in many parasite isolates and identified by size polymorphisms on PFGE, is thought to result frequently from deletions and insertions of DNA within subtelomeric sequences, a region shown to contain ordered repetitive sequence elements.

*2.1. The* P. falciparum Genome *Project Consortium*

Prior to the advent of yeast artificial chromosome technology, there had been relatively little access to the parasite's genome as its extreme AT content rendered inserts unstable in conventional bacterial plasmid clones. The estimated 80% AT-rich genome could, however, be stably maintained within the pYAC4 construct as demonstrated by the construction of a number of YAC (yeast artificial chromosome) libraries for different *P. falciparum* clones.[1] In 1993 a consortium of laboratories, distributed throughout the world, established the Wellcome Trust Malaria Genome Mapping Project with the aim of assembling YAC contigs across each chromosome as well as developing YAC, expressed sequence tag, bioinformatic and genetic mapping technology.[2] This consortium realised that sequencing of the entire nuclear DNA was a real possibility yet considered the endeavour fraught with difficulties due to the extreme bias in base content.

Complete genome sequencing has proved to be a powerful and efficient approach in accessing the complete gene complement for organisms as diverse as *Mycobacterium tuberculosis*[3], *Saccharomyces cerevisiae*[4] and *Caenorhabditis elegans.*[5] The advantages offered by such a tool in the investigation of human malaria eventually resulted in pilot projects being established in 1996 at three high-throughput genome centres, the Sanger Centre (UK), The Institute for Genomic Research (TIGR, USA)/Malaria Program, Naval Medical Research Center (NMRC) and Stanford University (USA) to establish whether sequencing the entire genome was possible. The Wellcome Trust, the Burroughs Wellcome Fund, the National Institute of Allergy and Infectious Diseases and the US Department of Defence provided funding for the pilot projects, and following their success these agencies agreed to fund the entire sequencing effort (see Table 1 for progress).[6] Associated with this work are a number of other groups supporting the efforts of the high-throughput centres in a range of activities including generation of chromosomal material, additional mapping information, testing bacterial strains more tolerant of AT-rich DNA and the provision of a repository for *P. falciparum* reagents (MR4, **http://www.malaria.mr4.org/**).

***Table 1.*** **Progress Summary for the Malaria Genome Project**

| Chromosome | Size (Mb) | Status |
|---|---|---|
| 14* | 3.4 | Late Closure |
| 13† | 3.2 | Closure |
| 12# | 2.4 | Late Closure |
| 11* | 2.4 | Closure |
| 10* | 2.1 | Closure |
| 9† | 1.8 | Shotgun in progress |
| 8† | 1.7 | Shotgun in progress |
| 7† | 1.7 | Shotgun in progress |
| 6† | 1.6 | Shotgun complete |
| 5† | 1.4 | Closure |
| 4† | 1.2 | Late Closure |
| 3† | 1.06 | Finished |
| 2* | 0.95 | Finished |
| 1† | 0.7 | Late Closure |

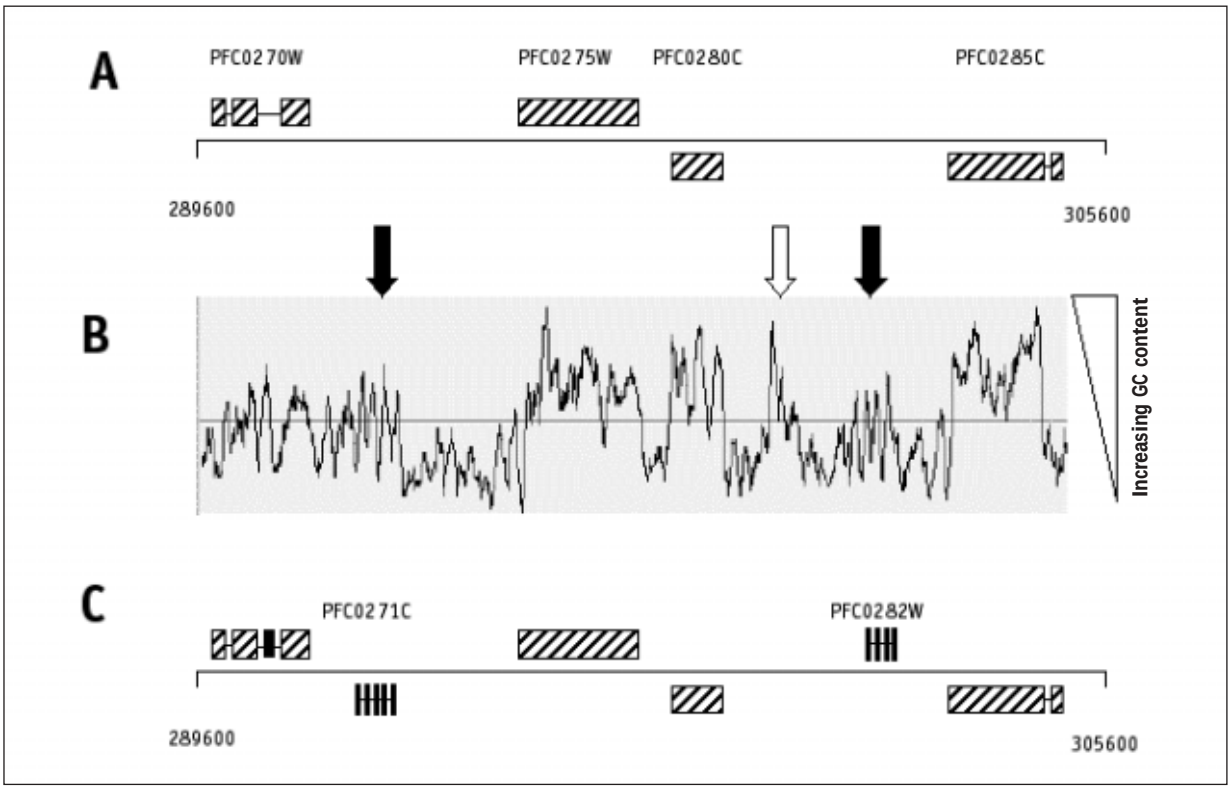
**The sequencing centres responsible for each chromosome are designated; *The Institue for Genomic Research/ Malaria Program, Naval Medical Research Center; †The Sanger Centre; # Stanford University**

A similar strategy is being used by all of the high-throughput sequencing centres, with individual chromosomes being excised from pulse field gels, cloned as small inserts into a double-stranded vector, and sequenced in the forward and reverse directions to generate read-pair information which is used in gap-filling. Sequence-tagged site (STS), simple sequence-length polymorphism (SSLP) microsatellite markers from the HB3xDd2 genetic cross,[7] together with the optical map[8] of ordered restriction fragments are used to position contigs on each chromosome, or to confirm that sequence data has assembled correctly. In addition, the groups at the Sanger Centre and Stanford use a shotgun skim (1-2 fold coverage) of YAC clones selected from the chromosomal YAC contigs generated by the original *P. falciparum* mapping project to help assign and order sequences originating from a particular chromosome region. All three sequencing centres aim for an error rate of less than 1 base in every 10,000 bases.

Once a section of chromosome sequence is finished it is analysed to identify a number of features including putative protein-coding regions, tRNAs and repetitive sequences. Database searches are performed to identify similarities to protein and expressed sequence tag (EST) sequences. Further analyses were performed in order to determine whether predictions have protein domains, signal sequences, putative membrane-spanning regions or any other distinctive features. A number of computing tools such as Hexamer/ Genefinder (R.Durbin; P.Green and L.Hillier, unpublished software), and GlimmerM[9] are available to assist in the identification of protein-coding regions, but many other gene prediction programmes exist or are currently being developed. These tools are efficient at identifying large single open reading frames, or genes with two or three relatively large exons. However, when predicting multi-exon genes, particularly those with small exons, the current generation of gene prediction programmes produces several different

gene models, with different programs producing conflicting data. [10, 11] These conflicting gene predictions are currently being tested experimentally by reverse-transcription PCR (RT-PCR) (Figure 1). Annotation of current and future *P. falciparum* sequences is therefore an ongoing process, with predictions and annotations being refined as more information, such as the RT-PCR and EST sequencing data, becomes available.

***Figure 1.* Testing gene models in *Plasmodium falciparum***




**Annotation of the *P. falciparum* genome is a continuing process. A region on chromosome 3 between bases 289600 and 305600 was initially shown to contain 4 ORFs (Fig. 1A, PFC0270W to PFC0285C (hatched boxes)). Analysis of the GC-content of this region (in overlapping 100bp segments) using the Artemis viewer indicated three further regions of higher than average GC-content, shown in Fig. 1B by the black and white arrows. Subsequent RTPCR analysis of asexual parasites identified two further genes (Fig. 1C, PFC0271C and PFC0282W (filled boxes)) as well as a modification to the PFC0270W gene model. Further analysis using other developmental stages may identify further genes, particularly associated with the GC peak shown by the white arrow.**

*2.2. Analysis of the* P. falciparum *genome*

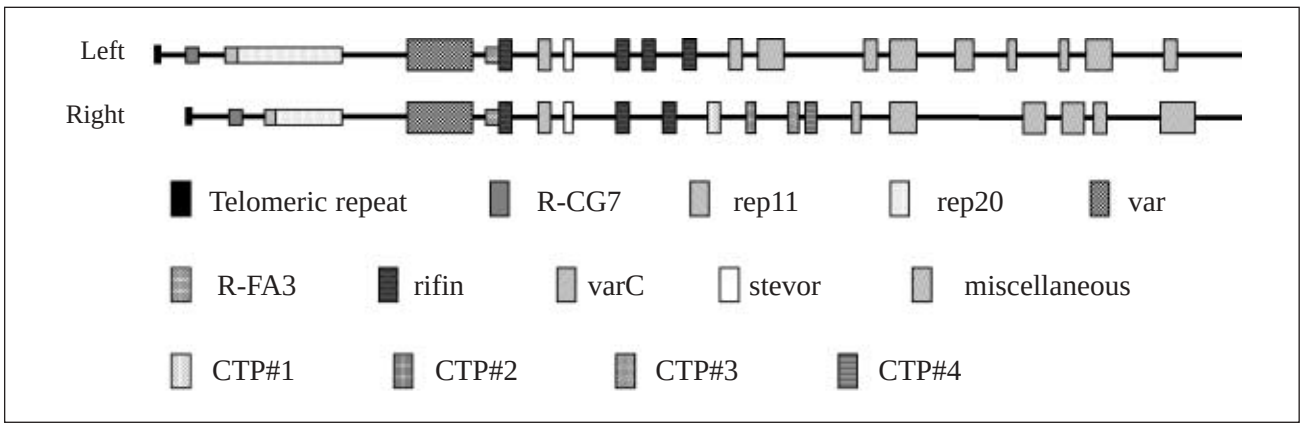
A total of 424 predicted protein-coding genes and 3 tRNA genes have been identified on the two chromosomes, 2 (TIGR/ NMRC)[12] and 3 (Sanger Centre),[13] completed to date. Approximately 37% (158 genes) of these genes have a readily identifiable homologue in another species. Such similarity data allows a function to be implied, with many of those identified being involved in parasite metabolism. Interestingly, comparing the sequence of the predicted proteins to their homologues in other species showed that the majority of *P. falciparum* proteins have insertions of low complexity sequence, often runs of a single amino

acid residue (typically asparagine, lysine or glutamic acid) or tandem arrays of a short peptide repeat sequence. Examples of such regions have been identified previously, and are polymorphic between different parasite isolates.

*P. falciparum* contains two organelles thought to have arisen through endosymbiotic events, mitochondria[14] and apicoplasts.[15,16] The unique nature of the apicoplast and the essential functions that it carries out make it a prime candidate for antimalarial drug development. Like many extra-nuclear elements, many of the genes for the organelle function have become nuclear-encoded. Examination of the predicted proteins encoded on chromosomes 2 and 3 identified several that have a putative apicoplast signal sequence. This information indicates that the apicoplast contains type II fatty acid synthase systems, typically associated with bacterial and plant plastids.[17,18] This observation is interesting in that it supports the hypothesis that the apicoplast is algal in origin and provides a very specific target for rational drug design. Other proteins likely to play a critical role in parasite metabolism are also easily identified from searches of the *P. falciparum* databases. Using this approach, Jomaa et al[19] identified two *P. falciparum* proteins with similarity to enzymes involved in the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway of isoprenoid synthesis, which is used in green algae and some bacteria, but not in animals. Antibiotics developed to inhibit this pathway have already been shown to have antimalarial activity in a rodent model system and *P. falciparum* in vitro culture.

Database searches allow the 'virtual' identification of falciparum homologues to proteins from other species (see above) but comparative analysis of sequence organisation[12, 13] has played a significant role in revealing subtelomeric coding sequences which would have otherwise gone unnoticed. A closer examination of the four *P. falciparum* subtelomeric sequences available shows that the order of both repetitive sequences and the variant multigene family members are conserved (Figure 2). Members of the var, rif, stevor and Pf60 multigene families are present at all four telomeres, and new telomere-associated multigene families have also been identified (conserved telomere-encoded proteins or CTPs). While var, Pf60 and stevor had already been relatively well-characterised, rif (repetitive interspersed family) clearly required a more detailed analysis.

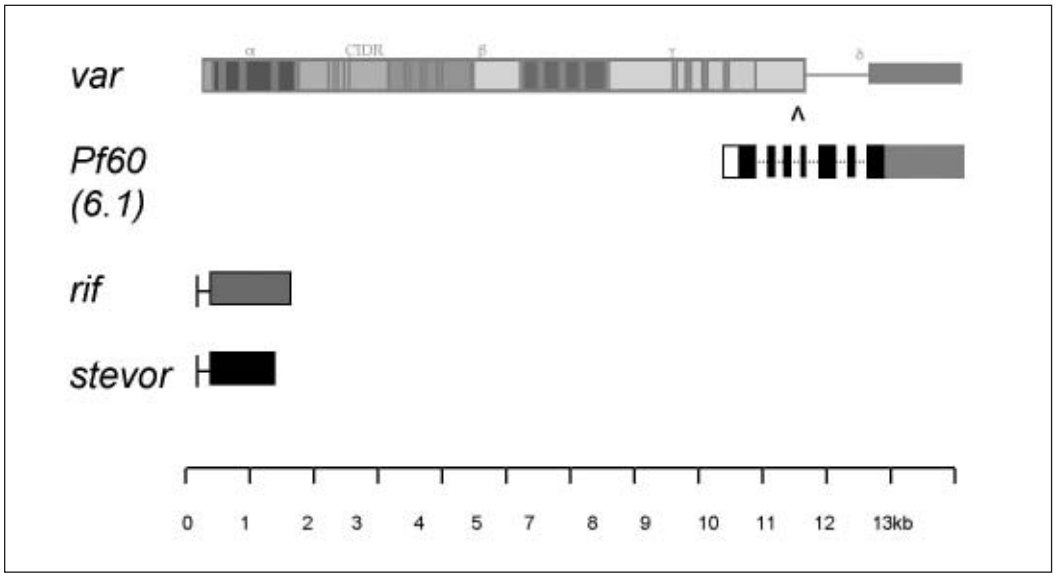
***Figure 2.*** **Sub-telomeric organisation of gene families in *Plasmodium falciparum***



**The telomeres of chromosome 3 are shown, with sequence repeats and multigene family members indicated as shaded boxes. A comparison of chromosome ends demonstrates that the order of tandem repeat sequences is conserved, although copy number can vary. Some conservation in the order and orientation of multigene family members can also be seen. The CTP genes (conserved telomere-encoded protein) are highly similar to a set a genes seen in the right subtelomeric region of chromosome 2.**

Var comprises a highly polymorphic family of roughly 50 copies per haploid genome.[20, 21, 22] It codes for PfEMP1 (*P. falciparum* erythrocyte membrane protein-1) variants expressed on the surface of the infected red cell from the late ring stages through schizogony. Each var gene is composed of two exons (Figure 3). Exon1 codes for the highly variable extracellular portion of PfEMP1, including between one and seven[23] Duffy-binding-like (DBL) domains, with at least one cysteine-rich interdomain region (CIDR). The 3' end of exon1 codes for the semiconserved transmembrane region, and exon 2 codes for the semiconserved cytoplasmic region of the protein. PfEMP1 is involved in cytoadherence of infected red cells to a range of different host receptors on endothelial cells and in binding to uninfected red cells, forming rosettes. The cytoadherent or rosetting phenotype of an individual parasite infected red cell has been correlated with severity of disease and disease pathology (reviewed in [24]).

***Figure 3.*** **Exon/Intron structure of sub-telomeric genes**




**Schematic of exon-intron structure for the two superfamilies, var/Pf60 and rif/stevor.This example of var exon1 contains four DBL-domains (indicated a, b, g, d) and one CIDR region. Regions of semiconserved sequence are indicated with dark-grey bars, otherwise the sequence is highly polymorphic. Transmembrane region indicated (^). Pf60 type 6.1 has 7 exons, the 7th being highly similar to var exon2. rif and stevor are each composed of two exons, and are of similar size.**

The Pf60 family members contain a 3' exon highly similar to the var exon2 sequence 25 26, and have multiple possible 5' exon organisations. One Pf60 protein, 6.1, is expressed in the nucleus of late asexual blood-stage parasites, is composed of 7 exons, and employs a mechanism for reading through an internal stop codon 27. Hybridisation-based analysis of Pf60 suggested 140 copies

per hapoid genome, but as the sequences are highly similar to var, an adjusted estimate would be roughly 90 Pf60 genes per haploid genome.
Rif was originally described as a ~1kb DNA fragment repeated in the falciparum genome, an anomalous open reading frame with no logical methionine start site.[28] There was little point in trying to hunt for upstream exons, since the dogma at that time was that most falciparum genes did not have introns. The sequence was spotted in between two var genes 22, but eventually, from early releases of genome sequence, rif was recognized as a relative of stevor.[29] This analysis identified the small upstream exon for both stevor and rif sequences (Figure 3), and showed that both sequence types contain predicted transmembrane regions which would orient the predicted protein as a loop on the outer surface of a cell membrane. The rif sequences are distinct from and much more polymorphic than stevor, with an estimated 200 copies of rif per haploid genome 13,[30] and roughly thirty-four members of the stevor family.[29] To date, it is uncertain where and when the stevor proteins are expressed,[31] although the relative lack of polymorphism suggests that they are not likely at the red cell surface. However, for rif, the large number of highly polymorphic copies suggested a location exposed to host immune/selective pressures. Indeed, it was shown that rif sequences code for clonally variant 35-44kD RIFIN proteins expressed on the surface of the infected red blood cell,[30] and that antibodies to the proteins are detectable in sera from immune individuals.[32] Although RIFINs and PfEMP-1 share the same cellular localisation, RIFIN function remains unclear. While var genes are transcribed during all ring stages of development, and rif genes are only expressed for a short time at the late-ring/early pigmented trophozoite stage, both proteins are detected on the red cell surface at roughly the same time (early trophozoites) (25).[33] Further studies are necessary to determine whether these proteins are functionally linked as well as physically linked within the genome.

The final gap to be sequenced on chromosome 3 covered a region of extreme [A+T] composition; 97.3% for 2.6kb. Subsequent comparison to the sequence of chromosome 2 identified a region similar in both composition and length. Both [A+T]-rich regions occur in gene-sparse areas of the chromosomes, in fact forming part of the longest intergenic region on chromosome 3. Closer analysis revealed the presence of chromosome specific repetitive sequences. Thus, their structure and extreme [A+T] composition suggest these regions as candidate centromeres. [A+T]-rich central cores are present in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* centromeres and the latter contain complex, chromosome-specific sequences. Although, to date, these regions have not been demonstrated to function as *P. falciparum* centromeres, a third example of this structure has recently been identified on chromosome 1.

## 3. Functional genomics

With an increasing number of pathogen genome sequences available, the impetus for "global" investigation has become stronger. The paradigm for these studies has been set by the yeast research community who have had access to the full genome sequence of *Saccharomyces cerevisiae* since 1996.

Although implicit in genomic sequencing, the development of techniques to study functional parameters across whole genomes has evolved into a new field of research termed "functional genomics". For *Plasmodium falciparum*, a number of technical difficulties remain but researchers are already applying functional genomics to the biology of this parasite.

*3.1. Bioinformatics*

The field of bioinformatics covers both sequencing and functional genomics in converting raw genome sequence data into useful information for the biologist. Probably the most important aspects are the search engines that can screen vast amounts of information for significant similarities between sequences and the algorithms used to predict protein-coding regions (see above). For the former, several sites exist on the web that will allow the user to search for homology to a test sequence (see Table 2), returning the results as multiple sets of alignments. Although there are some errors in the defining genes "in silico" it is clear that these techniques have generated an enormous number of potential gene functions that can be tested in the laboratory. Perhaps the easiest form of this are the annotations of complete chromosome sequences that are based on high-quality sequence information. The resulting tables of genes can be divided into those that have confirmed function in *P. falciparum*, sequences with homology to known genes in other organisms, sequences with homology to genes of unknown function in other organisms and sequences specific to *P. falciparum* of unknown function. One of the underlying assumptions is that the annotated sequence contains all the genes and that the predictions for the coding sequence are correct. Whilst this is largely true, there has been some discussion about "missing" genes and the incorrect identification of splice sites (see above). Therefore, an essential part of any bioinformatic system is the provision of software for browsing genomic sequence data so independent laboratories can make their own judgements on coding sequences.

Access to information is possible through the release of sequence data prior to publication, which has already made a significant contribution to malaria research. However, different web sites contain different parts of the sequence information from different chromosomes.

**Table 2. *P. falciparum* bioinformatic web-site URLs**

| | |
|---|---|
| **http://www.sanger.ac.uk/Projects/P_falciparum**<br>**http://www.tigr.org/tdb/edb/pfdb/pfdb.html**<br>**http://sequence-www.stanford.edu/group/malaria/index.html** | sequence data |
| **http://www.PlasmoDB.org** | "official" database of the Plasmodium Genome Consortium |
| **http://www.ncbi.nlm.nih.gov/Malaria** | genetics/ bibliography/ sequence data |
| **http://www.ebi.ac.uk/parasites/parasite-genome.html** | general site/ proteomics |
| **http://sites.huji.ac.il/malaria** | metabolic pathways |

The site at NCBI (see Table 2) has attempted to collate all the available information (as well as that for other Plasmodium species and the related apicomplexan parasite *Toxoplasma gondii*). A recent development, funded by the Burroughs Wellcome Fund, has established a full database (**http://www.PlasmoDB.org**). In its current form, PlasmoDB provides: views of finished and annotated sequence in both web-based and CD format; a relational database that should facilitate entry and analysis of expression data; a BLASTable database containing the most recent genomic sequence information (finished and unfinished); text-queriable results from a complete BLAST search of all *P. falciparum* data against GenBank + EMBL; and various other data-mining tools. In addition to the obvious interest of PlasmoDB to the malaria research community, WHO/TDR has made a commitment to provide on-line 'help-desk' support for PlasmoDB (and other parasite databases) through training of scientists/bioinformaticians from developing countries.

*3.2. Microarrays*

Microarrays are high-density arrays of DNA targets on glass or filter supports. These have been used in several studies, most notably for *S.cerevisiae*. There are essentially two formats for the DNA targets, namely DNA fragments (usually generated by PCR) or oligonucleotides. PCR-derived targets for *P. falciparum* have been produced from genome sequence data (D. Carrucci, pers. comm.) or by the amplification of sequences from a mung bean nuclease library. In the mung bean nuclease library study 34, arrays were made from a random library and screened with mRNA from asexual (trophozoite) and sexual (gametocyte) stages. The RNAs were labelled with different fluorochromes and allowed to hybridise to the arrays. Genes transcribed during asexual stages were labelled green, sexual stages labelled red and yellow signal (green + red) for genes that were active during both stages. From this single experiment the authors identified several developmentally regulated genes, including some which had been previously identified, confirming the efficacy of this approach. The potential for these types of experiments is great but will require careful data handling and analysis. Hayward et al[34] identified their candidate genes by sequencing the inserts after hybridisation but once the genome has been completely sequenced the address of each ORF (open reading frame) will be stored as part of the array information. An array covering all of the genes in the *P. falciparum* genome in duplicate would have approximately 12,000-14,000 spots, therefore every experiment would generate at least 14,000 quantitative data points per probe, in addition to the array baseline information. It is not hard to see that without a highly efficient database, the efficiency and accuracy of any analysis would be greatly reduced, particularly as transcriptional changes are likely to be defined by 'clusters' of genes[35] which makes the contribution of individual genes difficult to interpret. The database must be accessible by the research community in order to take full advantage of the results and, as seen for *C. elegans* (**http://www.wormbase.org**), a centralised database is essential.

Transcriptional analysis for *P. falciparum* is also being attempted by Serial Analysis of Gene Expression (SAGE;[36] D. Wirth, pers. comm.). This technique uses PCR amplification of mRNA to derive short sequence tags that are

unique to each gene. The tags are concatenated into long strings and cloned into suitable vectors. By sequencing many tags it is possible to not only identify what gene is being expressed but also to quantify the level of expression by recording the number of times specific sequence tags are present. The handling is more complicated than hybridisation to arrays but the major advantage of this technique is that relatively small quantities of material can be used which is particularly important where samples may be limited (e.g. field samples). For both techniques it is important that technology transfer, including not only the mechanics such as robotics and image analysis, but also an appreciation of the assumptions and limitations, is carried out. For example, neither technique can detect the products of alternative mRNA splicing events.

High-density arrays are not exclusively for use in transcriptional analysis but also have applications in genome analysis, particularly as oligonucleotide arrays. The high degree of specificity of oligonucleotide hybridisation allows a high level of discrimination, including single nucleotide substitutions. However their use is precluded prior to the completion of the *P. falciparum* sequencing project due to the cost of the chemical synthesis. One example of this type of approach comes again from *S. cerevisiae*[37] in which allelic variation throughout the genomes of two strains of yeast was identified solely by hybridisation of their genomic DNA to oligonucleotide arrays. The application of this technology to natural populations of parasites will be a powerful tool for molecular epidemiology. However, information from many example *P. falciparum* genomes will need to be collated in order to fully cover the true extent of highly variant and recombinogenic genes such as var.

### *3.3. Proteomics and Structural Genomics*

One of the greatest technical challenges in functional genomics concerns the analysis of protein expression (proteomics). Although genome-wide transcriptional analysis has produced very useful information, it is clear that the correlation between mRNA and protein levels is not perfect.[38] Techniques for the identification of proteins in general have developed rapidly over the last few years, but the major advance with regard to genome sequencing has been the use of mass spectrometry in protein analysis and characterisation through the combination of the ability to define the mass (and therefore amino-acid composition) of peptide fragments with a database of all the available peptide combinations derived from the genome sequence data (see[39] for review). Using these techniques it is possible to identify individual proteins from complex mixtures resolved by two-dimensional (2-D) electrophoresis or chromatography. This, allied to improvements in the reproducibility of 2-D electrophoresis, has facilitated differential analysis of protein composition from two populations of cells. Applications of this technology, for example in the analysis of the effect of drug treatments, have an important place in malaria research. Some practical difficulties also remain, particularly in the area of membrane proteins, which are poorly represented using classical proteomic techniques.[40] However, technology continues to improve (LS/MS/MS[41]) and the reward will be a clear definition of the spectrum of proteins involved in critical biological processes in the parasite.

Protein-protein interactions play an important role in cell biology. The tools to investigate this phenomenon have, again, been developed in yeast through the use of the "two-hybrid" system. In this, ORFs are fused to the Gal4 transcription-activation domain and screened by mating with specific coding sequences fused in turn with the Gal4 DNA-binding domain. Positive protein interactions are scored by the ability of the yeast progeny to grow on selective media. This procedure has recently been expanded to enable researchers to carry out a comprehensive analysis of the protein-protein interactions of approximately 6 000 yeast ORFs,[42] generating novel information as well as confirming previous specific screens. The extension of this technique to pathogen genome research will produce a further layer of functional information.

One of the goals of computational biology is the establishment of predictive biology, such that biological function of a protein will be evident based on primary sequence information. The clearest manifestation of this currently is in the field of structural genomics in which the three-dimensional structure of proteins is studied in the context of the amino-acid sequence. While much still needs to be done, the increasing number of resolved crystal structures for biological molecules has already resulted in the generation of rules/motifs that can be applied to searching for structural information.[43] As more information becomes available, including the integration of functional data in terms of active residues, these computer algorithms will improve.

### *3.4. Metabolomics and Vaccinomics*

One of the many outcomes from functional genomics has been the production of a new vocabulary to cover the many applications of this field. Thus the entire mRNA and protein complement of an organism have been termed the transcriptome and the proteome respectively. In a similar vein, the use of genomic information to facilitate studies of metabolic processes has been called metabolomics.[44, 45] Clearly the use of sequence similarities to identify components of known pathways will have a huge impact on research in this area, but functional screens have also been proposed. For example, by expressing all the predicted ORFs from a genome as heterologous fusion proteins it might be possible to screen for specific enzyme functions. This resource could also be used to determine host protective immune responses through immunisation with protein pools. Related studies have been called vaccinomics and are more closely associated with the use of DNA vaccine plasmids containing ORFs derived from the sequencing project to immunise experimental animals and screen for protection (in the case of pathogens).[46] Not only could this lead to the identification of vaccine candidates, but the resulting sera could also be used to determine the cellular location of each protein.

As the number of genomes that have been sequenced increases and researchers become more accustomed to thinking in "global" terms, the amount of information generated by functional genomics will expand rapidly. The challenges will then be to develop systems to analyse this information and to maintain a focus on the biological issues. Clearly one way in which this will be well served in malaria will be through studies on the par-

asites themselves facilitated by genetic crosses[47] and the technology of genetic manipulation (see below).

## 4. Transfection of *P. falciparum*

Transfection, or the introduction of exogenous DNA into the organism in vivo, potentially provides one of the most powerful tools for the analysis of the parasite's genome, allowing us to specifically address questions relating to gene function.

The ability to modify or disrupt components of the *P. falciparum* genome had eluded researchers for many years prior to the initial demonstration of luciferase expression in the chicken malaria model *P. gallinaceum.*[48] Following this report, transfection of *P. falciparum* was successfully carried out when a plasmid bearing the reporter gene encoding chloramphenicol acetyl transferase (CAT) was introduced into the readily cultured, intraerythrocytic stages.[49] Stable transfection and subsequent integration via homologous recombination into the genome of a plasmid bearing a pyrimethamine drug selectable marker (a mutant dihydrofolate reductase – thymidine synthetase (mDHFR-TS) gene) soon followed.[50] The plasmids described in these initial reports as well as a transgene expression system (expression of a reporter gene from a plasmid stably maintained by virtue of mDHFR-TS[51]) have provided the basis for all the subsequent studies made in *P. falciparum*.

Using both CAT and luciferase reporter genes a series of studies have addressed the structure and function of *P. falciparum* transcriptional units. *P. falciparum* promoters were shown to conform to a classical bipartite structure, a basal promoter being regulated by upstream regulatory factors, with transcript stability directed by 3' regulatory sequences.[52,53,54,55,56,57] Similarly it has also been found that the nuclear context in which promoters are placed play a key role in their function, suggesting that phenomena such as stage-specific gene expression and switching of expression between members of the var multigene family may rely on epigenetic factors such as chromatin assembly.[58,59]

More advanced work, utilising gene disruption and allelic replacement, have formed the basis of a series of recent reports examining aspects of *P. falciparum* biology such as cytoadhesion, gametogenesis and drug resistance. Disruption of the gene encoding the Knob Associated Histidine Rich Protein (KAHRP), located within electron-dense knob structures on the surface of infected erythrocytes show that this protein important in their formation.[51] Follow up studies demonstrated that the knob structure is critical in supporting Pf-EMP1 protein during its interaction with host receptors under fluid flow conditions. Pfg27, a protein expressed early following a parasite's commitment to sexual differentiation, was shown to be essential when disruption of this gene resulted in the abortion of transfectants early in gametogenesis, resulting in highly vacuolated and morphologically disrupted parasites.[60] Using an experimental strategy resulting in alterations to an open reading frame rather than disrupting it, the roles of mutations within genes that confer resistance to a wide range of antimalarial drugs have been investigated.[61] The introduction of mutations, associated with drug resistance in

epidemiological studies, into parasites bearing a drug sensitive background elegantly demonstrate the roles of dihydropteroate synthase in sulfadoxine resistance and P-glycoprotein homologue 1 (Pgh-1) in multi-drug resistance.[62] Moreover, these studies have also been used to demonstrate the reverse situation where mutations of cg2, a candidate chloroquine resistance gene, did not confer resistance in a sensitive parasite background, prompting the investigations resulting in the identification of a more promising candidate (David Fiddock and Tom Wellems, pers. comm.).

The primary limitation with this type of experimental strategy is that the intraerythrocytic stage parasites investigated are haploid. Targeted disruption of essential genes inevitably result in the death of the parasite, moreover, any effect on parasite viability will place that proportion of the population at a growth disadvantage. This may be partially overcome by selection or by the presence of alternative pathways that can overcome the growth disadvantage. Proposals for a consortium of laboratories, similar to the EUROFAN network for *S. cerevisiae*, to systematically "knock-out" every gene identified by the genome project have considered a wide variety of issues such as poor parasite viability, low transfection efficiency, the numbers of laboratories able to create mutants and the unit cost per gene knock-out.[63] Although there are still many issues to be resolved, it is agreed that some form of systematic analysis of the parasite's gene complement should take place. However, whether each laboratory would be allocated a share of the genome or whether a thematic approach (based on a laboratory's interest in a particular subject e.g. erythrocyte invasion, cytoadhesion or gametocytogenesis) is used still remains to be determined.

*4.1. Malaria model systems*

The lack of in vitro culture systems for most *P. falciparum* developmental stages and the ethical considerations necessary for the use of new world monkeys and chimpanzees highlight the advantages offered by model malaria parasite systems. Transfection of animal malaria models has opened up the entire parasite's lifecycle, including those in the invertebrate mosquito host, for critical analysis in a manner not possible with human parasites.[64] Moreover, the efficiency of the transfection process, facilitating double-cross over gene replacements, and the rapid selection of transfectants in vivo have resulted in a rapid accumulation of new insights into *Plasmodium spp.* biology.

Following the first reports of transfection in the rodent malarial model *P. berghei* in 1995[65] disruptions of the genes encoding circumsporozoite protein (CS)[66] and thrombospondin related anonymous protein (TRAP)[67] demonstrated their value in the investigation of function. Apart from their roles in host cell recognition and invasion, new roles in sporozoite formation and gliding motility were attributed to CS and TRAP, respectively. Recurrent biological themes have already been demonstrated, CTRP, a TRAP homologue, has been shown to have a role in ookinete motility and thus ability to infect mosquitoes. The readily available and immunologically well characterised hosts allow a full range of host parasite interactions to be investigated. Following the success of this work, transfection of two primate malaria mod-

els has been established at the Biochemical Primate Research Centre in The Netherlands. Both *P. knowlesi*[68] and *P. cynomolgi*[69] have been transfected with entirely heterologous plasmid constructs bearing a *Toxoplasma gondii* DHFR-TS drug selectable marker controlled by *P. berghei* regulatory sequences. One particular advantage of primate malaria models is that they allow investigation of transfected parasites within both their natural and artificial hosts. Within strict ethical limitations the use of these systems facilitates meaningful analyses of host-parasite interactions and will allow us to examine the mechanisms governing host immune response and provide an experimental model for the evaluation of vaccines.

Animal models share many features with their human malarial counterparts, such as life cycles, host-cell restrictions and immune responses. For example, *P. cynomolgi* infection of macaques is a particularly good model for *P. vivax* infection, sharing preference for reticulocytes and hypnozoite formation.[69] Although we have a substantial pool of knowledge for animal malaria models, particularly with respect to homologous vaccine antigens, we know comparatively little of these systems at the molecular level. Programmes for the sequencing of expressed sequence tag libraries as well as low-coverage genomic shotgun sequencing have already provided a fundamental boost to the application of animal models to human malaria biology and should be supported with larger scale sequencing activities. The capacity of animal models would allow intensive programmatic approaches to gene function through knock-out/tagging to be considered

Where species of Plasmodium such as *P. knowlesi* and *P. cynomolgi* provide models for malaria infection and immunity, the closely related Apicomplexan *T. gondii* provides a model for many aspects of Plasmodium biology.[70] Transfection systems are well developed in this parasite with high efficiency transformation and a number of selectable markers for both positive and negative selection.[71] This experimentally tractable system has been instrumental in tackling issues such as plastid structure and function,[72] host cell invasion, drug resistance, virulence and gene expression. Unlike *Plasmodium spp.* which exclusively integrate DNA molecules through homologous recombination, *T. gondii* also permits non-homologous integration, allowing the entire genome to be tagged for a phenotype-based approach to study gene function. For this Plasmodium will require a transposon based system of simple recognition site specificity, such as the Drosophila mariner element which efficiently transfers into a number of eukaryotic genomes including *Leishmania major*.[73]

Both low and high tech developments are needed to continue a systematic assault on the *Plasmodium* genome, such as improved culture methods of certain human and model bloodstage parasites, gamete development and efficient sporozoite culture. These, together with advances in transfection technology, such as new markers, methods to increase transfection efficiencies and adoption of the Tet-repressor system, will combine to make the use of transfection a more reliable and sensitive tool. However, only the coupling of these advances with the knowledge of the parasite's entire gene complement will allow both to be exploited to their full potential.

**Table 3. Genes modified or knocked out in *Plasmodium***

| ***Plasmodium* gene targeted** | **Phenotype** | **Ref.** |
|---|---|---|
| Pb CS | Inhibition of sporozoite formation, loss of infectivity | 66 |
| Pb TRAP | Sporozoites fail to glide and show reduced infectivity | 67 |
| Pb, Pf CTRP | Ookinetes non-motile and fail to invade midgut epithelia and develop into oocysts | 83 84 85 |
| Pf KAHRP | Infected-red blood cells "knobless" and have reduced binding to CD36 under flow conditions | 51 |
| PFG27 | Total (3' disruption) or significant (5' disruption) inhibition of gametocytogenesis | 60 |
| ***Plasmodium* gene modified** | **Manipulation and Phenotype** | **Ref.** |
| Pf DHPS mutation | Introduction of field-observed mutations give rise to sulfadoxine resistance when introduced | 61 |
| Pf pgh1 replacement | Introduction of field observed mutations give rise to mefloquine, halofantrine and quinine resistance. Also chloroquine in a strain specific manner | 62 |
| Pb TRAP | Replacement with Pf TRAP. Sporozoites can glide, invade salivary glands and are infectious. | 86 |
| Pb TRAP | Replacement with Pf TRAP mutants. TRM- sporozoites do not glide or invade salivary glands but remain infectious. Amut sporozoites do not invade salivary glands but remain infectious | 86 |
| Pb TRAP | Replacement with Pb TRAP mutant. Cyt DS and DL sporozoites do not glide normally, cannot invade salivary glands and are not infectious. Cyt/MIC-2 sporozoites can glide, invade salivary glands and are infectious | 87 |
| Pf Acyl Carrier Protein | Green fluorescent protein tag demonstrates need for bipartite leader sequence to correctly target protein to apicoplast and conservation of leader within apicoplasts | 80 |
| Pf MSP1 | Replaced C terminal with that of *Plasmodium chabaudi* MSP-1 demonstrates conservation of function and potential for immune variation | 80 |

*4.2. Comparative genomics*

Mention has already been made of the difficulties that can be encountered when annotating a complete genome, particularly assigning function to unknown genes and correctly identifying genes that contain numerous exons. Ultimately, the availability of a fully or extensively sequenced genome from a related species will be an invaluable tool that will facilitate an accurate assessment of the coding potential of a genome.[74] For malaria, studies on the chromosomes of the rodent malaria species demonstrated that there was little if any difference in gene linkage[75] and that large conserved linkage groups could be detected between these rodent malarias and *P. falciparum.*[76] Limited but more detailed analysis has revealed that the genome organisation is highly conserved in the internal non sub-telomeric regions of the chromosome.[77, 78] Thus the expectation is that alignment of orthologous chromosome regions will reveal detailed intron-exon boundaries, help identify orthologous, but polymorphic, antigen encoding genes, centromeres and identification of species specific genes. In this last regard the comparison with a more distant apicomplexan genome may also prove valuable. Although gene order may not be well conserved many salient features of apicomplexans will be revealed through genome comparisons, for example the apicoplast.[79, 8,0 81] The ease of transformation of *Toxoplasma gondii* and its suitability for cell biological studies combined with comparative genomics provides an excellent opportunity to illuminate malaria biology, develop drug targets[82, 19] and possibly vaccines.

## 5. Conclusion

The ability to completely sequence genomes has had a huge impact on the way in which scientific research is conducted. As the number of pathogen genomes that have been completed increases, the impetus for the development of techniques to utilise this information has increased. In parallel with the *Plasmodium falciparum* genome project, techniques such as transfection, microarrays and proteomics, as well as bioinformatic analysis, are already being developed and applied to enable researchers to address fundamental biological questions. The combination of these tools is expected to provide a predictive platform from which to launch hypothesis-driven biological research. Central to this expectation is continuing financial support for the further development of such techniques and resources that will exploit the genome sequence. Amongst these requirements are publicly accessible relational database development as well as detailed comparative and survey analyses that will provide an invaluable insight into parasite biology. It must be emphasised that the overall goal of such costly enterprises is to work towards a cure for malaria and the contribution of this technological approach could be immense. The current challenge to the research community is to continue the dissemination of this technology and to focus efforts on key aspects of the parasite biology in terms of the development of new drugs and vaccines.

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# Characterisation and sequencing of the *Trypanosoma brucei* genome

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***Abstract :*** *The nuclear genome of* Trypanosoma brucei *consists of at least 11 diploid megabase chromosomes (1-6 Mb), a variable number of intermediate-sized chromosomes (200-900 Kb) and 50-100 minichromosomes (25-100 Kb). The African trypanosome genome network was formed to coordinate the analysis and sequencing of the nuclear genome and consists of multiple research laboratories worldwide, contributing to different aspects of genome analysis. DNA sequencing is carried out at two high-throughput sequencing centres (TIGR and The Sanger Centre). The availability of the complete genome sequence and continued collaboration on post-genomic analysis will provide researchers with an extensive dataset. Analysis of the open reading frames, enzyme pathways, transcription patterns, etc. should allow researchers to make rational choices of potential drug targets for further experimentation.*

**ABBREVIATIONS**

**Mb**: megabase pairs
**Kb:** kilobase pairs
**TREU:** Trypanosome Research Edinburgh University
**GUTat:** Glasgow University Trypanosome antigen type
**VSG:** variant surface glycoprotein
**EST:** expressed sequence tag
**TIGR:** The Institute for Genomic Research
**GSS:** genome survey sequences
**BAC:** cloned DNA in a bacterial artificial chromosome
**P1:** cloned DNA in a bacteriophage P1 vector
**NCBI:** National Center for Biotechnology Information
**ORF:** open reading frame
**DB:** database

## 1. Introduction

African trypanosomes are unicellular parasites that replicate in the bloodstream and tissue fluids of mammals and in the tsetse fly. They cause disease in humans and their livestock in sub-Saharan Africa. Without treatment the disease is fatal, yet the drugs available for the treatment of human trypanosomiasis are very limited. Most were developed early this century and are not without risk due to their non-specific action in the human body. It is vital to develop drugs with greater specificity and efficacy. One approach to this task is to identify enzymes and pathways that are specific to the par-

asite, and to use this knowledge as a starting point for the discovery of drugs that target the parasite-specific enzyme(s). This was one of the rationales behind the decision to determine the nuclear genome sequence of an African trypanosome. Due to the hard work of a number of scientists, the support of the World Health Organization, increasing pressure from researchers worldwide and the pace of global developments in genome analysis and sequencing, small beginnings led to a coordinated research programme and high-throughput sequencing within three years.

The availability of the genome sequence will benefit many research programmes. Some of these benefits are already obvious, some will only become clear as more researchers begin to use the huge amount of available data. This paper will review the organisation of both the *Trypanosoma brucei* genome and the *Trypanosoma brucei* genome project, providing a guide to access to sequence data in both preliminary and final form.

## 2. The African trypanosome genome network

The *T. brucei* genome network comprises multiple research laboratories worldwide, contributing to different aspects of genome analysis and brought together by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). Membership is dynamic because, as priorities change, researchers with different skills are required. Many researchers contribute to the network without direct funding, and the network is dependent on the support and active participation of the research community. It is based on the commitment of its members to the idea that this huge task will progress most efficiently if the research community can work together.

## 3. The genome project reference stock

There is no perfect isolate that should form the basis of all molecular experiments on *Trypanosoma brucei*. It is also preferable that some genomic analyses are carried out on multiple stocks, for example karyotyping and random gene sequencing. However, we can only aim to sequence one genome in the near future. The cloned *Trypanosoma brucei* stock TREU927/4 (GPAL/KE/70/EATRO1534) was derived from a population of trypanosomes isolated from a tsetse fly in Kenya and was chosen as the reference genome for a variety of reasons, some optimal and some pragmatic.

The original stock exists and the history of its isolation is documented. It was not isolated from an infected human, but laboratory tests indicate that it has intermediate resistance to human serum (C.M.R. Turner, pers comm.). It is pleomorphic and may be replicated as bloodstream or procyclic form in laboratory animals, tsetse flies or in culture.[1, 2] It has been used as a parent in laboratory-controlled genetic crosses with other stocks and cloned hybrids have been isolated,1 allowing the creation of a genetic map. A high-quality, arrayed genomic library of the megabase chromosomal DNA of TREU927/4

already existed,[3] and was used to determine the structure of a megabase chromosome. A culture-adapted line has been generated with greater stability of variant antigen types, and this grows to a higher density in culture (TREU927/4 GUTat 10.1) (C.M.R.Turner, pers comm.). Both 927/4 and its derivative 10.1 may be transformed with foreign DNA. It has the smallest nuclear genome of all *T. b. brucei* or *rhodesiense* stocks examined so far with approximately 10Mb less DNA,[2] reducing the amount of funding and sequencing required quite considerably. However, it is capable of completing the life cycle, indicating that all vital genes are present.

## 4. Organisation of the *Trypanosoma brucei* genome

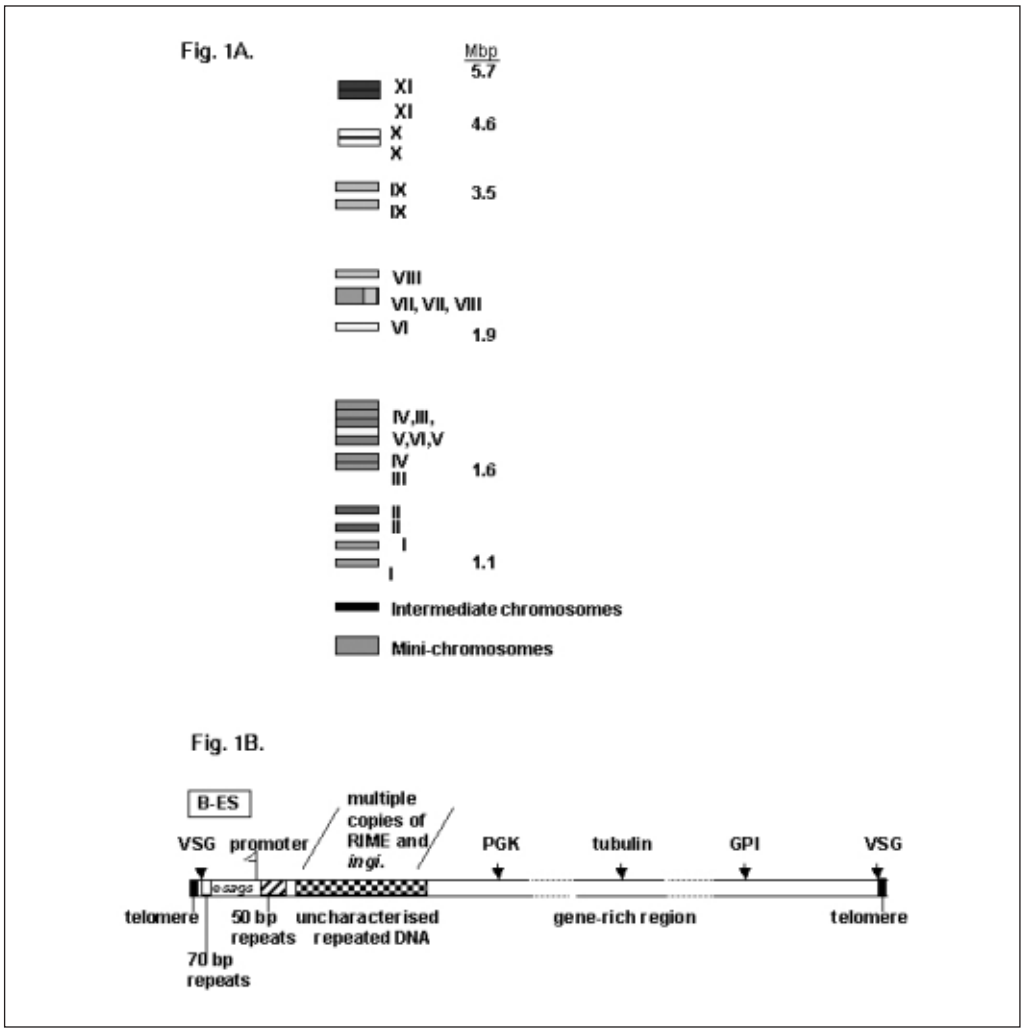
The nuclear genome of *Trypanosoma brucei* contains three size classes of chromosomes.[4] There are at least 11 pairs of diploid megabase chromosomes, ranging in size from 0.9 Mb to over 6 Mb.[2] Most or all of the expressed genes are located in these chromosomes (with the exception of some variant surface glycoprotein genes(VSGs)). Many, possibly most, genes are transcribed in polycistronic transcription units.[5] Some chromosome ends carry VSG expression sites. Only one site is active at one time, resulting in a uniform protein coat on the surface of the parasite. An active VSG may be replaced by a different VSG, or a different expression site may become active, resulting in antigenic variation.[6,7] This is a very effective way for the parasite to evade destruction by the host immune system and results in the characteristic fluctuating parasitaemia and accompanying fever. There are a variable number of intermediate-sized chromosomes of indeterminate ploidy that also carry VSG expression sites,[8] and multiple minichromosomes containing non-transcribed VSG and simple repeat sequences.[9]

The genome network has prioritised the sequencing of the megabase chromosomes, as these contain the greatest variety of genes. However, other studies have provided information on regions of the genome involved in antigenic variation [e.g. [6]], including the mini- and intermediate chromosomes and further investigation of the DNA content of these molecules could be considered in the future.

### *4.1. The megabase chromosomes of TREU927/4*

The chromosomes of African trypanosomes can be resolved by pulsed field gel electrophoresis. The megabase chromosomes are diploid, inherited in a Mendelian fashion[10] and have been assigned Roman numerals in order of increasing size in TREU927/4 (Figure 1).[11] Most of the chromosomes differ in size from their homologues by up to 15%.

**Figure 1**




**A. The molecular karyotype of the megabase chromosomes of genome project reference stock, TREU927/4. The sizes given in megabase pairs are estimated from the relative mobilities of *S. cerevisiae* and *S. pombe* chromosomes in PFGs.**

**B. The structure of chromosome Ia of stock TREU927/4 [14]. B-ES, bloodstream-form VSG expression site; VSG, variant surface glycoprotein gene; esags, expression site-associated genes; RIME, ribosomal inserted (putative) mobile element; ingi, putative mobile element; PGK, phosphoglycerate kinase; GPI, glucose phosphate isomerase.**

*4.2. Size polymorphism in megabase chromosomes*

Homologous chromosomes differ substantially in size between cloned stocks and considerably more than reported in other protozoan parasites. Karyotyping of 12 stocks revealed the greatest size variation in chromosome I, which is 0.9 Mb in one cloned laboratory hybrid and 3.6 Mb in laboratory strain 427. [2, 12, 13] All chromosomes show some variation, yet all cDNA markers remain in identical syntenic groups in all stocks studied.

*4.3. Chromosome structure*

The first chromosome maps of *T. brucei* megabase chromosomes revealed the compartmentalisation of the chromosome into several distinct sections[14]: (1)

sequences associated with bloodstream-form and metacyclic VSG expression sites:
(2) repetitive DNA found on most chromosomes, within which most of the previously described putative transposons (RIME, and ingi)[15, 16] are located,
(3) a gene-rich, non-repetitive section (Figure 1B). Comparative maps of homologous chromosomes from other stocks reveal that all sections show size variation.[14]
Although these (and other) studies have revealed remarkable plasticity of the trypanosome genome, they also reveal a significant level of conservation of genome structure. Initial apprehension that the genome was so polymorphic as to make sequencing of one strain of little use for cross-comparison to other strains have been assuaged. However, further studies should determine what role this remarkable plasticity is playing in the survival strategy of the trypanosomes.

## 5. Sequencing of the *Trypanosoma brucei* genome

*5.1. Random sequencing of cDNAs*
Rapid gene discovery was achieved in the early phase of the genome project by sequencing of randomly selected cDNA clones (Expressed Sequence Tags, ESTs).[17, 18] At the time of writing, there are 5017 *T. brucei* ESTs in the public databases from four different cloned stocks of *T. brucei.*[19] Most of these sequences were generated from cDNA clones of bloodstream-form mRNA. There has been no concerted effort to produce large EST datasets from each of the life-cycle stages as it was decided to divert effort and resources to sequencing of genomic DNA. The determination of life-cycle stage-specific expression will be undertaken by other methods (see section 6.2). In addition to providing the sequence of many novel *T. brucei* genes, the ESTs have provided a rich source of markers[2] and will aid sequence annotation.

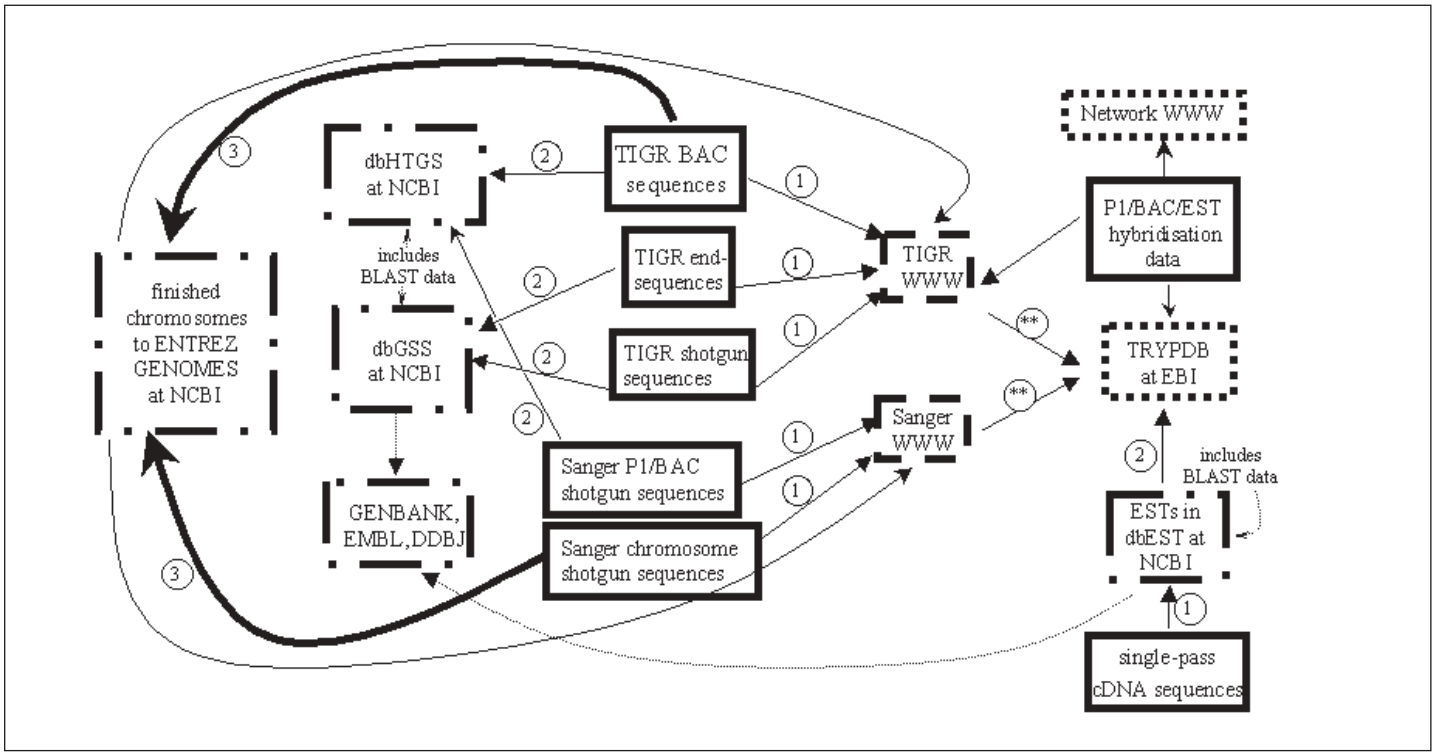
*5.2. Random sequencing of genomic DNA*
In a pilot project, approximately 500 random genomic clones were sequenced[20] to show that this led to equally efficient gene discovery, due to the lack of introns and the close spacing of genes in the *T. brucei* genome. Therefore, it was decided that a portion of the funds obtained for high-throughput sequencing at The Institute for Genomic Research (TIGR) should be allocated to sequencing of random, short pieces of DNA (Genome Survey Sequences, GSS). Sequencing of both ends of almost 25 000 clones (almost 50 000 short sequences) has provided a total of 24.47Mb of the TREU927 genome (to be increased to approximately 29 kb).[21] A proportion of these sequences derive from the ends of large genomic clones (in bacteriophage P1 and bacterial artificial chromosome (BAC) vectors), and these contribute to the mapping and sequencing of whole chromosomes (section 5.3) by providing paired markers of ca. 500bp every 2.5kb across the chromosomes (excepting the minichromosomes). Many researchers have reported finding *T. brucei* homologues of known genes in the random end-sequences, and this has generated a great enthusiasm for the rapid provision of more such

sequences. In response to requests from the community, the Sanger Centre has agreed to provide a further 47 000 single-pass sequence reads to aid gene discovery and to facilitate the completion of contiguous chromosome sequences (initiated August 2000).[22]

*5.3. Sequencing of chromosomes*

Sequencing of chromosome I of *T. brucei* strain 927/4 GUTat 10.1 commenced at the Sanger Centre in 1998. Sequence was obtained from shotgun clones of chromosomal DNA eluted from a PFG (> 7 X coverage) and from mapped P1 clones (1 X coverage), a combination of methodologies pioneered by the malaria sequencing consortium. While approximately 75% of the chromosome sequence was contiguous by year 2, 25% of the chromosome has proved difficult due to repetitive DNA: genes in VSG expression sites, retrotransposons, and tandem repeats (Figure 1B and [14,23]). Nevertheless, completion was anticipated by the end of 2000. Following the random sequencing phase (section 5.2 above), TIGR commenced the sequencing of chromosome II in 1998. This sequence is derived from BAC clones, mapped to chromosome II using cDNAs from the EST project and sequenced to 7 X coverage. The end-sequences determined in the first phase of the project (section 5.2) allow the selection of BACs with minimum overlap for maximum efficiency. Completion of chromosome II is also expected by the end of 2000, and sequence from BACs mapped to chromosomes IV and VI have also been provided.[24] Preliminary (and therefore probably incomplete) annotation is provided by the sequencing centres prior to completion.[23,24] It is best to follow progress by regularly monitoring both sequencing centre WWW sites (Figure 2).

**Figure 2. Access to *Trypanosoma brucei* DNA sequence data**



**The diagram shows the internet and ftp sites at which DNA sequence data are deposited.**
**1. Single-pass, unannotated genomic sequence data are deposited first on the websites at the sequencing centres. EST sequences are deposited first into dbEST at NCBI by individual research laboratories.**
**2. The genomic sequences are deposited in batches in the public databases at NCBI, and BLAST search results are provided.**
**3. The Sanger Centre submits preliminary annotations of large sections of unfinished chromosomes to EMBL**
**4. Fully finished chromosomes consist of a single contig with complete ORF annotation and are deposited in all public databases, and in the genome database at NCBI at the time of publication.**
**** All sequence information will be downloaded into a web-accessible relational database at the Sanger Centre, as a collaboration between the sequencing centres. This DB will contain all types of T. brucei genome data.**

**TIGR WWW: http://www.tigr.org/tdb/mdb/tbdb/index.html**
**Sanger WWW: http://www.sanger.ac.uk/Projects/T_brucei**
**dbHTGS: http://www.ncbi.nlm.nih.gov/dbHTGS/index.html**
**dbGSS: http://www.ncbi.nlm.nih.gov/dbGSS/index.html**
**dbEST: http://www.ncbi.nlm.nih.gov/dbEST/index.html**
**Entrez Genomes: http://www.ncbi.nlm.nih.gov/Entrez/Genome/org.html**
**GENBANK: http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html**
**EMBL: http://www.ebi.ac.uk/embl/index.html**
**DDBJ: http://www.ddbj.nig.ac.jp**
**Genome Network WWW: http://parsun1.path.cam.ac.uk**

Most importantly, the battle to secure funds to finish the genome has seen significant progress in 1999-2000. The Sanger Centre has sufficient funds from the Wellcome Trust (UK) to finish chromosomes I, IX, X and XI, thus bringing their contribution to approximately half the genome. TIGR has provisionally adopted chromosomes II–VIII, also approximately half the genome, and is awaiting a funding decision.

*5.4. Access to sequence data*

The fields of genomics, databases and bioinformatics are dynamic. It can be difficult for researchers to feel confident that they are taking full advantage of the DNA sequence as it becomes available and, at a later stage, when more extensive analysis and annotation has been carried out. Figure 2 gives some guidelines to where a sequence may be found and what status it has at different stages.

cDNA sequencing has been carried out in individual research laboratories and the sequences are made available in batches via the database for expressed sequence tags (dbEST) at the National Center for Biotechnology Information (NCBI).

All genomic sequence data are made available immediately via the websites at the respective sequencing centres (TIGR and Sanger Centre). These are single-pass sequences and no error correction nor annotation are offered at this stage, but this is a very important resource for researchers who are looking for genes in *T. brucei* for which there are homologues in other organisms. Search engines are provided, allowing researchers to look for sequences with high similarity to the gene they seek. (TIGR sequences may also be searched at[25] and TIGR has recently provided data on significant similarities to genes in the databases[26,27]). At intervals sequences are submitted in batches to the public databases at NCBI. Random genomic shotgun sequences and end-sequences of genomic clones (section 5.2) are submitted to the database for

Genome Survey Sequences (dbGSS). Sequences of shotgun clones derived from whole BACs (section 5.3) are submitted to the database for high-throughput genomic sequences (dbHTGS) in three stages (at 3 X and 7 X coverage, and at completion). All these data will eventually be mirrored in the GENBANK, EMBL and DDBJ databases, ensuring that all available *T. brucei* sequences may be found in a single database. However, there are some advantages in looking at the data in the specialised genome databases, as annotation is more extensive and sequence similarity assignments are provided.

On completion of a chromosome sequence, the most careful annotation is carried out. Great emphasis is laid on ensuring the sequence is accurate and contiguous (some areas of ambiguity may be tolerated in the final sequence, if too many resources are required to provide final clarification), and on trying to identify all open reading frames (ORFs) (researchers should be aware that, however careful the annotation, some protein coding genes may not be predicted from sequence analysis alone.) The analysis of the chromosome is published in a peer-reviewed journal and the sequence with full annotation can then be viewed with the Entrez browser at NCBI. The sequencing centre responsible for the sequencing of the chromosome also places the same data on its website.

In an ideal world it would be possible to carry out searches of all data at a single site but the curation of such a database would necessitate delay in access to the data. Immediate access to raw, unfinished sequence is highly prized by a research community waiting impatiently for gene sequences and the sequencing centres have responded to this need. This is only really possible via deposition on home websites prior to batch submission to NCBI. It is therefore important for researchers to consider which databases contain the most valuable data (e.g. the public genome databases because of their extensive annotation, or the sequencing centre databases because they may contain sequences not yet processed for transfer to NCBI) and in some cases to perform searches at several sites.

Nevertheless, it is planned to create a database to contain all genomic information (hybridisations, mapping, primary sequence annotation from the sequencing centres, secondary (ongoing) sequence annotation, references, transcription data, etc., etc.) This is likely to be a relational database using SQL (Structured Query Language) and hosted by the Sanger Centre in collaboration with TIGR and the *T. brucei* network. A similar database is already under construction for *Plasmodium*.[28] Genome databases of this type are long-term projects, providing a single site to review ongoing genomic and functional genomic analyses, and as such serve a different function to the rapid access sequence deposition on the sequencing centre websites. News on progress will be posted on relevant websites.

The field of bioinformatics is changing rapidly and the databases described here will not remain static. NCBI, EBI, the sequencing centres (and others) are actively developing better analysis tools, and it is necessary to watch their websites for new innovations and developments.

*5.5. Access to biological resources*

All biological resources used by the genome network are available to researchers on request, including the TREU927/4 stock and 10.1 derivative, high-density filters of genomic libraries, genomic DNA clones, cDNAs and karyotype blots.[29] The genome website provides addresses and email links. The genome network insists that hybridisation and sequence data derived using its resources are returned to the database curator for inclusion in the genome database.[29, 30]

## 6. The future

*6.1. Sequencing and analysis*

To make full use of the substantial investment in genome sequencing it is necessary to complete the task, to ensure we have complete information on all enzyme pathways. High-throughput sequencing becomes more efficient as new methods and strategies are developed, and these will be utilised in our efforts to ensure the funds secured enable us to complete the sequencing of the entire genome. It is also now absolutely imperative to invest substantial effort into bioinformatics to make the data accessible and informative, to stimulate new ideas in the search for novel approaches to combat the disease.

*6.2. Post-genomics*

The African trypanosome genome network has initiated discussions on the "post-genomic" agenda. This requires careful coordination and the involvement of appropriately skilled researchers. At the 1999 and 2000 network meetings, lists of priorities were drawn up,[31] including microarrays for transcription analysis, techniques for random gene knockouts, RNAi and phenotype characterisation, and the use of the genetic map for positional cloning of genes (some to proceed in collaboration with other kinetoplastid genome networks). Some recommendations of particular interest for the readers of this booklet include identification of trypanosome-specific genes essential for infection and development, characterisation of molecular structures and metabolic pathways, elucidation of unique trypanosome-specific mechanisms of gene regulation and RNA processing, and analysis of the protein profile of the parasite to identify functionally important genes (proteomics). These approaches could lead to the identification of novel drug targets, but only if those researchers skilled in and committed to the development of drugs offer their services to the global collaboration and contribute to the post-genomic projects of the future.

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# Overview of bioinformatics and applications in drug discovery

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***Abstract :*** *The concepts of bioinformatics and target analysis are discussed in the context of the drug discovery process. The unprecedented opportunities offered by the flood of genomic data, filling both public and commercial databases, are being exploited by the pharmaceutical industry to increase the chance of revealing new drug targets amenable to exploitation in the treatment of a wide variety of diseases. The informatics challenge lies in data interpretation and reduction to practice. Target discovery does not demand costly generation of structural data but is well placed to exploit such data where it is available. However, much can be gained by sequence search and pattern recognition particularly in respect of partial sequences, such as expressed sequence tags (ESTs). Examples of target discovery strategies from the cyclooxygenase (COX) and phosphodiesterase (PDE) gene families are presented, both to illustrate potential opportunities and to highlight critical decision points in antiparasitic drug discovery.*

## 1. Introduction

The analysis of DNA or protein sequence information follows the same rules from whichever organism the data are derived. In presenting this overview, the author has focussed on the analysis of human genes because that is his area of expertise. In Section 5, some thoughts are presented on the use of bioinformatics in antiparasitic drug discovery, drawing mainly on experience derived from participating in an antifungal target discovery programme, as well as reflecting some discussions held at various WHO conferences. The emphasis is on strategies and practical application to drug discovery. Of

itself, bioinformatics is a long way from the end product of the pharmaceutical company – a small molecule chemical. Rather, it is at the front end along with genomics, often impacting at a point before a protein has even been identified as a "drug-able target". Nevertheless, bioinformatics has a central role in the discovery process of most pharmaceutical companies today and the purpose of sections 2-4 is to illustrate, in a short space, why this is so.

## 2. Bioinformatics – gateway to the genome

Bioinformatics is the application of information technology to the domain of biology. Significant resources have been devoted to the generation of data in the few years since DNA sequencing was automated and sequence data became a commodity. In the public domain, data has been generated from genomic DNA, mRNA transcripts and ESTs. The rapid generation of DNA sequence data has led to parallel increases in the size of protein sequence databases. This is a result of our ability to predict, more or less accurately, the coding sequence of genes and, thus, to predict the protein product of the genes. Sequence similarity searches, on both the DNA sequence and the predicted protein sequence, lead to predictions of protein function on the basis of observed sequence similarity. Rigorous phylogenetic analysis of full-length sequences enables the degree of confidence in the relationships between members of a set to be established. At this point members of the set can be defined as homologous or not. The essential components that are brought together in the discipline of bioinformatics are a) data collation; b) information integration; and c) knowledge generation.

### *2.1. Data collation*

Sequence data generation occurs in laboratories all over the world. Normally, high-quality validated DNA data are deposited in databases centred in the USA (GenBank),[1] Europe (EMBL data library)[2] and Japan (JIPID).[3] For protein sequence data, submissions are generally made to the Protein Identification Resource (PIR),[4] while databases such as SWISS-PROT[5] maintain high-quality annotation of a relatively restricted set of protein sequences. Along with these databases, others fill gaps in data coverage by extracting translations of sequences from the primary nucleotide databases (e.g., TrEMBL).[5] From the analyst's point of view, these are all primary databases. Secondary databases are those that take a higher level view and typically use some form of pattern recognition technique to classify sequences (e.g., PROSITE,[6] PRINTS,[7] Pfam,[8] etc.). Clearly, a wide range of datasets exists and these must in turn be collated for use in the commercial environment. The collation activity is intensive and ever present, owing to the need for comprehensive coverage for most analytical needs.

Data diversity:

An area of rapid growth in bioinformatics is the ability to deal with many different types of data in addition to DNA/protein sequences and secondary "pattern" databases. These include data from: transcript expression, expression chips, protein expression, protein interactions, protein structure, interaction of small molecules with proteins, metabolites, etc. It is the increas-

ing diversity of data that will provide the major stimulus to database growth in the post-genome era.

*2.2. Information integration*

Bioinformatics has become almost synonymous with integration of biological information; however, this is a somewhat biased view. The bioinformatics community has quickly assimilated the benefits of the internet-based approach to integration. However, there is also a database approach to integration exemplified by, for example, the PRINTS[7] database. As our knowledge of biological systems grows, our integration technologies must also grow to mirror the complexity of the systems we wish to model. This will require a mix of database and network techniques, as well as the development of new networking protocols taking into account the specific characteristics of the biological data.

*2.3. Knowledge generation*

Interpretation is the biggest challenge facing bioinformatics. Current algorithms are excellent at rapidly searching for sequence similarities in large databases. However, the result is a list of matches (termed a "hitlist") that must be assessed by a specialist trained in the science of sequence analysis. Even the use of more advanced tools, such as those supplied to search the secondary databases, demand expert intervention to reduce the results to a form usable by the non-specialist. The effect of noise in the hitlist is critical and, especially where assessment of similarity is not trivial, distinguishing biological significance from statistical significance is evidence of a thoughtful approach.

Ultimately, it is the remit of bioinformatics to understand the origin of the data, annotate and store the information, make it available for retrieval and analysis, reduce complexity and thence drive forward discovery programmes. At Cambridge Drug Discovery (CDD), these ideas are implemented in a proprietary database, TargetBASE™, which builds on the accumulated knowledge about targets that have been used in successful drug discovery programmes and can now be used to predict suitability of related genes as drug targets. Through incorporation of chemistry data, where available and relevant, TargetBASE™ builds up relationships that span the discovery process, ultimately influencing decision-making in approaches to new therapeutic areas.[9]

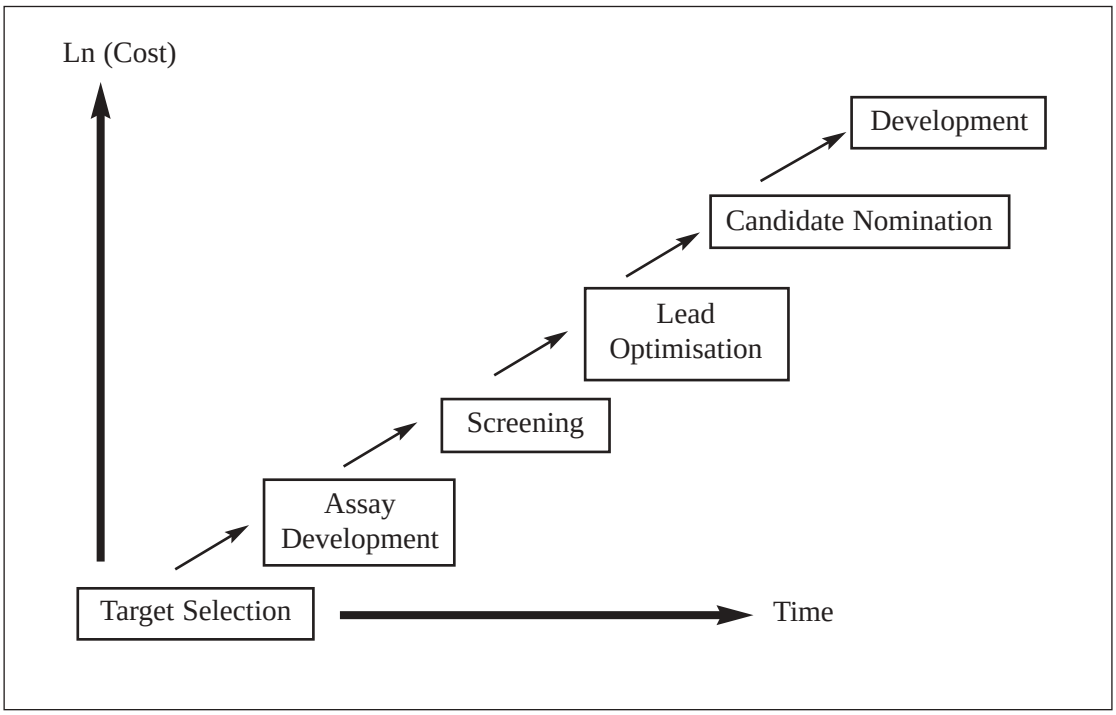
## 3. The drug discovery process

The process of discovering drugs has been somewhat serendipitous in the past. Pharmaceutical companies have preferred targets that have a precedent and where some validation in terms of utility as a target has been demonstrated. High-throughput screening approaches rely on the use of assays that can be miniaturised and automated, together with a library of structurally diverse compounds (typically ~200 000 compounds are required). The result of the screen may be a selection of small molecules that show high affinity for the target and that are amenable to chemical modification to increase

selectivity, eliminate toxic effects and become prototype drugs (leads). Once the requirements of the discovery aspect are met, the lead becomes a candidate drug and begins on its progress through the stages of clinical development and, hopefully, registration with the authorities, prior to marketing and sales. At various points along the way, patents are filed to help protect the investment of the pharmaceutical company in the average 10 year life-cycle of the new drug. Often 3-4 years are spent in the discovery phase.

In an effort to develop new drugs in areas of unmet human need, a significant emphasis is currently placed on discovery of new drug targets. This can be a somewhat risky strategy because it adds to the length of the discovery process, thereby increasing costs for each new chemical entity (NCE). The length of this time penalty depends upon how novel the target is and also on how much critical validation information is available. The general expectation is that a proportion of the genes in the complete human genome will be similar enough to known targets that they will provide new therapeutic opportunities without significantly distorting the discovery investment profile ("development by analogy"). It is clear that several large pharmaceutical companies have already invested heavily in acquisition of genomic data, much of which relates to potential novel targets for which development by analogy will not work. The effect of this strategy is to trigger substantial investments in complex validation technologies that may prevent a high proportion of truly novel potential targets getting to the assay development stage. Simple economics suggest development by analogy is the way forward. The stages in the discovery process are summarised in Figure 1.

**Figure 1. Stages in the drug discovery process**



## 4. Role of bioinformatics in drug discovery

The relationship between bioinformatics and drug discovery is synergistic. The tools of bioinformatics are harnessed to prioritise targets on the basis of their relatedness to known targets. The straightforward cases that can employ "development by analogy" are quickly progressed, while the more interesting, potentially very costly, novel targets should be outsourced for development. These latter will finally re-emerge when a package of data, including hits or leads (by way of validation), can be licensed-in to feed the development pipeline. Probably the most significant investment in new target discovery is development of appropriate assay technology.

The richest source of new targets is the human genome, which consists of about 3 billion bases of DNA coding for about 100 000 genes. The total number of gene products (including alternatively spliced variants) is unknown. Only a fraction of these genes will make good drug discovery targets. Currently, with around 15 000 unique human genes in publicly available databases, only just over 400 are identified targets of drugs currently on the market.[10] There is thus a clear opportunity for discovery of new targets for use in therapeutic approaches in areas of high unmet human need.

The availability of structural information for a variety of proteins has made an impact in the modelling of target-inhibitor complexes and the techniques of lead optimisation can benefit greatly from such modelling approaches. The fact remains, however, that most drug targets are membrane bound and extrapolation of techniques (such as structure prediction and fold compatibility searching) from the soluble protein universe to the membrane bound universe is not to be undertaken lightly.

As we have seen, there are a wide variety of categories of data available for analysis. In terms of target discovery, availability of ESTs (in the form of transcript images) from Incyte and the public domain has made it possible to evaluate gene expression at the mRNA level in a variety of tissues in both the normal and diseased states. The use of transcript imaging assumes a simple relationship between transcript expression and protein expression; however, for many common drug targets (including G-protein-coupled receptors, the most ubiquitous family of drug targets) this relationship is not applicable. Whilst comparison of transcript expression levels leaves much to be desired (including a sound mathematical model for the analysis of random samples from cDNA libraries), the use of ESTs in discovering new examples of known target gene families can be fruitful in its own right.

Two examples of the use of bioinformatics in defining strategies for target discovery will be outlined here: first, an EST analysis approach to discovery of a potential novel cyclooxygenase (COX) gene family member; second, use of genomic information in assessing therapeutic possibilities in the PDE4 gene family.

### *4.1. Partial sequences*

Given a database of ESTs and the sequences of human COX-1 and COX-2, can we assess the possibility of the existence of a third family member? The motivation for such a query is that non-steroidal, anti-inflammatory drugs

(NSAIDs), such as aspirin, cause unwanted side-effects (including gastric lesions) in long-term therapy.[11] COX-1 protects the gastric mucosa while COX-2 is involved in inflammatory responses throughout the body. Development of selective COX-2 inhibitors is possible; however, a selective putative COX-3 inhibitor could represent a backup route with improved selectivity profile. When searching for ESTs that are similar to known family members, account should be taken of tissue localisation data, the similarity of known homologues, and the possibility of polymorphisms. The use of animal models (i.e. ESTs from rodents) can also highlight missing members of known human gene families. This is classic gene discovery, using standard searching tools on partial sequences, that moves one step further into target discovery because of the existence of drugs for close gene family members.[12]

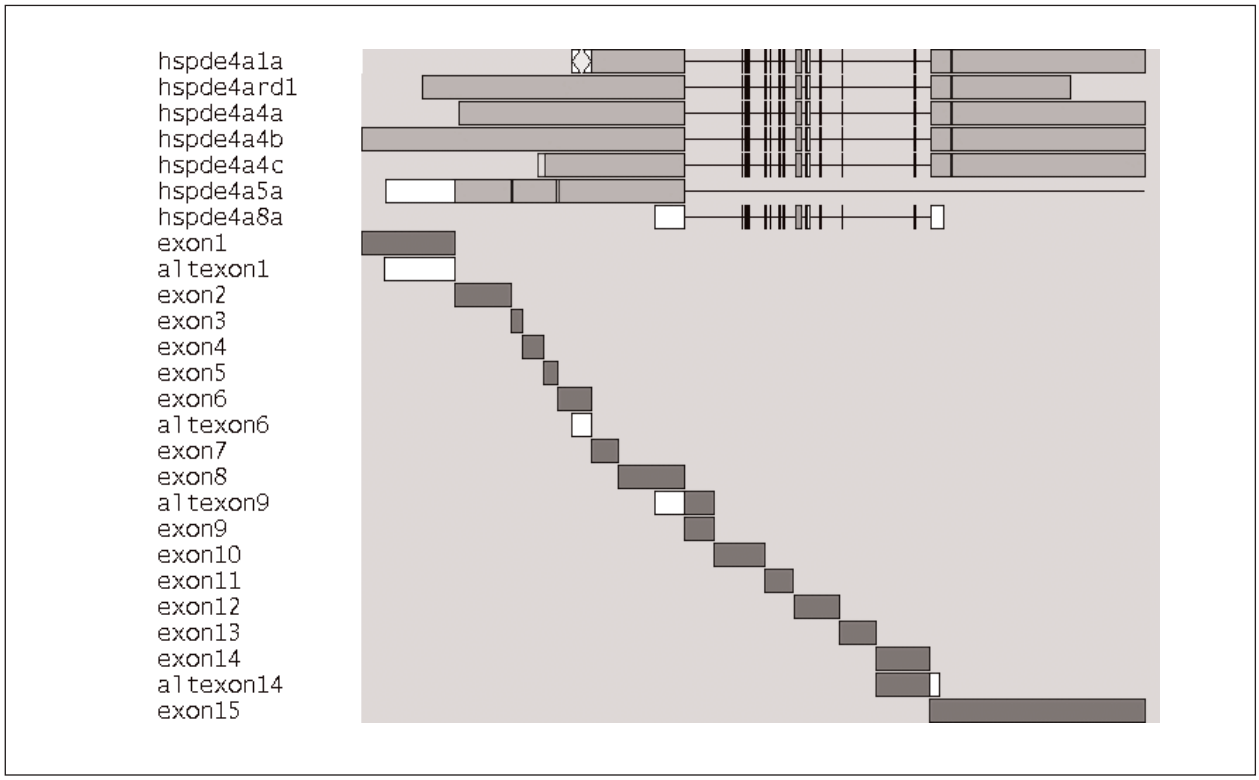
Another approach to the same problem is that of transcript profiling. Here, we are not simply establishing the existence of a previously unknown gene family member but also establishing a quantitative relationship between the numbers of genes expressed at the mRNA level between different tissues. Comparison of the changes in these mRNA expression levels between healthy and diseased tissue is used to predict potential new targets. The underlying mathematical model of random sampling from cDNA libraries should be fully understood before embarking on such projects, in case erroneous judgements are made. Certainly, the approach has yet to be validated by equivalent experiments at the protein expression level, or by drugs on the market resulting from target discovery using transcript profiling.

*4.2. Genomic sequences*

Phosphodiesterases are members of a ubiquitous, multi-gene enzyme superfamily that operates by turning off cAMP and cGMP signalling. They regulate cell activation in a wide range of biological systems. The human PDE4 subfamily has four members with emerging therapeutic roles in CNS disorders, inflammation and smooth muscle relaxation. The catalytic domain is highly conserved across the PDE4 subfamily. Several N-terminal splice variants have been sequenced. By evaluating the exon structure of the genomic DNA (see Figure 2) it is possible to reconcile the known splice variants and also to predict the possibility of further variants that have not been sequenced and deposited in publicly available databases. Prediction of the function of such hypothetical variants is not trivial, however, as sub-cellular localisation of the protein is achieved through protein-protein interactions between the variable N-terminal region and specific proteins in different tissues.

As the complete human genome sequence becomes available, accurate prediction of exons will become a more pressing issue. The value of ESTs allied to genomic data in validating such predictions should not be underestimated.

**Figure 2. The human PDE4a gene structure, illustrating N-terminal variation in relation to exon distribution in the gene.**




## 5. Conclusions for anti-parasitic drug discovery

So far, we have focussed on bioinformatics as the discipline relates to the analysis of human gene families. Extension to parasite genomes is clearly possible and illustrated in subsequent presentations. The tools for all the searching and prediction techniques are the same. We tend to think of the control of parasites as a matter of searching for sequences not represented (thus far) in the human genome and assessing them for essentiality in knock-out experiments. It should be borne in mind when hunting for new potential targets that selectivity can be obtained between human and fungal forms of the same enzyme (e.g., fluconazole inhibits a pathway common to both yeast and human but remains a very successful antifungal agent).

Furthermore, selectivity only demands the mutation of one residue in the binding region of the drug. The comparative analysis of sequences from human and parasite genomes takes on a highly sensitive aspect when this exquisite sensitivity is taken into account. Pathways previously thought intractable, owing to their presence in both human and parasite genomes, become more attractive once the fine detail of sequence relationships has been thoroughly worked out. An intimate knowledge of the active site will make it easier to predict whether amino acid residue differences are relevant for selective inhibitors design.

Oral availability is also an issue, especially when treating large populations. Approaches that depend upon the inhibition of proteases, for example, may suffer from poor oral availability because of the presence of homologues in the gut, or other non-specific binding events. It is crucial always to bear in

mind the form of the end product even during the target analysis stage. While it is probably not practical to suggest an intravenous drug on a daily basis for such populations, a periodic oral formulation could be more appropriate, generating higher patient compliance, while at the same time being more economic both to manufacture and administer.

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# DNA chips: Applications in pharmacology

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***Abstract :*** *Microarray assays allow massive parallel data acquisition and analysis of gene expression on a genomic scale. DNA chips provide a quantitative measure of the RNA molecules present in any cellular extracts and allow the comparison of the gene expression profiles in different biological samples. As a model, we used Affymetrix chips to analyse host human macrophage cell genetic response after infection with Listeria monocytogenes by monitoring the expression of 6800 genes. This bacterial pathogen was shown to modulate expression of several hundred host cell genes. Most of these genes have never been described as associated with Listeria infection. Clustering these genes in functional groups provides new informative insights on the mechanisms by which the Listeria exploits mammalian cell functions to grow. Building a comprehensive framework, the identification of these genes would provide new specific targets for future therapy. Allowing such a broad approach, chips technology would then dramatically enlarge pharmaceutical strategies in drug discovery.*

## 1. Introduction

*Listeria monocytogenes* (LM) is a facultative intracellular bacteria that causes bacteromia and CNS infections, including meningitis, meningo-encephalitis and brain abscess, septicaemia, gastrointestinal symptoms (nausea, vomiting), intra-uterine infections resulting in spontaneous abortions, both in humans and domesticated animal.[1] LM is a gram-positive bacterium able to penetrate and grow in professional phagocytes and non-phagocytic target

cells such as endothelial cells.[2] Upon infection, a vigorous cellular response is induced resulting in pleiotropic biological changes associated with regulation of host cell gene expression. Macrophages respond to LM infection by transient activation of signal transduction pathways, leading to specific modulation of gene expression, and to the subsequent alteration of specific protein synthesis that will either hinder or favour the ongoing infection. As a model to understand the cellular response upon parasitic infection we have analysed the gene expression pattern of THP1 cells before and after Listeria infection. To do so we have used the DNA chips technology that enables the profiling and a comparative analysis of gene expression in any biological sample.[3-7] A quantitative analysis of the transcriptome is here based on monitoring "hybridization signatures." The concept behind DNA chip or microarray technology is similar to that underlying Northern and Southern blots. A probe is hybridized to multiple defined cDNAs which have designated spots on solid phase of chips. These cDNA targets can be either bacterial colonies, PCR-products from cDNA clones or sets of oligonucleotides designed to assay a gene of interest. The probe is usually a complex mixture of cDNA fragments generated from total cell or tissue mRNA by reverse transcription and labelling. This complex probe contains thousands of different messengers RNA species, each characterized by a different expression level. The fluorescence-labelled probe fragments bind to their appropriate partners and the intensity of emission can be assessed by a laser. This technology has a great potential as it allows the simultaneous analysis of thousands of genes: using this method, global gene expression can be scored then compared between two populations. Such an approach provides a new access to the key area of disease and drug discovery as the different tested biological samples can be, for example, healthy versus infected, normal versus tumorous, or normal versus drug-treated. As altered gene expression patterns are expected to occur with disease development, the characterization of expression differences underlying a pathological process might be useful as diagnostic or therapeutic tool. Here, we used Affymetrix chips containing as many as 272 thousand groups of oligonucleotides in an 1.6cm$^2$ area, each feature contains approximately ten million oligonucleotides to monitor the expression of 6800 human genes.[8] We then analysed host human macrophage cell genes that are differentially transcribed following infection with the bacterial pathogen Listeria monocytogenes.

## 2. Materials and methods

1,25-dihydroxyvitamin D3/retinoic acid-treated THP1 cells were infected with LM, and two hours after infection RNA was extracted for global quantitative gene expression analysis on a chip.

Messenger RNA was first isolated from cells using the guanidinium isothiocyanate method. Double-stranded cDNA was then synthesized using the Gibco BRL Superscript Choice system according to the manufacturer's instructions. In vitro-transcription was used to produce biotin-labeled cRNA from the cDNA, with the MEGAscript T7 kit, Ambion using Biotin-11-CTP and Biotin-16-UTP (Sigma, 1:3 labelled to unlabeled). The cRNA was fragmented

before the hybridization steps onto the chip. All the protocols used are those recommended by GeneChip, Affymetrix.

## 3. Results and discussion

As a step toward understanding the complex biological framework resulting from a parasitic infection, we have analysed the gene expression patterns and searched for differences between normal and infected cells. We then report large-scale measurement of gene expression of the human monocytic THP1 cell line infected by *Listeria monocytogenesis* using different technologies.

*3.1. Global criteria analysis definition*

In order to validate modulation of gene expression observed using the DNA chip technology we have first performed a robust analysis of different hybridisation parameters and also compared the results obtained using different technologies. First, to examine the reproducibility of hybridisation signals obtained by the oligonucleotides DNA chips, we hybridised the same complex probe sample to two different sets of arrays, and found a good reproducibility between repeated measurements (r=0,99). To assess differences due to the preparation of the complex probe rather than the hybridisation and reading steps, we also hybridised samples independently prepared from the same pellet of THP1 cells. A close correlation was observed (r=0,96). While not as good as that obtained in the simple hybridisation reproducibility experiment, this might reflect differences in extraction and in vitro-transcription reactions. In large-scale gene expression measurements, sensitivity of mRNA detection is a crucial parameter, because most of the mRNAs are present as a few number of copies per cell. The general signal distribution pattern obtained with the DNA chip technology was in agreement with typical mRNA population distribution patterns, as most signals were skewed towards the low intensity values. We then compared the relative signals obtained by DNA chip technology and by two macroarrays technologies, and clearly demonstrated a higher detection sensitivity of the DNA chips technology, as it detected 3- to 6-fold more genes, with a limit of detection 4- to 7-fold lower (data not shown).

LM infection of the THP1 cell line induced substantial cellular trauma so that a great number of genes were modulated: either up- or down-regulated (on average 166 and 248, respectively). The fold change parameter (FC) is the most indicative parameter for comparing gene expression modulation with one another. The FC distribution indicated that 80% of the down-regulated genes displayed a low FC (<3), compared to only 27% of the up-regulated genes. To select the most likely to represent LM-infection regulated gene candidates, we empirically tested different criteria to make further selection and assessed the reproducibility of expression variations using the oligonucleotide technology. The first criterion to be tested was the FC. In standard large-scale measurement studies, FCs above a 2-, 3- and even 4-fold variation have been considered as significant, based on the assumption that the low FC values are more difficult to reproduce. Nevertheless, we may note that

FC as low as 1.8 could however be reproduced by Northern blotting experiments (data not shown). An aspect of the Genechip software is to allow the identification of regulated genes whose expression was undetectable in one of the two tested samples. In that case, the FC was calculated by dividing the hybridisation intensity value for the gene in the sample where it was detected as present by a minimal value of 20 which approximates the limit of detection and thus the calculated FC most likely represents an underestimation of the true FC. Moreover, we frequently observed in reproducibility experiments that while the absolute assigment of a given gene (increased or decreased) was reproducible, the absolute value of the FC could vary between experiments, and therefore it might be rejected from the analysis when too stringent criteria were applied. All these considerations taken together, although a poor reproducibility of low FC was expected, we decided to use a non-stringent criterion in the analysis whereby all the genes with a FC of at least 1.8 were considered.

In the overall analysis, in addition to the FC, the intensity signal has also to be considered. We observed that intensities values of less than 60 are less reliable than high intensity values and should be cautiously considered when a variation of gene expression was detected. We thus decided to select only the genes whose intensity was above 60 in the control sample when we considered the decreased genes and the genes whose intensity was above 60 in the LM-infected sample when the increased genes were considered.

Using these different criteria as a rule, we have investigated the reproducibility of variation of gene expression in simple hybridisation reproducibility experiments, in sample-to-sample variation experiments, and in infection-to-infection variation experiments. In addition to low FC and/or low intensities, the lack of reproducibility in these cases reflected the use of different physical chips or of independently prepared samples, and the introduction of a biological variation in the analysis. As expected the level of reproducibility decreased when a new source of variation was added. The best reproducibility was observed with the up-regulated genes rather than the down-regulated genes, probably reflecting a wider distribution of the increased genes FC.

Another important issue of large-scale measurement studies was the agreement between the different available technologies and the data reliability. Therefore we compared the hybridisation signatures of DNA chip technology and two high-density macroarrays. Excepted differences concerning sensitivity discussed above, we found a close correlation between the chip and the macroarray data : 10 genes detected as being increased by the DNA chip platform belonged to the list of the genes also assayed by the macroarray platforms and were also found increased. Moreover, four modulated genes tested in Northern Blot experiments were all confirmed (data not shown). Taken together, these data pointed out the reliability as well as the high sensitivity of the DNA chip technology.

Previous work using Northern blot or D-PCR strategy have attempted to characterize the differentially expressed genes in the macrophage host cells.[9] Our study allowed the reproducible identification of 73 up-regulated genes and 24 down-regulated genes as the consequences of LM infection and showed

that 87 of these differentially expressed genes are newly identified LM modulated genes (Figure 1). The effectiveness of our approach was reinforced by the ability to successfully evidence the mRNA for many known LM induced genes among all the genes modulated.

*3.2. Cross-talk between LM and THP1 host cells*

The development of a parasitic infection is the result of a series of molecular changes occurring in the cell. To avoid the antibody response, complement and the effector cells, each intracellular pathogen species has developed different strategies to exploit host cell functions for infection and growth.[10] Functional clustering of the newy described genes here provides new insight into the mechanisms by which the Listeria exploits mammalian THP1 cell functions to grow in the phagocytic host cell. We thus observed a complete different reprogramming of host cell regulatory pathways in response to the LM infection. Several sets of gene products promoted LM growth while others contributed to the host cell response in promoting listericidal host cell activity.

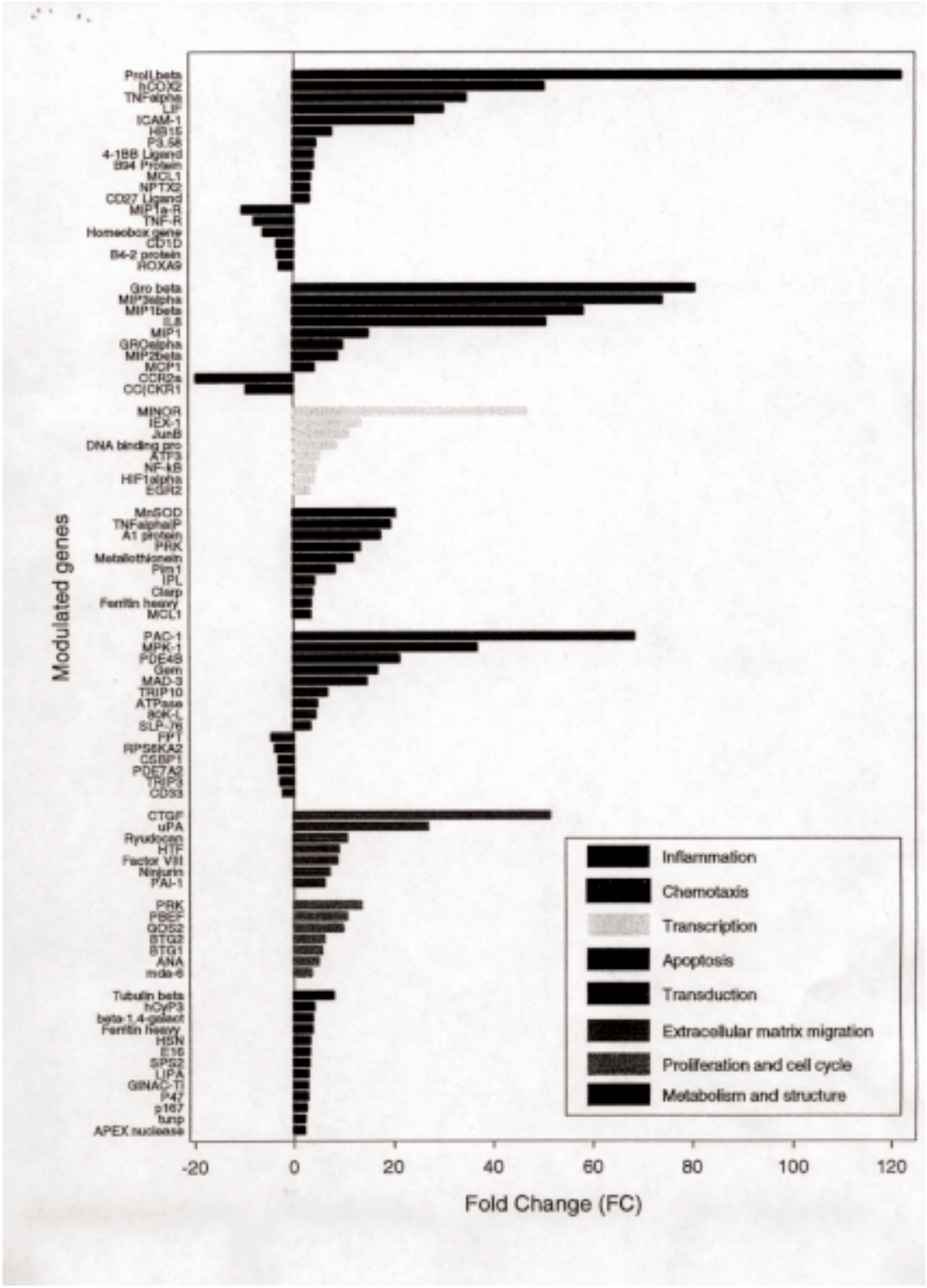
The identification of the genes whose expression was modulated by Listeria infection was based on the reproducibility of the variations observed in at least three experiments (two of them were two independent infections of THP1 cells). We have identified 73 up-regulated genes and 24 down-regulated genes. These genes were presented on Figure 1 where the gene expression variation was given by the average of the FC observed in three experiments. Some genes whose expression was found to be as increased were also assayed by macroarray technologies. All of these genes were confirmed by the chips platform and the macroarray platform. FC-values identified by both technologies were closely correlated. We have also examined the agreement of the oligonucleotide technology results with Northern blot assays. Indeed, taking four representative genes that were differentially expressed: TNFa, MIP1a and MIPb (up-regulated) and HHCPA (repressed), the Northern blot analysis showed a close correlation between the fold change data obtained in the nucleotide microarray and the intensity variation in the Northern blot measurement (data not shown).

Further validation of the analysis was highlighted by reproducing already known LM infection modulated genes. Among the 97 listed genes in Figure 1, at least 10 genes have previously been described as LM infection-induced genes. The other 87 remaining genes are newly described LM regulated-genes. For example an up-regulation of IL1b, TNFa, MIP1a, MIP1b, MCP1, IL8, ICAM-1, MKP-1 and the inhibition of TNFR-I have already been shown to be induced by LM infection. Based on their known function or structure-predicted one the differentially modulated genes may be clustered to a functional point of view. As expected the most modulated genes encoded proteins involved in the inflammation process: pro-IL1, TNFa and COX-2, chemotaxis and activation of effector cell processes : IL8, MIP1b, GROb and MCP1.

Furthermore we highlighted other unexpected functionally related gene families that encode proteins involved in :

1) migration and remodeling the clot and extracellular matrix : uPA, uPAR, HTF, CTGF, Factor VIII, PAI-1, ryudocan and ninjurin,

**Figure 1 : Summary of DNA chips data. The modulated genes fold change following *Listeria monocytogenes* infection is plotted. Genes are clustered according to their known function.**



Furthermore we highlighted other unexpected functionally related gene families that encode proteins involved in :
1) migration and remodeling the clot and extracellular matrix : uPA, uPAR, HTF, CTGF, Factor VIII, PAI-1, ryudocan and ninjurin,
2) transcription : IEX-1, EGR-2, HIF-1, ATF3 and NF-kB,
3) transduction : PAC-1, MKP1, PDE4B, Mad-3 and Gem,
4) cell cycle and proliferation : G0S9, G0S2, BTG1, BTG2, ANA and mda-6,
5) apoptosis: MnSOD, TNFaIP, A1 protein, Pim1, MCL-1 and Clarp,
6) Cell metabolism and structure : b galactosyltransferase, GlcNAc-TI, actin-bundling protein, tubulin and tunp. A few other modulated genes had not been described at all and their function has to be determined (11 genes). The physiological implications of these regulations following LM infection should now be investigated. All the modulated genes we observed induced two responsive strategies taking place in the host cell. On the one hand the host cell developed an immune response strategy to hinder the parasitic development, on the other hand the bacteria pathogen elicits metabolic processes to counteract the host cell response and grow. Besides the inflammatory host cell responses including cytokine and chemokine production, LM induced expression of several genes that either counteract the inflammation process or exploit the normal cellular mechanism to favor its own metabolism. To limit the inflammatory response, LM was found to repress the transcription of the chemokine receptors encoding genes and class I and class II MHC genes to interfere with the presentation of antigens. Other survival strategies performed by this pathogen are induced growth arrest of the host cell and the induction of anti-apoptosis genes. Another important cluster of induced genes includes those involved in remodeling the intracellular matrix to favor migration of the pathogen. The accurate identification of the implicated genes is a mine of information. The overall transcriptional response of the THP1 cell is now under study to build a comprehensive framework that defines the interactions of the host and the pathogen, fate of the infected cell and the LM pathogenesis.
Profiling the gene expression patterns in THP1 cells and comparing two biological samples before and after the LM infection provides global and accurate information for building a comprehensive framework to interprete LM pathogenesis. Such an approach, here performed to analyse the interrelations between the host cell and a pathogenic bacteria could be enlarged to all biological samples where there is a significant difference in expressed genes. Comparing normal and tumorous samples, normal and treated sample, diseased and drug-treated ones could provide access to crucial information for new drug discoveries and the development of new diagnoses and therapies.

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# Bacterial targets and antibiotics: Genome-based drug discovery

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***Abstract :*** *The responses, at the molecular level, of bacterial cells to changes in their environment are extremely complex. The unravelling of these complex processes is a goal that until a few years ago would have been unthinkable. However, the sensitivity of the new technologies for what is termed "global analysis" is constantly being improved and we will soon be in the position where we are able to decipher the entire programme of events initiated by a given stimulus. The forerunner of these "new" technologies is the more than 20-year-old use of 2D-PAGE. The introduction of commercially available fixed pH gradient gels, improvements in mass spectrometry and the large number of genomic DNA sequences deposited in the databanks have dramatically transformed the usefulness of this technology. We are using this methodology combined with transcriptomics to determine the modulation of gene product and expression levels induced by various classes of antibiotics in an attempt to gain insight into the mechanisms of new classes of chemicals arising from the screening of compound libraries.*

## 1. Introduction

The ever-increasing frequency and spectrum of infections resistant to currently available antibacterials raise the question as to the feasibility of keeping pace with the rate at which drugs become ineffective. With the appearance and spread of multi-resistant and, in fact, untreatable tuberculosis[1] we are effectively returning to the pre-antibiotic era. Risk assessment for a number of infectious diseases such as cholera, diphtheria and respiratory tract infections, including tuberculosis, will have to be reconsidered and the strategies to combat the causative pathogens re-evaluated.[2]

The lack of innovation in antibacterial treatment is clearly reflected by the diminishing interval in time between when a new drug enters the market and the appearance of resistance to it in one or more different strains of bacteria. An intrinsic problem here, is that the new antibiotics are all, in fact, modifications of an already relatively limited number of existing classes of compounds. This phenomenon and the lack of new antibiotics with novel mechanisms of action can partly be explained by the reduced efforts in antibacterial research in the 1980s. It was believed that the existing repertoire of antibiotics would result in the extinction of a number of infectious diseases and thus bacterial infections were no longer considered a problem. Research efforts and financing by both the major pharmaceutical companies and public health organisations were shifted to other more pressing medical problems.

Fortuitously, the revival of interest in this problem has coincided with an explosion of technological advances originally initiated by the programmes to sequence genomic DNA. Miniaturisation, automation and bioinformatics are changing our concepts regarding the way we can approach antimicrobial research. Instead of studying the expression of individual genes, operons or even stimulons, we are now able to follow the entire expression pattern of an organism. The major stimulus for this change of concept in the prokaryotic field was the publication of the fully assembled genome sequence of *Haemophilus influenzae*.[3]

Various tools for the efficient utilisation of sequence information have been developed in the past few years. Comparative analysis of different genomes has identified many orthologues and paralogues that can be classified as protein families and genetic manipulation has revealed many genes that are essential for the growth of organisms in defined media[4] or in vivo.[5,6] The first of these tools is obviously dependent on the sequence information whereas the second is only facilitated by the information. Two technologies, proteomics and transcriptomics, which constitute the "readout" for the response of a cell to changes in its environment, would be totally impractical without the knowledge of the complete genome sequence.

Clearly classical biochemistry will be required to confirm the findings from the above mentioned technologies but together they form the basis of an approach towards the understanding of cellular networking. This is of significance if we are eventually to understand how a cell functions as an entirety.

## 2. Proteomics

The term ‘proteomics’ is defined as the study of the protein complement expressed by a genome[7] or perhaps more realistically, the protein complement expressed by a genome at a given time. In practice, this study consists of two parts: 1) separating and identifying the expressed proteins and (2) the analysis of the changes in their expression levels, which reflect the response of the cell to its environment. Apart from the availability of genome sequences, this technology has grown in its usefulness because of the improvements in the reproducibility of the gels used for 2D-SDS PAGE and because of the increase in the sensitivity of mass spectrometry.

Formally, slight variations in the technical preparation of the gels prepared for isoelectrophoresis often led to major differences in the migration of the denatured polypeptides resulting in ambiguity and difficulties in comparing experimental findings. Today, however, commercially available fixed pH gradient gels allow for the reproducible comparison of gels within and between laboratories. Visualisation of the separated polypeptides is now the limiting factor. For example, in a standard 2D PAGE gel, with a pI range between 3.5 and 9.5 it is theoretically possible to display ~70% of the proteins that could be synthesised by *H. influenzae*. In practice, however, only 30–40%, depending on the method of visualisation, can be observed. The major reason for this is not that the other proteins are not synthesised but that they are synthesised in such low abundance and there are limits as to how much protein can be loaded onto a gel!

*2.1. Mass Spectrometry*

Identification of the polypeptides represented by the spots was formally, and in some cases still is, achieved by N-terminal sequencing, which requires levels of polypeptide in the 10-100 picomole range. Recently this methodology has been largely superseded by fragmentation of the polypeptide with proteases having specific cleavage sites and the subsequent determination of the molecular masses of the resulting peptides using Mass Spectrometry. Comparison of the experimentally determined masses to a database of masses calculated, in silico, for all possible open reading frames (ORFs) in a genome, usually, and practically always in the case of bacterial genomes, results in the identification of the polypeptide. The sensitivity of the MS has improved to the point where only femtomoles are required for mass determination (because of losses in the preparation of the sample, ~100 nanomoles are required per spot), thus allowing the identification of most spots observed in a Coomassie blue image. However, preparative gels run for the isolation of polypeptides for identification purposes result in patterns that are, in some regions of the image, extremely difficult to match to those produced by analytical gels. It is therefore necessary to accumulate the spots from analytical gels for the accurate identification of many spots.

*2.2. Signal intensity*

The image acquired using Coomassie blue displays the total amount of any individual protein in the experimental sample. As there is heterogeneity in the physiological state within a culture and different proteins have different rates of turnover, changes in the relative intensity revealed using Coomassie blue are diluted out. This is also true when the proteins are visualised using autoradiography after continuous metabolic labelling with 35S L-methionine. We have found that a 2-3 minute pulse of 35S L-methionine provides sufficient label and sensitivity to visualise changes in the protein synthesis patterns resulting from the cells' responses to differences in their environment. An example, using 35S L-methionine for the quantification of changes resulting from the treatment of *H. influenzae* with novobiocin, is shown in Figure 1. It should be realised that the changes that we measure do not discriminate between differences in synthesis and a combination of synthesis and degra-

dation. Experiments, which are in progress, involving a pulse followed by a chase of unlabelled L-methionine should help to resolve such problems.

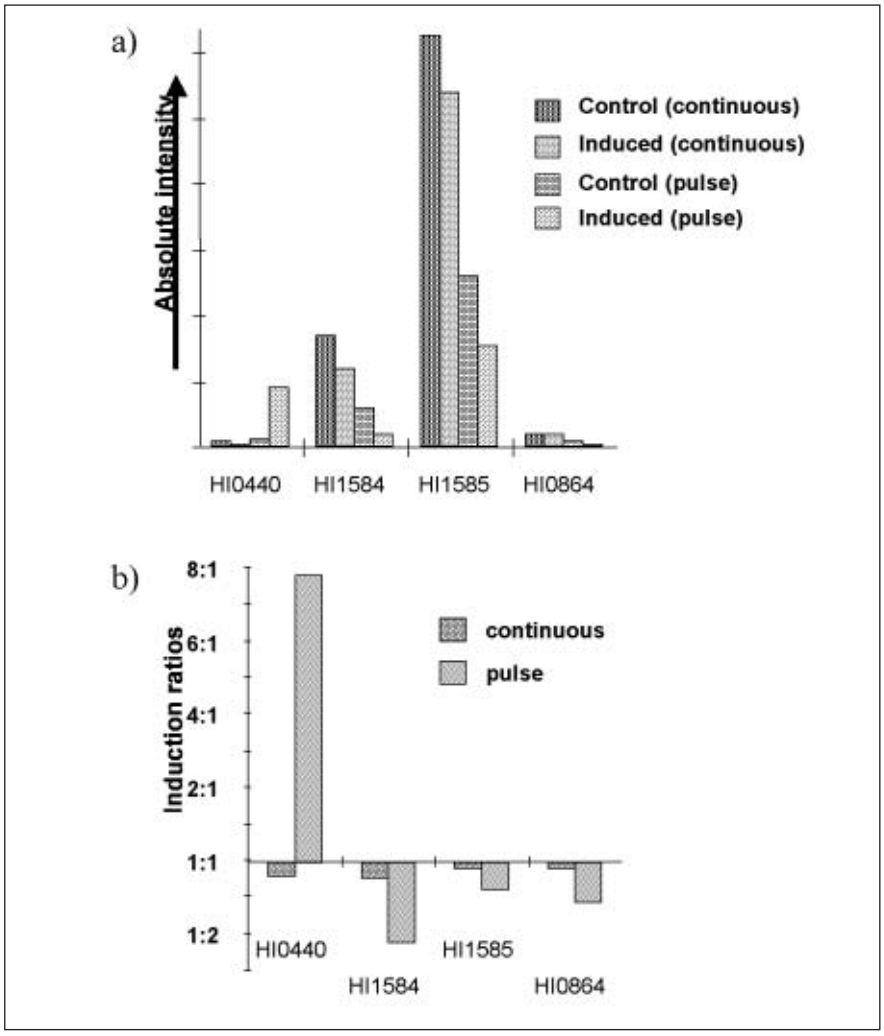


**Figure 1. Comparison using either continuous or pulse metabolic labelling of H. influenzae with 35S L-methionine. Cells were treated or not with novobiocin for 30 minutes, either with 35S L-methionine included in the medium or added as a 3-minute pulse after 27 minutes. Protein extracts were run in 2D-PAGE and the PhosphoImager images from the dried gels were matched and quantified. The intensities of four spots are shown as a) absolute values and b) the calculated ratios of treatment to no treatment.**

*2.3. Pattern comparisons*

Exposure of cells to sub-lethal levels of antibiotics elicits survival responses that are characteristic for any particular antibiotic. This property is being utilised in an attempt to characterise the mode of action of antimicrobials identified in various whole-cell assays and to validate the target for compounds synthesised in the chemical programmes. Proteomics is used as an aid in understanding which response is being induced. Using the appropriate software it is possible to compare the patterns of unknown responses with a database of patterns produced using antibiotics for which the mode of action has been established (in this context the term "pattern" refers not only to

the presence or absence of a spot but also to the relative intensity of the spots). The "established" responses in the database, although showing a common pattern of changes for different antibiotics acting upon the same target, also contain superimposed secondary responses, which cannot be explained with reference to the "known" target. These secondary responses most probably represent the mechanistic differences in the action of the antibiotics but perhaps also indicate secondary targets. We have recently encountered an example of the latter situation in a compound selected in vitro for its activity against DNA gyrase. A secondary in vivo response could be discerned from a comparison of the patterns that indicated inhibition of protein synthesis. This activity could be confirmed in an in vitro transcription/translation assay.

## 3. Metabolic pathways

This comparison is a useful first step in determining the response as it does not rely on the identification of the spots and therefore uses all the information contained within the gel image. However, as the goal is to discover and validate new targets, it is clear that there will be responses for which there is no comparable pattern in the database. Although the number of spots that have been identified is limited it is still possible to visualise, albeit with gaps, the changes in the enzyme levels within the major metabolic pathways. The information gained from such analyses coupled to what can be learnt from the literature about the regulation of these pathwa of antimicrobial compounds. In these cases a detailed analysis of the expression patterns with respect to the identified spots is required. In a recent study we examined the response of *H. influenzae* to the inhibitors of dihydrofolate reductase (trimethoprim) and dihydropteroate synthase (sulfamethoxazole).[8] We chose these particular antibiotics as proof of concept as the downstream effects of tetrahydrofolate starvation, for example: inhibition of dTMP and L-methionine biosynthesis, have been examined in detail for *E. coli*.[9,10] Most of the enzymes, involved in L-methionine biosynthesis, are sufficiently abundant that they can be identified with confidence in the gel pattern. The changes in the intensity of the representative spots could be measured and are shown for trimethoprim in figure 2. Such a graphical representation could be extended to the enzymes involved in gluconeogenesis and pyruvate biosynthesis. It was not, however, possible to identify, with any degree of confidence, enough of the spots representing the enzymes involved in pyrimidine biosynthesis to construct any meaningful figure. Proteins that were found to be diagnostic for the two responses, most notably several of the stress proteins as previously noted for *E. coli,*[11] cannot be assigned to any particular pathway. This study demonstrated that responses showing similarity in one or more pathways to an already determined antibiotic target pattern, but deviating in others, can be an indication of a second target in the same pathway. An explanation for all the various changes that were observed is at present not possible. These studies are still in their infancy and much more experience is required to enable the dif-

ferentiation of secondary effects from the primary effects of inhibition. A further consideration when examining the response of the cell to an antibiotic is the specificity of the antibiotic. For example, quinolones, inhibitors of DNA gyrase, have been shown to inhibit topoisomerase IV[12] as well as, at higher concentrations, translation.[13]

The scarcity of information concerning the pyrimidine pathway is indicative of the limitations in the sensitivity of the methodology. On a preparative scale it is possible to assign spots representing almost half of the polypeptide products encoded within the genome. However, as mentioned above the difference in the migration of the polypeptides in preparative gels when compared with analytical gels excludes, in many cases, a direct transfer of this information. Pooling the excised spots from many analytical gels is at present the only reliable method of verifying the assignation of a spot. Undoubtedly, the sensitivity of mass spectrometry will be further improved so that all visible spots, even in analytical gels, can be annotated.

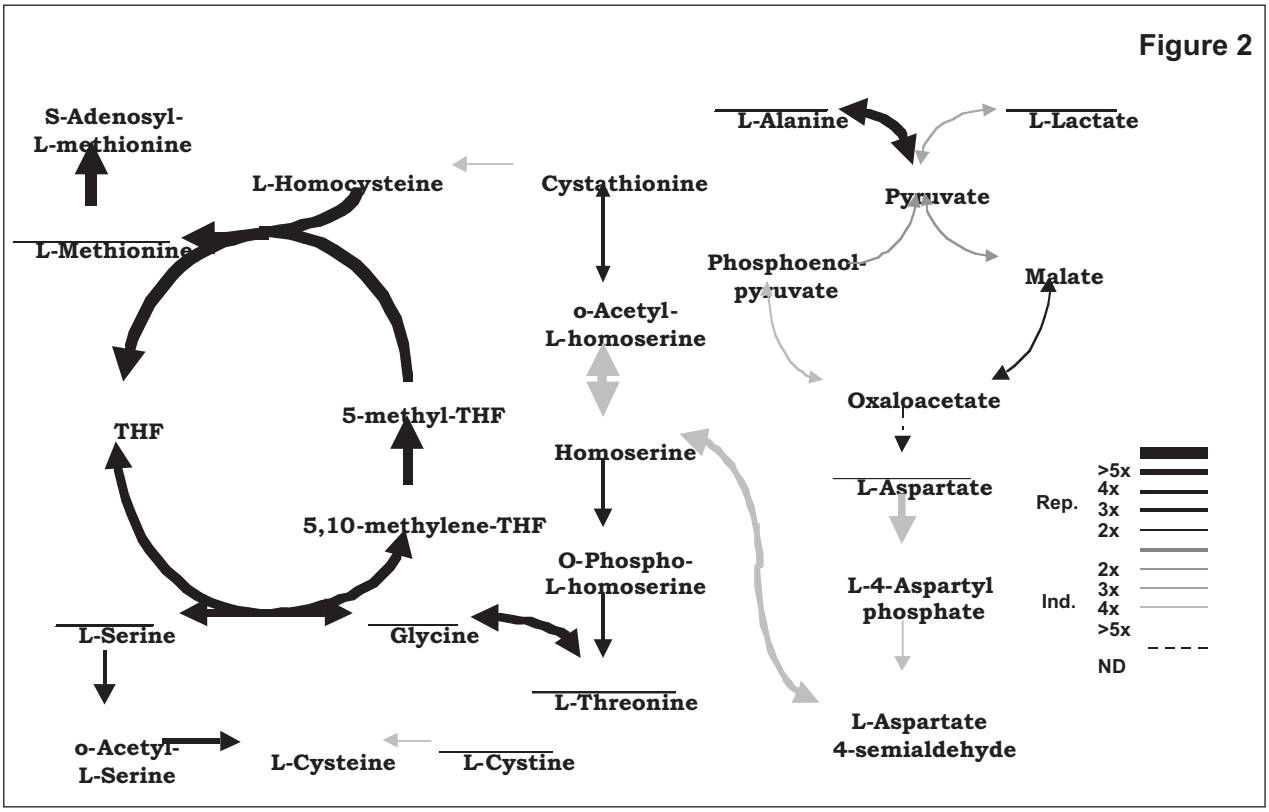


**Figure 2. Visualisation of the induction pattern of the enzymes involved in L-methionine biosynthesis after treatment with trimethoprim. *H. influenzae* were pulse labelled with 35S L-methionine for 3 minutes either with or without treatment with trimethoprim for 30 minutes. Protein extracts were run in 2D-PAGE and the PhosphoImager images from the dried gels were matched and quantified. The relative ratios of the intensities of the spots (with or without treatment with trimethoprim) were calculated and the values visualised graphically using a colour code. This code is converted to a grey scale/thickness code for the convenience of publishing.**
**Stippled arrows indicate information from the ratios of mRNA levels.**

## 4. Combining proteomics with transcriptomics

A complementary approach to proteomics is transcriptomics. In an early experiment we compared the relative ratios between exponentially growing cells and cells in stationary phase for a small number of protein levels to those of the corresponding mRNAs determined by Northern blotting. The ratios determined for the protein levels were much greater than those determined for the mRNA levels. However in the case of a similar experiment using trimethoprim as an induction agent, the ratios of a set of proteins synthesised from mRNA known to be transcriptionally regulated resulted in the expected higher ratio values for the mRNA. Recently we have begun to measure the mRNA levels using hybridisation to oligonucleotide arrays (Gene Chips from Affymetrix) thereby complementing the results that we obtain in the proteomics experiments. This technology has the advantages that the gene identity is known for all signals (at an appropriately high stringency) and that all-possible ORFs can be arrayed. At present a disadvantage is the amount of culture required to produce enough RNA for the analysis. This is particularly disadvantageous in the analysis of the responses of bacteria to novel compounds, which are of limited availability. From an initial analysis of our results it is also clear that some of the mRNAs corresponding to proteins identified in the gels are of too low abundance to be confidently quantified, again emphasising the complementary nature of the two technologies.

## 5. Conclusions

In conclusion proteomics is an important technology in the field of genomics, which when coupled to transcriptomics, provides a solid basis for the analysis of the networking of the bacterial response to its environment. It is clear that both technologies produce vast amounts of data and that appropriate software must be developed to process this data. We have made a start in this direction in that all experimental data is kept in an Oracle database from which we can access it using various commercial, e.g. MineSet, or in-house software that can display the data in a visual form.
The information obtained from these technologies, as with that obtained from gene disruption and computer assisted comparative analysis, is primarily of use in the formation of hypotheses that facilitate the design of further experimentation in genetics and biochemistry.

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SECTION III

# Compound acquisition and rationale for drug development

# Combinatorial Chemistry : A new tool in the search for biologically active molecules

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***Abstract :*** *Biological screening of large numbers of chemical compounds remains the favourite path leading to the discovery of new and original active molecules. Recent technological progress, including the automation of biological essays, has increased significantly the number of screened compounds in a given time. Pharmaceutical companies, chemical collections, traditionally obtained from plant extracts or from chemical synthesis, are no longer able to provide a sufficient diversity of new compounds for the discovery of new drug candidates. Peptide chemistry has practice in synthesis on solid support, automation, deep analytical methods and large variety of building blocks. Taking benefit of this practice, this field introduced the concept of combinatorial chemistry.*

*Combinatorial chemistry gave the chemist the opportunity to take up the challenge and generate quickly a large number of chemical compounds by condensing a small number of chemical entities together in all combinations defined by a given reaction sequence. For the medicinal chemist, combinatorial chemistry became a useful tool acting at two different levels of drug research. First of all, it allows the chemist to build up libraries of chemical compounds in order to find a "hit", that is to say a lead compound. Secondly, combinatorial techniques can be used to optimize the lead compound, generating families of related structures.*

## 1. Principle of combinatorial chemistry

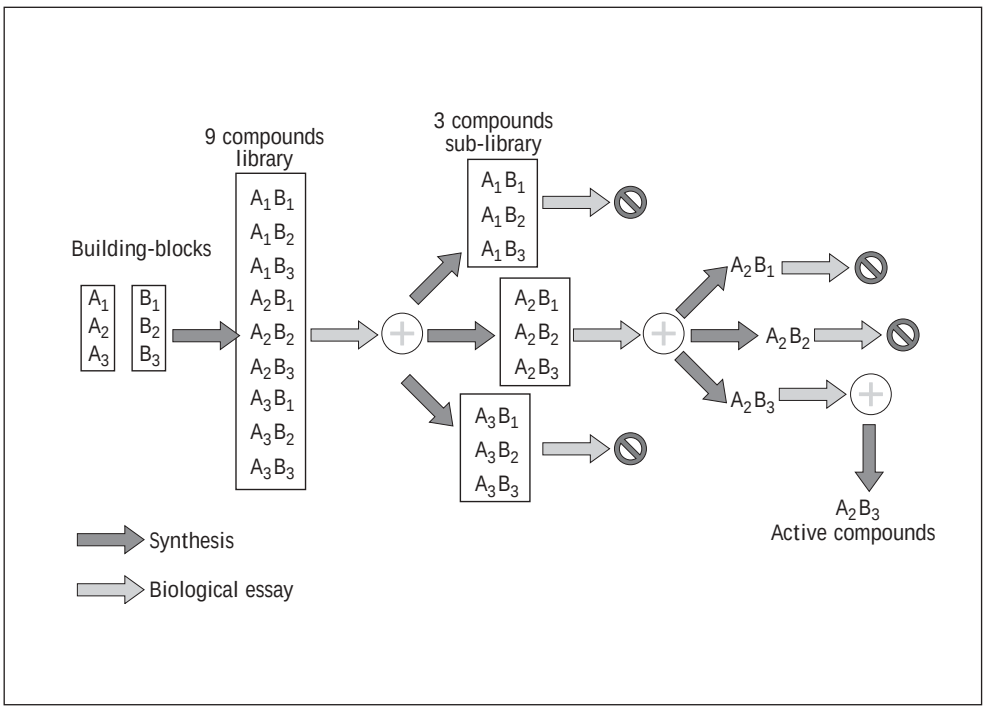
In traditional organic chemistry, two chemicals A and B react together leading to an unique compound A-B. In contrast, in combinatorial chemistry a

set of type A compounds (A1, A2,….An) reacts with a family of type B compounds (B1, B2,….Bm). These reactants are called *building blocks*. All the combinations of type AxBy chemicals will be generated in this way. This new set is called a library.

The key-step of combinatorial techniques resides in the identification of the active compound(s) among the library. The way in which libraries are built up determines the identification procedures that must be applied. So, two different strategies can be distinguished: synthesis of mixtures of compounds or parallel synthesis in which compounds are synthesized at the same time but separately.

## 2. Synthesis of mixtures

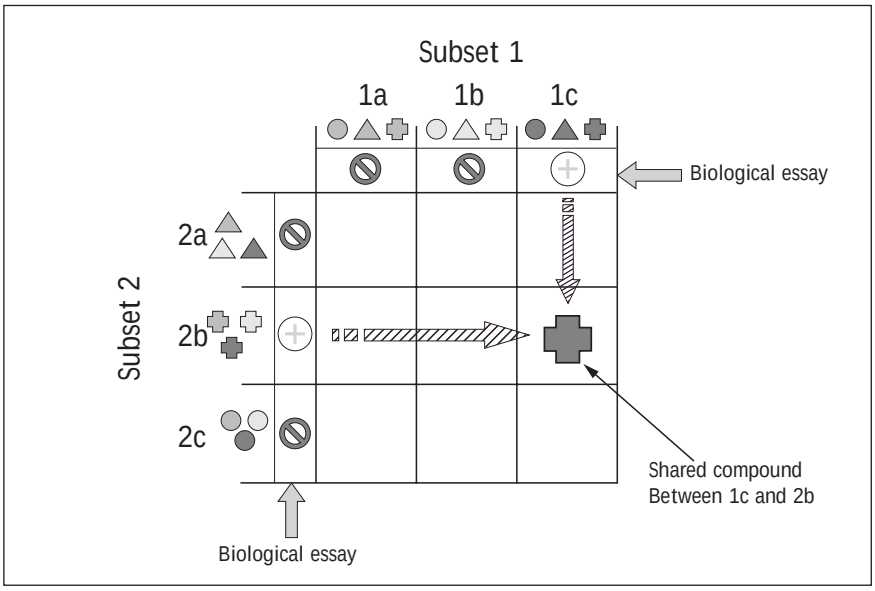
Whatever is the strategy employed to generate the mixture, the identification of the potentially active compound relies on the division of the library into smaller sets called sub-libraries. The simplest logical method developed by Houghten et al is called iterative deconvolution.[1] An example of a 9-compound library is illustrated below (Scheme 1).




**Scheme 1**

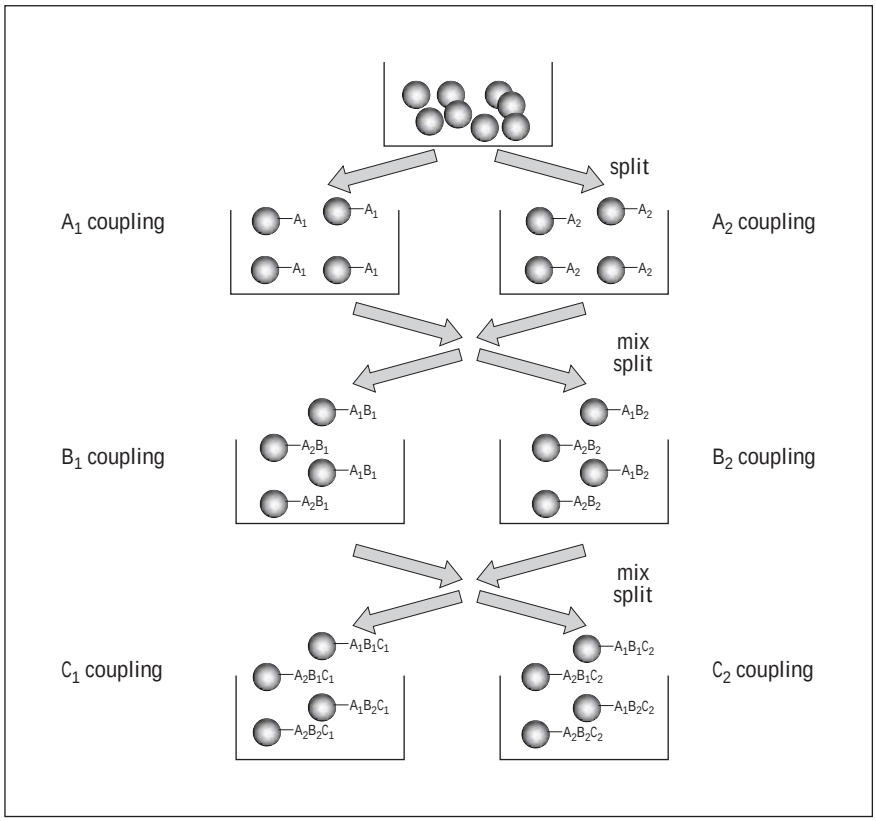
If a biological activity is detected for the whole library, three sub-libraries will be synthesized each obtained by the combination of one single building block of the first family and the whole set of the second one. Each of these sub-libraries will be evaluated separately leading to the synthesis of three separate compounds, among which is the active one. Unfortunately, this kind of process implies the synthesis of numerous sub-libraries for each detected biological activity.

An alternative method had been designed by Deprez et al. to facilitate identification of an active molecule within a mixture. This method called orthogonal libraries[2] relies on two "orthogonal" subsets of sub-libraries both containing all the compounds of the library. However, the synthesis is organized in such a way that any sub-library of the first subset shares one and only one chemical compound with any sub-library of the other subset. This property allows a direct identification of an active compound as illustrated below (Scheme 2).




**Scheme 2**

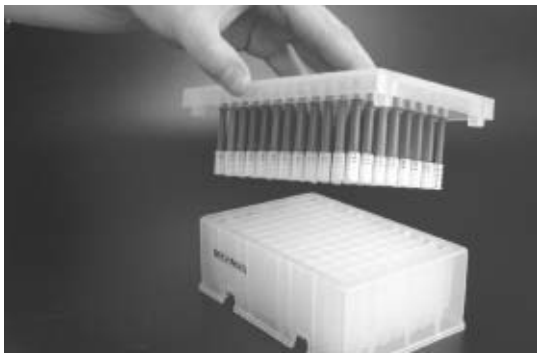
One of the problems linked to the synthesis of mixtures of compounds is the difficulty in controlling the composition of the library. Indeed, some compounds can be more abundant in the mixture than others, depending on the reactivity of the different building-blocks. The "split and mix" solid-phase synthesis method had been developed to overcome this drawback.[3]
Before each combinatorial step, the resin is split in a number of batches equal to the number of building-blocks introduced at this stage of the synthesis. Each of these resin batches reacts separately with a single building block. An excess of reagents can be employed to reach reaction completion. At the end of the step, the resin batches are mixed carefully together to obtain an homogenous distribution. The resin can be split once more in a number of batches equal to the number of building-blocks of the next step. At each stage of the synthesis, a given resin bead is in contact with a single building-block. Each bead follows a unique path and carries only one chemical species (Scheme 3).




**Scheme 3**

## 3. Parallel combinatorial synthesis

It is often difficult to find the more active molecule in a mixture library and to have an accurate idea of the exact composition of the mixture. For this reason the parallel combinatorial techniques, both in liquid and solid-phase, became very popular with medicinal chemists. The principle is very simple: parallel libraries are designed in the same way as their mixture counterparts but this time, the compounds are synthesized separately at the same time. This strategy has been fully supported by the recent development of robots and automatic synthesizers originatly design for peptide synthesis and is actually the more popular methodology to generate a large variety of compounds. However, some parallel techniques need little automation. One of them is the Multipin technology whose strength is the use of a new type of pellicular solid-support called "crown".[4] Crowns are tiny plastic objects whose surface is grafted with a wide range of polymers and linkers. Crowns can be fixed on and inert plastic stem which can be attached in an 96-well array format that fits perfectly with microtitre plates (Scheme 4). Ninety six different reactions can be done just by encasing the crowns in the wells containing reagents. Shared steps such as washing are done by dipping the 96 crowns in solvent-fulfilled tank. This kind of easy-to-use technology is useful to quickly generate middle sized libraries or to optimize reaction conditions by screening a wide range of solvents, concentrations, temperatures, and so on.



**Scheme 4**

## 4. Conclusion

Whatever the technique used, combinatorial chemistry becomes a powerful tool for the design of libraries of compounds and for the discovery of unexpected active material. Beyond the simple synthesis of large collection of compounds, the challenge for the medicinal chemist is to imagine and to optimize new reactions in order to be able to generate a wide molecular diversity. Robotization and automation of analytical methods to analyze a large number of samples in a minimum amount of time is the complementary challenge in the race for the discovery and optimization of new drug candidates.

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# Target discovery and validation with special reference to trypanothione

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***Abstract :*** *A number of criteria must be met before an enzyme or receptor can be regarded as a suitable target for drug discovery and drug development. This article considers the various approaches to target discovery, critically examines the criteria for target selection and compares the strengths and weaknesses of target validation by chemical and genetic methods. Particular reference is made to the prospects of drug development against trypanothione reductase, a target common to diseases caused by species of trypanosomes and the leishmania.*

## 1. Introduction

With the advent of whole genome sequencing, for each individual pathogen hundreds, if not thousands of potential drug targets are emerging. As discussed elsewhere in this book, resources for discovery of drugs against tropical diseases are extremely limited and thus the current challenge is to narrow down the list to a handful of the most promising targets. One way of achieving this is to consider criteria of what constitutes a good drug target in a parasite.

## 2. Characteristics of an ideal drug target

First, it should be obvious (though often ignored) that a drug target should be essential for either the survival or the growth of the pathogen in the host stage of the life cycle. The appropriate life-cycle stage must be considered, since host environmental cues (physiological, nutritional, chemical and immunological responses) can have a profound effect on gene expression and

consequently susceptibility to drugs. Inhibition of a target that is essential for survival is generally preferable to one that is required for growth, since the former is likely to have a cytotoxic effect whereas the latter will have a cytostatic effect.

The second criterion to be considered is that the target should be sufficiently different between host and parasite to allow selective inhibition. Ideally, the target should be unique to the parasite, but this is relatively rare in eukaryotic pathogens[1]—trypanothione and ovothiol in trypanosomatids, hydrogenosomal metabolism in trichomonads and haem detoxification in malaria parasites are good examples. Common targets that differ between host and parasite are relatively common and cover many areas of nucleic acid, protein, carbohydrate and fatty acid metabolism. Indeed, it has been stated that as many as one third of all genes are potential targets simply because a single amino acid change in the active site region of an enzyme can have a profound effect on the affinity of an inhibitor for its target. However, this is a gross oversimplification. Many of these can be immediately discounted as suitable targets, since a single point mutation in the pathogen gene could instantly render the organism resistant to the drug!

A third criterion for the selection of a drug target has to do with the technological demands of the subsequent stages of drug discovery (see article by J.J. Martin). For example, for structure-based drug design the target has to be amenable for study at the molecular level; and for high throughput screening one has to have convenient, cheap and sensitive assay methods that are suitable for miniaturisation and automation. Soluble enzymes that can be readily produced as active recombinant proteins are generally preferable to complex multi-component mixtures or membrane-associated molecules.

Some consideration should be made with regard to the nature of the substrate or ligand recognised by the enzyme, transporter or receptor. Recognition sites for small molecules less than about 200 Da generally limits the range of chemical diversity and three-dimensional space that can be explored in inhibitor design. Likewise, the need for a negatively charged compound for tight binding (e.g. phosphorylated compounds) may lead to difficulties in delivery of a potent inhibitor to the target in the intact cell. Finally, the structural uniqueness of the physiological ligand or substrate itself should be born in mind. For example, selection of a nucleotide or co-enzyme binding site of a unique target for inhibitor design, runs a higher risk of unexpected toxic side effects by inadvertent inhibition of a totally unrelated co-enzyme dependent enzyme in the host.

Another key trend in the pharmaceutical industry relates to the growing realisation that identification of inhibitors with drug-like properties should be introduced at an early stage in drug discovery, thereby eliminating candidate molecules that are unlikely to fulfil the requirements of the later stages of development (e.g. oral availability, toxicity). Lipinsky's 'rule of five' and other computational methods are often used for the prediction of 'drug-likeness'.[2,3]

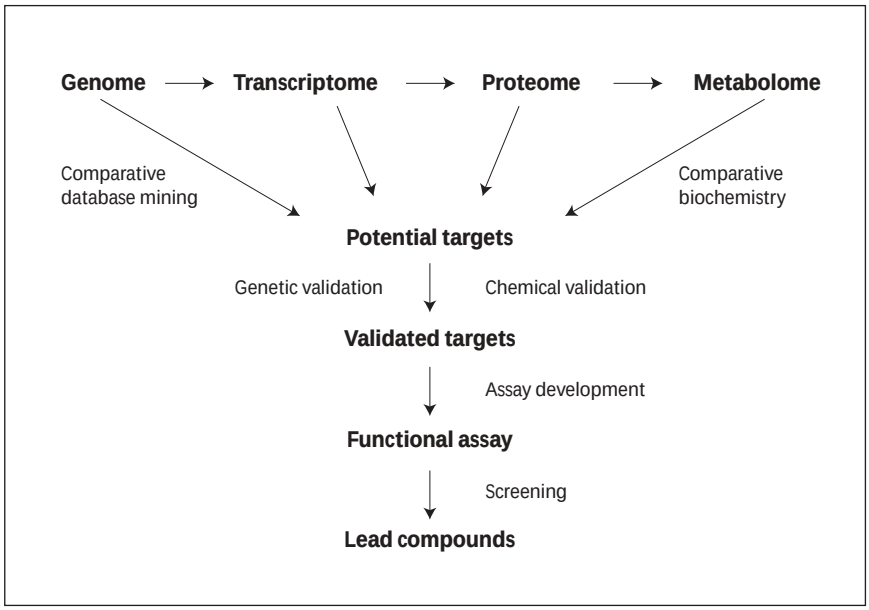
The above considerations should be regarded as guidelines rather than hard and fast rules. Not all drug targets meet these criteria. For example, ornithine decarboxylase in *T. brucei gambiense* is slightly less sensitive to inhibition by

difluoromethylornithine (DFMO, Ornidyl) than the human homologue! Nonetheless, the better a target adheres to the above criteria, the more likely a pharmaceutical company can be persuaded to consider investing time and energy into a drug discovery programme.

*2.1. Target discovery*

In the pre-genomic era, potential antimicrobial drug targets were identified through basic research (e.g. comparative biochemistry or molecular biology) or applied research (e.g. drug mode of action or drug resistance). In the emerging post-genomic era, these classical biochemical and molecular genetic methods are now being augmented with 'database mining' using bioinformatic techniques such as comparative genomics, proteomics and metabolomics (Figure 1). Database mining of the *Plasmodium falciparum* genome project has been particularly fruitful with the identification of the DOXP pathway (isoprenoid biosynthesis)[4] and, within the apicoplast, ribosomal protein synthesis[5,7] and type II fatty acid biosynthesis[8,9] as potential chemotherapeutic targets. These and other areas of parasite biochemistry and molecular biology are now under active investigation to fully characterise key components both structurally and mechanistically and to compare them with the isofunctional components of the host for metabolic, mechanistic or structural evidence for potentially exploitable differences.

**Figure 1. Key steps in the identification of novel drug targets and lead inhibitors.**




It should be noted that database mining is a useful adjunct to existing tools, but is unlikely to completely supplant traditional methods of target discovery in the immediate future. Bioinformatics cannot readily predict non-protein targets such as complex carbohydrate structures (e.g. bacterial cell wall as the target for penicillin), membrane lipids (e.g. amphotericin) or detoxification mechanisms such as haem incorporation into haemozoin (e.g. chloroquine and artemisinin); neither can it predict the functions of enzymes associated with unique metabolic pathways (e.g. ovothiol[10] or trypanothione [11]).

A substantial proportion of putative open reading frames in parasite genomes have no homology with genes from other species, yet these may have essential and possibly unique functions thereby rendering them potential drug targets. The construction of metabolomes (the sum total metabolic pathways in an organism) may be useful in identifying pathways in which one or more enzymes are apparently absent. If the entire genome of a pathogen is known, then the absence of enzymes in a pathway whose existence has been demonstrated by classical biochemical methods should immediately prompt further investigation to identify these enzymes since they are likely to have substantial differences from those of the mammalian host.

*2.2. Target validation*

There are two principal methods by which a potential target can be demonstrated or 'validated' for the drug discovery process: chemical and genetic. Where possible both approaches should be employed as they can yield valuable complementary as well as confirmatory information.

Validation by chemical means involves the use of drugs or lead compounds to provide experimental evidence that specific inhibition of a target leads selectively to cessation of growth or death of the parasite. Not only can lead compounds (e.g. antimetabolites) be employed to verify the vital nature of a particular metabolic or signalling pathway, but also can identify unexpected toxic consequences arising as a consequence of inhibition. For example, a substrate analogue could be involved in lethal synthesis of a toxic end-product (e.g. allopurinol incorporation into nucleic acid), or formation of a lethal complex (e.g. fluoroquinolones forming a ternary complex with DNA gyrase and DNA). Likewise, a chemical ligand binding to a receptor can have unexpected effects on downstream signalling pathways. Chemical tools can also identify non-protein targets such as bacterial cell walls, haemozoin and membrane lipids as mentioned above. In addition, the chemical approach also has the advantage of assessing the likelihood of successfully delivering a drug to the correct intracellular compartment. However, specific inhibitors may be missed simply because they are unable to penetrate cellular membranes. Another drawback is that lack of specificity may lead to erroneous conclusions about the importance of a particular target. Comparison between drug-resistant and drug-sensitive parasites can also provide important clues; however, the diverse mechanisms by which resistance can arise[12] means that such findings are hardly ever conclusive.

Genetic validation is regarded by some as the most definitive method for demonstrating that a drug target is essential for growth or survival of a pathogenic organism.[13] However, essentiality does not mean that the target is sufficiently different from that of the host to allow selective inhibition. The precise techniques employed depend on the genetic tools available for any given parasite (e.g. availability of inducible or non-inducible expression vectors; choice of drug-selectable markers) and the genetic and physiological properties of the organism under study (e.g. gene copy number, ploidy; ease of culture in defined media; susceptibility to drug selection; ease of transfection). In this respect, genetic manipulation of leishmania and trypanosomes is generally easier than that of malaria, although techniques for

genetic manipulation of *Toxoplasma gondii*[14] can provide invaluable information about probable outcomes in malaria.

At the DNA level, targeted gene deletion by homologous recombination with a gene conferring resistance to a toxic drug or experimental compound will completely ablate expression of that particular allele. If the organism is diploid or haploid with more than one genetic locus for the potential target, then multiple rounds of transfection with gene-deletion constructs and selection with multiple drug-selectable markers will be required. Various outcomes of such experiments have been observed and, as discussed below, must be interpreted with caution.

First, the ability to obtain viable organisms in which expression of a target is completely abolished is suggestive that the target is not essential for growth or survival and therefore probably not a drug target. However, such phenotypes must be demonstrated in the appropriate life-cycle stage and life-cycle environment. Indeed, if the phenotype of such a null mutant can be predicted, it may be possible to obtain viable cells by growth in medium containing an appropriate supplement. For example, viable mutants of African trypanosomes and leishmania lacking ornithine decarboxylase activity can be obtained by selection in medium supplemented with putrescine, the product of this enzyme.[15,16] It can also been argued that certain drug targets will be missed by this approach. For example, when a target converts a pro-drug into an active toxic metabolite (e.g. allopurinol misincorporation into nucleic acids). While this may be true, it could also be argued that the likelihood of developing drug resistance by complete loss of the target or pathway is fairly high and therefore not the best choice in the long-term for investment of large amounts of time and money for drug discovery.

In situations where chromosomal null mutants cannot be obtained, this negative result is suggestive, but not absolute proof, of the essential nature of the target. Further evidence can be obtained by 'rescue' of chromosomal null mutants by expression of another copy of the target (sometimes from a related species) either on an episomal vector or at another chromosomal locus. Unfortunately, this reveals no information as to precisely what level of enzyme activity is compatible with growth and survival and therefore what level of inhibition has to be achieved by drug treatment. Inducible or repressible gene-expression systems, such as the tetracycline inducible-system developed by Clayton's group for the African trypanosome,[17] are urgently required for other parasitic systems.

Stable overexpression of drug targets can also be informative. For example, if one already has a potent and selective inhibitor available, then the predicted phenotype would show a correlation between overexpression and decreased sensitivity to the compound. However, it should be borne in mind that if the target is only one component of a complex metabolic or signalling pathway, then the expected phenotype might not be evident (see article on metabolic control analysis by Michels and colleagues in this volume for details).

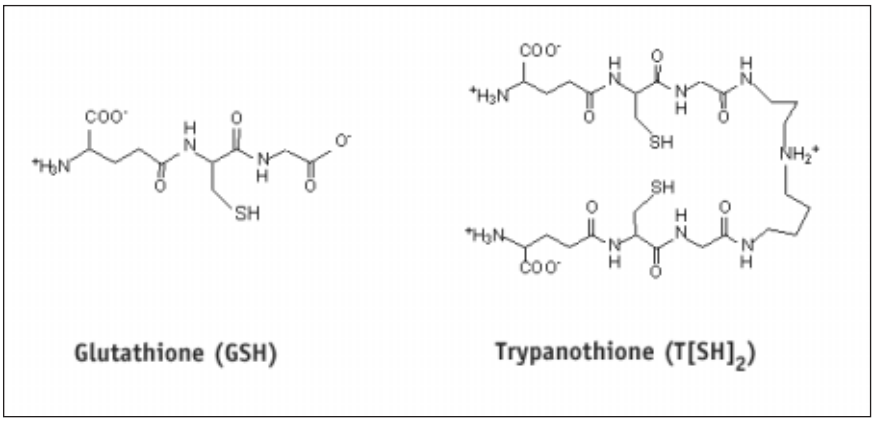
At the RNA level, expression of a target can be modulated by transient or stable expression of anti-sense RNA, by uptake of antisense oligonucleotides[18] or by RNA interference (RNAi) using dsRNA.[19,20] Better methods are required

to effectively deliver antisense oligonucleotides and dsRNA into all cells in a culture, particularly in malaria. Stable expression of antisense RNA or dsRNA using inducible systems is preferable to constitutive expression, since transgenic organisms lacking an essential biochemical component generally cannot be selected – only 'escape mutants' can be recovered making interpretation difficult.

Where the mechanism of an enzyme or a component of a signalling pathway is known at the molecular level then it is sometimes possible to engineer dominant-negative mutant proteins that interact with the wild-type protein to disrupt its normal function. As described below, this has been used with some success against trypanothione reductase.[21]

## 3. Genetic and chemical validation of trypanothione as a drug target

Trypanothione[11] was discovered in the 'pre-genomic era' through investigations on the mode of action of arsenical drugs and the glutathione biosynthesis inhibitor, buthionine sulfoximine (BSO) against African trypanosomes. This metabolite is unique to trypanosomes and is absent from mammalian cells, where glutathione is the major low-molecular-mass thiol.




The thiol-dependent redox roles of trypanothione include the maintenance of the correct intracellular reducing environment for enzymatic activity and antioxidant functions such as the removal of peroxides or the trapping of free radicals (see reviews[22,24]). It also has specific roles in deoxyribonucleotide synthesis[25] and reduction of dehydroascorbate.[26] In Leishmania species, it is implicated in resistance to trivalent antimonial drugs[27,28] and, in some parasites, it may also serve as a reservoir for polyamines or glutathione.[29,30] The principal form found in the parasites is the dithiol, which is consumed in redox metabolic reactions to form trypanothione disulphide. The disulphide form is rapidly reconverted to the dithiol by the NADPH-dependent enzyme trypanothione reductase (TryR), a homologue of glutathione reductase. Although trypanosomes contain glutathione and thioredoxin-like proteins, they apparently lack glutathione reductase and thioredoxin reductase activity; thus, the trypanothione/trypanothione reductase

system appears to substitute for both glutathione /glutathione reductase and thioredoxin/thioredoxin reductase systems present in most other organisms. The biosynthesis and principal functions of trypanothione are summarised in Figure 2, which also emphasises the central role of TryR in thiol-redox reactions.

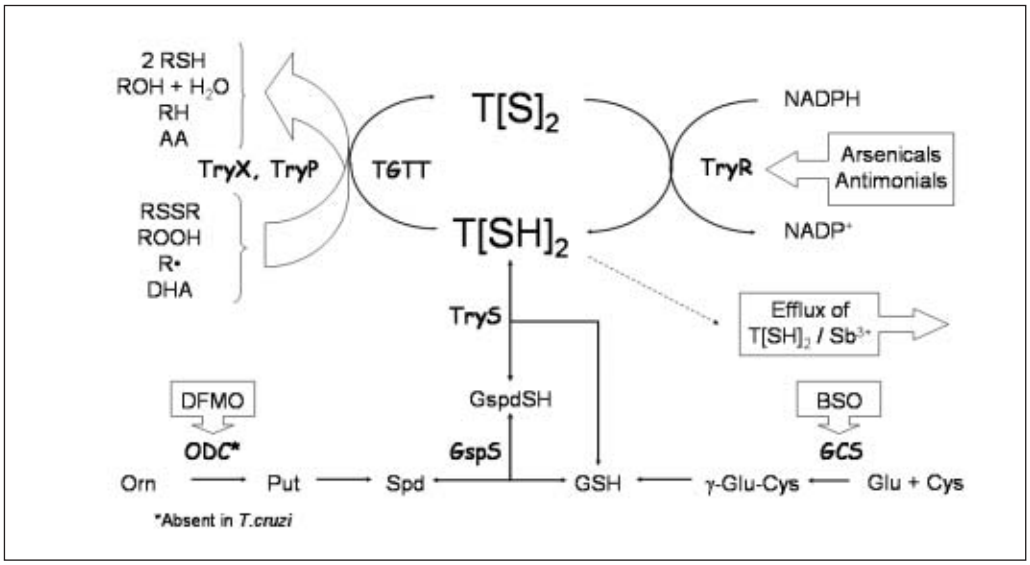



**Figure 2. Biosynthesis and protective roles of trypanothione (T[SH]2). Inhibitors that interfere with trypanothione metabolism are boxed. The key enzymes that are potential or actual drug targets are: trypanothione reductase (TryR), tryparedoxin (TryX), tryparedoxin peroxidase (TryP), trypanothione: glutathione thiol transferase (TGTT in *T. cruzi*), trypanothione synthetase (TryS), glutathionylspermidine synthetase (GspS), ornithine decarboxylase (ODC in *T. brucei*) and g-glutamylcysteine synthetase (GCS). Other abbreviations: RSSR, any disulphide; ROOH, any hydroperoxide; R∑, any radical; DHA, dehydroascorbate; AA, ascorbic acid.**

Chemical and genetic approaches have been used to examine the importance of trypanothione metabolism for the growth and survival of trypanosomes and leishmania. Inhibitors of polyamine and glutathione biosynthesis (e.g. DFMO and BSO) lower intracellular levels of trypanothione. Exposure to nifurtimox or benznidazole is also reported to decrease intracellular levels of trypanothione.[31] The trivalent arsenical, melarsen oxide, forms a stable adduct with trypanothione, which in turn is a competitive inhibitor of TryR.[32] Trivalent antimonial compounds are time-dependent inhibitors of TryR[33] and elevated trypanothione biosynthesis is associated with resistance to antimonial drugs in laboratory-derived strains.[28] Various experimental inhibitors of TryR also have lethal activity against trypanosomes and leishmania in vitro (see review [34]). DFMO and arsenicals are synergistic in killing trypanosomes in vivo and DFMO and BSO reverse antimony-resistance in vitro. However, despite this impressive list of activities, many of these effects could be non-specific and therefore unrelated to the microbicidal activity of these drugs and experimental compounds. In view of these uncertainties, several genetic approaches have been used to specifically examine the role of TryR.

The first approach involved overexpression of TryR in *T. cruzi* and *L. donovani*. Although enzyme activity was increased by up to 8-10 fold, such cells grew

normally and showed no increase in resistance to H2O2 or drugs thought to act via oxidant stress.[35] However, when intracellular thiols were oxidised by exposure to diamide, overproducing cells were able to recover normal thiol levels more rapidly than wild-type cells. It was concluded that the rate-limiting step in peroxide metabolism must be elsewhere in the pathway. The subsequent discovery by Flohe's group that metabolism of peroxides involved two additional proteins (tryparedoxin and tryparedoxin peroxidase[22]) accords with this observation.

The second approach involved the expression of anti-sense RNA from a plasmid in *T. cruzi*.[36] Unexpectedly, TryR activity was unchanged in cells over expressing anti-sense RNA. Further investigation revealed that these represented escape mutants in which a proportion of the plasmids had undergone rearrangement of the insert from antisense to sense orientation, thereby effectively neutralising the antisense effect.

The third approach involved the expression of a dominant-negative mutant *T. cruzi* TryR in *L. donovani*.[21] Functionally active TryR is a dimer with three key residues required for catalysis: a pair of cysteine residues provided by one monomer and a histidine residue provided by the second monomer of the dimer pair. Overexpression of a doubly crippled mutant (C53A/H461Q) depleted TR activity in recombinant cells by up to 85% and these cells showed a significant impairment in their ability to regenerate dihydrotrypanothione from trypanothione disulfide following oxidation with diamide. Nonetheless, trans-dominant mutant recombinants were still capable of maintaining a reduced intracellular environment during cell growth in culture and were able to metabolise hydrogen peroxide at wild type rates in vitro. Importantly, however, amastigotes that expressed the trans-dominant mutant enzyme displayed a decreased ability to survive inside activated macrophages in a murine model of Leishmania infection. The apparent inability of *Leishmania* to modulate the expression of active TR homodimers in response to the expression of trans-dominant mutant protein suggests that specific inhibitors of this enzyme should be useful anti-leishmanial agents, provided enzyme activity could be inhibited by more than 85%.

The fourth approach used gene disruption or gene replacement technologies in various *Leishmania* spp.[37,38] Although a single allelic replacement in diploid clones resulted in a 50% reduction in enzyme activity, null mutants could not be obtained, due either to chromosomal rearrangements or to gene amplification. Only when cells were supplemented with an extra-chromosomal copy of TryR could homozygous chromosomal null mutants be obtained. As above, cells with a 50% reduction in TryR levels showed an impaired ability to survive within activated macrophages. These results indicated that TryR is essential for the growth and survival of leishmania parasites. A more sophisticated approach was employed in *T. brucei* using conditional knockouts.[17] Recombinant cell lines were generated where an additional copy of TryR was inserted into a separate locus under the control of a tetracycline-inducible promoter and the two wild-type alleles were replaced with the drug resistant marker NEO. In the presence of tetracycline, these cells had a normal growth phenotype with TryR activity increased up to 4-fold. However, on withdrawal of tetracycline, TryR levels fell to less than 10% of controls,

resulting in cytostasis followed by cell death. This was associated with loss of virulence in mice and hypersensitivity to H2O2 in vitro. Escape mutants were also observed where expression of TryR was recovered via an unknown mechanism.

## 4. Suitability of trypanothione reductase as a drug target

As discussed above, essentiality of an enzyme or a receptor does not necessarily mean that a target is amenable for drug development. The following examines the potential strengths and weaknesses of TryR as a case for further drug development.

The molecular architecture and chemical mechanism of TryR is known in considerable detail (e.g. see [39,40] as is the case for the human homologue, glutathione reductase. Although the human and parasite enzymes share similarities in overall secondary structure and kinetic mechanism, they show a pronounced ability to discriminate by over 1000-fold between their cognate substrates, glutathione disulphide and trypanothione disulphide, respectively.[24] The molecular basis for this remarkable two-way discrimination involves size, charge and hydrophobicity/hydrophilicity of the active sites, reflecting the physicochemical properties of the substrates. Thus, the trypanothione disulphide-binding site in TryR is larger than that of glutathione reductase, contains a hydrophobic cleft for recognition of the polyamine moiety of trypanothione and a negatively charged glutamate side-chain that effectively repels glutathione disulphide from the active site. Conversely, glutathione reductase has a smaller active site and a positively charged region that together effectively exclude trypanothione from its active site. Structures of both enzymes and their respective substrates have been described.[39,41] making structure-based drug design an attractive proposition. Numerous lead inhibitors with moderate binding constants (~1 mM) have been identified for TryR including various tricyclics, substituted polyamine analogues and aminodiphenyl-sulphides (see review[34]). High-resolution structures of inhibitors bound to TryR are urgently required to aid in further refinement of these leads.

With regard to screening, recombinant TryR from *T. cruzi* can be readily produced in active form in large amounts (>106 assays from 2 l culture).[40] A convenient colourimetric microtitre plate assay has been developed in this laboratory, which is suitable for automated high-throughput screening. This has undergone pilot tests in collaboration with Glaxo Wellcome (now GSK); a test screen of 25 000 compounds has identified 100 promising leads for further development.

One potential pitfall of TryR as a drug target needs to be considered. The intracellular concentration of trypanothione varies between 0.3 mM in the African trypanosome to about 3.0 mM in *Leishmania spp*. It is estimated that > 99% is present as the reduced form (the product of the TryR-catalysed reaction) and thus the intracellular concentration of the disulphide form (the substrate for TryR) lies between 3-50 mM under normal steady state conditions. However, as soon as TryR is inhibited, T[S]2 will start to accumulate due to continuing oxidant processes in the cell. It is not known at present

what ratio of T[S]2 to T[SH]2 is growth inhibitory or lethal to the cell, but no major changes in thiol levels were observed when TryR levels fell to about 90% of controls. Nonetheless, to take a worse case scenario, where half of the T[SH]2 must be converted to T[S]2 before death occurs, then it can be easily calculated that competitive inhibitors with Ki –values in the nanomolar range will be required to maintain inhibition at >90% in response to accumulating T[S]2. This is by no means insurmountable, but suggests design of irreversible inhibitors may be a better strategy for drug development.

## 5. Conclusions

The currently available chemical and genetic data strongly indicates that TryR is essential for growth and survival in African trypanosomes and leishmania and therefore a candidate drug target. Studies are underway to confirm that this also applies to *T. cruzi*. Since TryR has one known catalytic function, namely the reduction of T[S]2 to T[SH]2, then this also suggests that its substrate, trypanothione, is also essential. Definitive proof of this hypothesis awaits the results of knockout and inhibitor studies on the unique biosynthetic enzymes, glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS) shown in Figure 2. More chemistry input from both academia and industry is required to refine the currently available leads and turn them into serious drug candidates.

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# Targets for antimalarial drug development derived from basic research

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***Abstract :*** *Metabolic pathways in the malaria parasite offer a rational basis for drug development. Enzymes of pathways that are unique for the parasite are thought as evident targets. In this brief examination, various "old" and novel targets are inspected: 1. The unique process of ingestion and digestion of haemoglobin, including the postranslational processing of the involved proteases. 2. The inhibition of the detoxification of ferriprotoporphyrin IX. 3. Interference with antioxidant metabolism of the infected erythrocyte. 4. The metabolism of methionine and the biosynthesis of polyamines. 5. The biosynthesis of folate. 6. The use of iron chelators aimed at ribonucleotide reductase deserves further efforts. 7. The new pathways being discovered to be associated with the apicoplast – an organelle that is unique to the parasite. 8. The biosynthesis of ferriprotoporphyrin IX. With the completion of* Plasmodium falciparum *genome project, novel pharmacogenomic methods may reveal additional, hitherto unforeseen targets for the development of new drugs.*

## 1. Introduction

The use of parasite metabolic processes as potential targets for the development of antimalarial drugs has been recently reviewed (1). Due to space limitations, in this review I shall only briefly discuss "old" targets that got little attention, and novel targets that have emerged only recently.

## 2. Haemoglobin degradation

During its asexual development the parasite endocytoses into the food vacuole a considerable portion of its host cell cytosol that consists of 95%

haemoglobin (Hb). Although the process of ingestion is well known at the ultrastructural level, the molecular details are unknown, as are the process of pro-plasmepsins and HRP proteins delivery to the endocytic vesicles. This process is unique to the parasite, and therefore deserves close scrutiny as it may provide novel targets. Specific parasite aspartic, cysteine and a novel metal proteases digest the ingested in Hb digestion in an ordered manner.[2] The genes have been cloned and expressed in heterologous systems. Specific inhibitors have been identified and shown to inhibit parasite growth in culture and in vivo, and the combination of plasmepsin and falcipain inhibitors was shown to be synergistic.[3] Non-peptide inhibitors of falcipain were designed through molecular modeling,[4] and new lead compounds were selected as plasmepsin II inhibitors from a combinatorial library.[5] Based on selective inhibition, it has been suggested that the proteolytic processing of pro-plasmepsins may be mediated by specific enzymes. So far, the concentrations of protease inhibitors needed for antimalarial therapy in vivo seem to be too high to avoid toxicity to the host.

*2.1. Detoxification of ferriprotoporphyrin IX*

Large amounts of ferriprotoporphyrin IX (FPIX) are produced during Hb digestion. Only a fraction of this FP is polymerized into the apparently harmless haemozoin by a process mediated by histidine-rich proteins,[6] while the rest exits the food vacuole to be degraded by glutathione.[7] Both processes are inhibited by chloroquine and amodiaquine, while quinine and mefloquine inhibit only the first one.[8] While efforts have been directed at the synthesis of more efficient inhibitors of FPIX polymerization,[9] most screening tests were done in absence of HRP. A new direction should be proposed due to the involvement of glutathione-dependent degradation of FPIX, and assays for drug specificity are available. The degradation of FPIX probably provides iron to the parasite. In view of the vast amounts of iron that are thus produced, it may be valuable to look into the mechanism of compartmentalization and neutralization of this potentially noxious transition metal. Interference with this process may provide a new chemotherapeutic approach.

*2.2. Redox and glutathione metabolism*

Oxidative stress is defined as an imbalance between antioxidants and prooxidants in the favour of the latter. Subtle oxidative pressure as that impinged by phagocytes and polymorphonuclear leukocytes[10] can kill the parasite because infected cells are initially under oxidative stress by reactive oxidative species produced by the parasite itself. H2O2 produced during Hb digestion exits the vacuole to be detoxified by both parasite and host cell catalase and glutathione peroxidase (Gpx).[11] Superoxide anions can also be produced by the mitochondrial respiratory chain and other oxidases, and are dismutated to H2O2 by the specific mitochondrial Mn-SOD and cytosolic Fe-SOD. Genes of parasite SOD and Gpx have been cloned and specific parasite catalase activity has been demonstrated.[12] Prooxidants[13] and compounds interfering with glutathione metabolism[14] have been suggested for years as potential antimalarials, but none has emerged as a plausible drug. Gpx links the antioxidant defense to the metabolism of glutathione. The parasite has

the ability to synthesize glutathione de novo and even to supply it to the host cell.[15] The gene for *-Glu-Cys synthetase has been cloned,[16] displaying variable numbers of the YQS(N/L)LQQQ motif in different geographical strains. The significance of this motif for enzyme activity remains to be established. Buthionine sulfoximine inhibits this enzyme as well as parasite growth in vivo.[16,17] This compound is presently tested in the clinic to overcome glutathione-dependent drug resistance of cancer cells (18). Two genes coding for glutathione reductase (GR) have been cloned from *P. falciparum.*[19,20] The first gene was subsequently shown to code for thioredoxin reductase.[21] BCNU (1,3-bis (2-chloroethyl)-1-nitrosourea) inhibits GR and parasite development,[22] and is also used in cancer therapy. Significant differences in the sequence of the ligand and drug binding site between the human and the parasite genes, may pave the way for the development of new drugs.[14] Other compounds can deplete glutathione by adduct formation that is mediated by glutathione transferase. It should be emphasized that glutathione depletion induces apoptosis.

Another system that may be involved in antioxidant defense is the thioredoxin/thioredoxin reductase (TrxR) couple. TrxR catalyzes the transfer of electrons from NADPH to Trx, which in turn acts as a reductant of disulfide-containing proteins and most significantly ribonucleotide reductase. The gene coding TrxR has been cloned and expressed[23] and the sequence of the active site was characterized.[21] The gene coding for Trx has been recently identified on *P. falciparum* chromosome #3.

## 3. The methionine cycle, methylation and polyamines

The methionine cycle in parasites has recently received attention since it is central for providing substrates for methylation of DNA, RNA, proteins and phospholipids, and for the synthesis of polyamines.[24] Although the basic pathways resemble those of their mammalian host, some enzymes and metabolites are unique to parasites, and therefore constitute potential targets for chemotherapy. Seleno-methionine, sinefungin and neplanocine inhibit the conversion of S-adenosylhomocysteine to homocysteine (that serves as a substrate for the generation of tetrahydrofolate from 5'-methyltetrahydrofolate) by the specific hydrolase. The methionine recycling pathway provides some targets for antimalarial compounds,[25] and 5'-methylthioribose kinase demonstrably converts some suicide substrates into potent toxic compounds.[26]

Polyamines are essential for cell proliferation as inducers of conformational changes in DNA and RNA and as modulators of chromatin structure.[27] They are produced from adenosylmethionine, an intermediate of the methionine cycle and putrescine, or can be supplied by the host. Several inhibitors have been developed as potential anticancer agents against ornithine decarboxylase, adenosylmethionine decarboxylase, spermidine and spermine synthetases, but very few have been tested against malaria parasites. DL-*-difluoromethylornithine, and inhibitor of ornithine decarboxylase that mediates the synthesis of putrescine (the substrate for spermine and spermidine synthesis) is a cytostatic antimalarial that specifically inhibits the synthesis

of some proteins,[28] but extracellular polyamines reverse this inhibition. Nothing is known about the transport of polyamines in infected cells. It is likely that the combination of transport inhibitors and the growing number of inhibitors of polyamine biosynthesis[29] may provide a useful antimalarial concoction.

## 4. Folate metabolism

Tetrahydrofolate is an essential coenzyme that contributes one carbon groups for the synthesis of amino acids and nucleotides. Enzymes involved in folate metabolism are traditional targets for antimalarials. Mutations in dehydrofolate reductase (DHFR) and dehydropteroate synthase (DHPS) that confer resistance to pyrimethamine and sulfadoxine (SDX) occur in a stepwise, progressive accumulation of mutations in DHFR and DHPS.[30] Sometimes, no correlation is observed between in vivo and in vitro sensitivity, because subphysiological levels of pABA and folate are used in the latter. The variations in folate/pABA levels in patients may contribute to drug resistance. There are indications that folate uptake may vary among parasite strains, and this would obviously contribute to resistance as exogenous folate rescues parasites from inhibition by SDX.[31] Another interference in drug action may result from the ability of the parasite to salvage from the host cell exogenous methyltetrahydrofolate (folinic acid) that can be used for the regeneration of tetrahydrofolate,[32] and its addition can rescue parasites from the inhibition by cycloguanil and WR99210.[33] These aspects of resistance have not been fathomed.

Combination of DHFR and DHPS inhibitors is synergistic. Low-level resistance of DHPS are sometimes associated with high resistance to combinations: synergy may depend primarily on pyrimethamine response or other mechanism(s) contribute to SDX resistance. Molecular modeling of DHFR has contributed to the development of new pyrimethamine derivatives,[34] and to the identification of new lead compounds.[35] Transformation of *P. falciparum* with human DHFR has revealed that proguanil does not target the parasite enzyme as its metabolic product cycloguanil does.[36,37] Through the use of this system, it is becoming evident that inhibitors of folate metabolism may have additional targets than the specific enzymes that they inhibit. This system also provides for screening of new DHFR inhibitors.

## 5. Ribonucleotide reductase and iron chelators

Ribonucleotide reductase catalyzes the reduction of ribonucleotides to 2'-deoxyribonucleotides. Genes encoding for the 2 subunits of this heterodimeric enzyme have been cloned and sequenced. The activity involves the transfer of electrons from NADPH through thioredoxin/glutathione reductase to thioredoxin/glutathione (see above) and to the enzyme's catalytic site. A tyrosyl radical that catalyzes the replacement of the C-2 OH of the ribose ring by H, is also present in the catalytic site and is maintained by an adjacent iron center. Iron chelators have been considered as putative

inhibitors. Although different chelators were active in culture and in mouse malarias, and new chelators are constantly tested,[38] their clinical efficacy has yet to be determined. There is always the imminent danger that the use of chelators may exacerbate anemia that is one of the hallmarks of falciparum malaria.

## 6. The apicoplast and the shikimate pathway

Malaria parasites, like other apicomplexans, have a specific organelle that resembles plant plastids. As the host does not have such organelles, the biochemical processes that take place in the parasite apicoplast could serve as distinct and specific targets for novel antimalarials. Indirect evidences suggest that *P. falciparum* expresses the shikimate pathway that is characteristic of plant plastids.[39,40] The activity of four of the six enzymes of shikimate biosynthesis could be detected in *P. falciparum*. However, the absence of a leader sequence in the chorismate synthase[39] and its phylogeny[41] suggest that the apicomplexan enzyme is cytosolic -like in fungi. Even if the shikimate pathway is not related to the apicoplast, it prevails as a specific target for novel drugs. The elucidation of the role of the parasite plastid may provide additional targets. This is supported by the ability of thiostrepton (a thiazole-containing antibiotic) to specifically bind to the malarial plastid rRNA and to inhibit parasite growth.[42]

The shikimate pathway could be the source of p-aminobenzoic acid (pABA) needed by the parasite for the synthesis of folic acid. This synthesis is achieved by the following sequence of reactions: shikimate * shikimate-5-phosphate * 3-enolpyruvyl-shikimate-5-phosphate * chorismate * 4-amino-4-deoxychorismate * pABA. The activity of the enzymes involved, i.e., aminodeoxychorismate synthase and aminodeoxychorismate lyase, has been detected and the genes of both enzymes as well as aroE which codes for chorismate synthase have been identified.

There are, however some difficulties with the emerging picture: First, both pABA and folate are present in the serum of normal humans. Both can enter into infected cells, and their presence antagonizes the action of sulphus drugs (inhibit DHPS) and of pyrimethamine and cycloguanil (inhibit DHFR). It is hard to understand why the drugs are so effective when the products of the inhibited enzymes can be obtained from the host. Whereas pABA can rapidly enter into the infected cell and be incorporated into metabolic intermediates,[43] nothing is known about the transport of folate. In this context, it is worth mentioning that different strains of *P. falciparum* take variable times to adapt to grow on folate-containing medium that is devoid of pABA, and that the reversal of antifols activity in the presence of folate is not always related to innate, gene-mutation-related resistance.[31] These observations suggest that the transport of folate into infected cells may determine the sensitivity of parasites to sulfonamides in addition to the observed point mutations dihydropteroate synthase, a matter of great importance that deserves a deep scrutiny.

Chorismate, the end product of the shikimate pathway, is also a precursor of the biosynthesis of coenzyme Q (CoQ; ubiquinone). CoQ plays a pivotal role

in the mitochondrial respiratory electron transfer, that in plasmodia is essential in the synthesis of pyrimidines as well as in other functions.[44] The other precursor of CoQ is mevalonate. As inhibitors of hydroxymethyl-glutaryl-CoA reductase that is involved in mevalonate synthesis, also inhibit parasite growth.[45] *Plasmodium spp.* incorporate [14C]pHBA into ubiquinone. Incorporation of shikimate has not been tested. Atovaquone, hydroxynaphthoquinones, and some degradation products of primaquine may be competitive inhibitors of CoQ-mediated electron-transfer. The remarkable sensitivity of electron-transfer at CoQ to salicylhydroxamate, an inhibitor of CN--insensitive oxidase, may explain the synergistic action of atovaquone and SHAM.[46]

## 7. Biosynthesis of ferriprotoporphyrin IX

Although vast amount of ferriprotoporphyrin IX (FPIX) are produced when the intraerythrocytic parasite digests its host cell hemoglobin, this FPIX is apparently not available for the synthesis of parasite haem-proteins. Parasites contain several haem-proteins and the ability to label them in *P. falciparum* with [2-14C]glycine and [4-14C]aminolevulinate (ALA), indicates that the parasite is able to synthesize FPIX de novo.[47] Since [1-14C]glutamate was not incorporated, it was concluded that the FPIX biosynthesis was similar to that of liver and erythroid cells. Several enzymes of FPIX synthesis were assayed: *-aminolevulinate synthase (ALA-S) was shown to be specifically expressed in *P. falciparum* and *P. berghei* [48] and the gene of the first species has been characterized.[49] Other enzymes, such as ALA-dehydrase, coproporphyrinogen oxidase and ferrochelatase are imported from the host cell through an apparent specific receptor, that could serve as drug target (although the parasite may have its own enzymes). In *P. berghei*-infected mice, a high expression of these enzymes is induced in host erythrocytes by infection. A similar induction is probable also in falciparum malaria. The extremely high concentration of the ALAS inhibitor succcinylacetone needed to inhibit *P. falciparum* in culture (IC50=2 mM), hints to the possibility that the parasite can use some of the host-derived FPIX for synthesis of haem-proteins. Haem arginate is used as a stable source of FPIX for the treatment of various porphyrias that result from decreased activity of FPIX biosynthetic enzymes. Haem arginate also inhibits ALA-S, and could be tested for its antimalarial action although it is expected at the same time to inhibit FPIX production in the host's bone marrow.

## 8. Conclusion

Due to time and space limitations, several other essential processes that could serve as drug targets, have been omitted. Such are glycolysis (where recent molecular studies have indicated that lactate dehydrogenase and triose-P-isomerase could serve as specific targets), purine and pyrimidine syntheses, DNA and RNA syntheses, the regulation of the cell cycle, protein trafficking and secretion, mitochondrial functions, cytoskeleton maintenance, proteasome and ubiquitin-mediated protein degradation, phospho-

lipid metabolism, transport of substrates and waste products and signal transduction. The tremendous effort that has been invested in basic biochemical and physiological research in malaria, has provided exciting insights into the intracellular life of the parasite, but disappointedly few lead antimalarial compounds. Fortunately enough, ethnic medicine continues to provide us with new drugs. It is hoped that modern approaches in drug development such as functional genomics, proteomics and screening of combinatorial libraries that are becoming useful in drug development, will transcend into malaria research. The malaria genome project provides not only the expected information about genes that code for elements involved in the various processes described above, but also some surprises. Such are the genes that are related to the synthesis of amino acids (the parasite was considered hitherto to get all the amino acids from globin degradation or from the host), to the synthesis of ATP by mitochondria (the parasite was considered to depend exclusively on glycolysis for ATP production), and to the synthesis of fatty acids (considered to be supplied from the host). These apparent "inconsistencies" as well as the myriad of supposedly redundant genes (rifin, stevor, Ser/Thr protein kinase, etc.), must await the verification of their expression. It should be remembered that gene expression varies with parasite stage and progression through the life cycle, and the elucidation of their functional meaning of the genes at each phase in conjunction with their use as potential drug targets will hopefully get a refreshed impetus.

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# Protein prenylation in trypanosomatids: a new piggy-back medicinal chemistry target for the development of agents against tropical diseases

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***Abstract :*** *Protein prenylation is a recently discovered protein modification involving the attachment of 15-carbon farnesyl and 20-carbon geranylgeranyl groups to eukaryotic proteins. We have shown that protein prenylation occurs in the trypanosomatids* T. brucei, T. cruzi, *and* L. amazonensis. *We have detected the enzyme that attaches farnesyl groups to proteins, protein farnesyltransferase (PFT), in the cytosol of these parasites and have purified* T. brucei *PFT to homogeneity. PFT farnesylates the cysteine SH of a tetrapeptide sequence at the C-termini of proteins (CaaX, where C is cysteine, a is usually but not necessarily an aliphatic residue, and X is a variety of amino acids). CaaX mimetics that are potent PFT inhibitors are also potent at killing trypanosomatids in vitro. Since CaaX mimetics are being intensively developed as anti-cancer agents, we can take advantage of the wealth of pre-clinical data on these agents as we develop these compounds as anti-trypanosomatid agents (piggy-back approach).*

## 1. Introduction

In the mid-1980s, the author M. H. Gelb together with Prof. J. A. Glomset discovered protein prenylation in mammalian cells.[1] This protein modification involves the attachment of 15-carbon farnesyl or 20-carbon geranylgeranyl groups to the C-terminal cysteine residues of a specific set of proteins (Figure 1). Many of these prenylated proteins are small GTPases including Ras, Rab, Rac, and Rho that play a role in cellular signal transduction and intracellular vesicle trafficking. The functions of protein prenyl groups are

not fully understood, but they play a role in binding proteins to membranes and possibly to direct interactions with other proteins.[2]

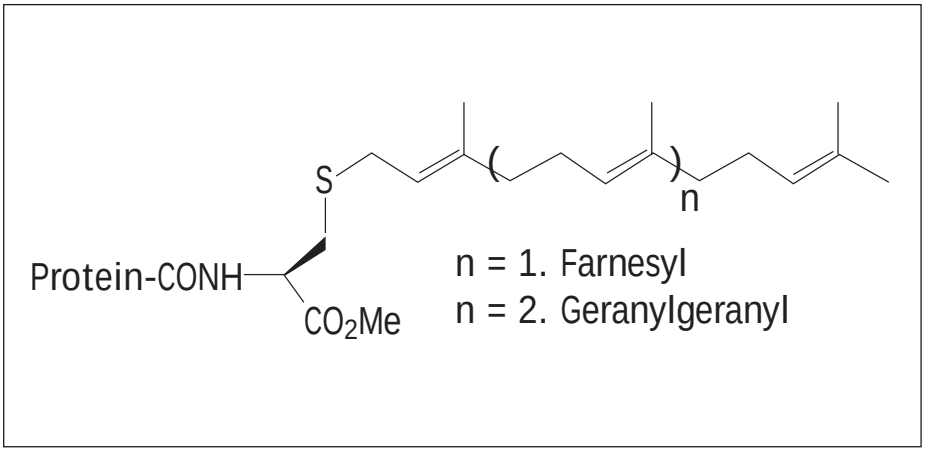



**Figure 1. Structure of the farnesyl and geranylgeranyl groups attached to proteins. Note that the prenylated cysteine is the C-terminal residue (aaX is removed) and its a-carboxyl group is methylated.**

The farnesylation of Ras is absolutely essential for the ability of this protein to transform mammalian cells.[3] Thus, there is currently intense medicinal interest in protein prenylation. The enzyme that attaches the farnesyl group to proteins, protein farnesyltransferase (PFT), is a target for anti-cancer drugs. In fact, several hundred potent PFT inhibitors have been reported over the past few years.[4] Schering-Plough and Janssen Pharmaceuticals are conducting Phase-I clinical trials with PFT inhibitors for the treatment of cancer since such compounds are much less toxic than expected, and they cause shrinkage of tumors implanted into animals. Several additional companies are gearing up for clinical trials.

There are three protein prenyltransferases in mammals.[5,6] PFT transfers the farnesyl group from farnesyl pyrophosphate (FPP) to the cysteine SH of the C-terminal protein sequence CaaX (a is usually but not necessarily an aliphatic residue, and X is usually S, M, Q, A). Gelb, and independently Pat Casey (Duke Univ.), were the first to identify and purify protein geranylgeranyltransferase-I (PGGT-I), the enzyme that transfers the 20-carbon geranylgeranyl group from geranylgeranyl pyrophosphate (GGPP) to CaaX (when X is L or F). Finally, PGGT-II (also called Rab geranylgeranyltransferase) attaches two geranylgeranyl groups to both cysteines at the end of Rab proteins. The X-ray structures of rat PFT and the complex of the enzyme with substrates have been solved.[7,8] After prenylation of the CaaX sequence, the last three residues (aaX) are removed by an endoprotease. The new C-terminal S-prenylcysteine is methylated on its a-carboxyl group (Figure 1).

## 2. Trypanosomatid protein prenylation as an ideal drug target

There is tremendous need for new drugs against trypanosomes, leishmania, and filarial parasites. These organisms combined afflict an estimated 150 million people in tropical regions. Existing drugs against trypanosomatids are either lacking, ineffective, or highly toxic. Based on the following studies, we have developed a working hypothesis that inhibitors of trypanosomatid protein farnesylation represents an ideal target for the development of agents against these devastating tropical diseases.

We, and independently Mark Field in the UK, showed that prenylation occurs in *T. brucei,*[9,10] and we found that it also occurs in *T. cruzi* and *Leishmania mexicana amazonensis* (*L. amazonensis*).[11] Prenylation also occurs in *Giardia lamblia*[12] and *Schistosoma mansoni*.[13] T. Egwang at Med Biotech Labs in Kampala discovered protein prenylation in *Brugia malayi* and *O. volvulus*. We showed that *T. brucei* has PFT, and that this enzyme obeys different substrate specificity rules than the mammalian homolog. Thus, it has been possible to prepare PFT inhibitors that are selective for the parasite enzyme.

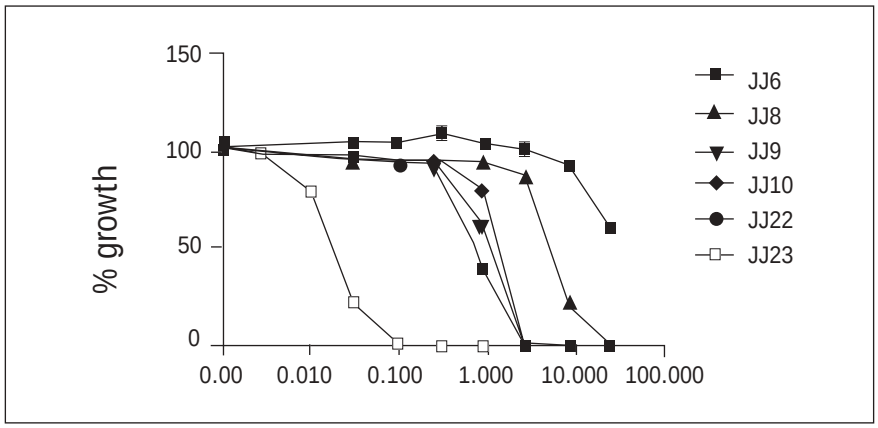
Over the past year, we have purified *T. brucei* PFT 90 000-fold to homogeneity.[14] Partial amino acid sequencing indicates that we have identified the two PFT subunits. Interestingly, the parasite PFT subunits are ~50% larger than the mammalian homologs. The full-length a-subunit of *T. brucei* PFT has been cloned, and efforts are underway to clone the b-subunit.

We have collected about 250 PFT inhibitors from a number of laboratories and tested them on *T. brucei* PFT. The relative potencies of the inhibitors against the parasite and mammalian enzymes are quite different, which underscores the likelihood of being able to make parasite-selective inhibitors (absolute specificity is not required, see below). We have identified 3 PFT inhibitors that inhibit *T. brucei* with an IC50 of 0.5-1.5 nM. These compounds are a series of mimetics of the CaaX prenyl acceptor and have been synthesized by Hamilton's group at Yale University. On a relative scale of drug production cost, these PFT inhibitors are inexpensive to synthesize, an important consideration for anti-trypanosomatid agents. The group of S. Sebti has shown that these compounds display good pharmacokinetic and anti-tumour properties in rodents. We showed that these inhibitors are able to block incorporation of 3H-mevalonic acid, the precursor of prenyl groups, into a subset of *T. brucei* prenylated proteins (likely, those that are farnesylated rather than geranylgeranylated). Low nanomolar amounts of these CaaX mimetics block the growth of bloodstream *T. brucei* (Figure 2) and of *T. cruzi* amastigotes growing in 3T3 host cells (not shown). These compounds are among the most potent anti-trypanosomatid agents known to date. Remarkably, these compounds and PFT inhibitors in general are not toxic to uninfected mammalian host cells when tested up to 50 mM. Other CaaX mimetics plotted in Fig. 2 are less potent PFT inhibitors and are correspondingly less potent at arresting the growth of parasites. As we prepare for studies with parasite infected mice, we can take advantage of the wealth of published pharmacokinetic data on PFT inhibitors so that we will test only those compounds that are bioavailable to parasites in experimental animals.

The advantage of "piggy-backing" on a large amount of medicinal and clini-

cal data that exists for PFT inhibitors is extremely important for the development of drugs to treat parasites that infect people in developing countries. It may also be noted that killing trypanosomatids with PFT inhibitors appears to occur by a well-defined mechanism, unlike many reported anti-trypanosomatid agents which act by unknown mechanisms. Finally, PFT may be a good target for the development of antimalarial agents.[15]

**Figure 2. Inhibition of T. brucei bloodstream form growth by CaaX mimetics. The most potent compound (ED50 ~ 30-50 nM) is also the most potent inhibitor of T. brucei PFT in vitro.**




## 3. Trypanosomatid GTPases

GTPases in mammalian cells control critical processes including growth regulation (Ras family), cytoskeletal organization and cell cycle progression (Rho family), and vesicle trafficking (Rab proteins). Since most GTPases are prenylated and since most prenylated proteins in eukaryotic cells are GTPases,[2] the study of protein prenylation and GTPases in go hand in hand. We and others are beginning to identify some of the GTPases in trypanosomatids. Engman's lab has cloned CaaX-containing DnaJ chaperone proteins from *T. cruzi*,[16] and Hide's lab cloned a *T. brucei* Ras/Rap-like protein).[17] The Lopes lab has also characterized a full length genomic clone of a *T. cruzi* Rab GTPase that is most homologous to mammalian Rab11. This protein, which is encoded by a single copy gene, contains a C-terminal CC sequence and thus is likely to be doubly geranylgeranylated by PGGT-II.

## 4. Future studies

Once both subunits of *T. brucei* PFT have been cloned, we will attempt to overexpress this enzyme so that milligram amounts become available for structural studies including x-ray crystallography. The high resolution structure in comparison to that now available for mammalian PFT[7] will enable a structure-based approach to further improve the potency and selectivity of trypanosomatid PFT inhibitors. Efforts are also underway to clone the PFTs from *T. cruzi* and *L. braziliensis* and to overexpress these enzymes.

Although we have detected PFT in the cytosol of *T. brucei* and *T. cruzi* (the *T. brucei* enzyme has been purified to near homogeneity as noted above), so

far we have not been able to detected PGGT-I activity in cell-free extracts from these parasites, although PGGT-II activity toward Rab proteins has been detected. This leads to the working hypothesis that proteins that are normally geranylgeranylated in mammalian cells via PGGT-I may be farnesylated by PFT in trypanosomatids. Since PGGT-I inhibitors are very toxic to mammalian cells (see for example[18]), blocking the putative farnesylation of homologous proteins in trypanosomatids by PFT inhibitors may be the basis for their toxicity to these organisms. The continued investigation as to whether trypanosomatids have PGGT-I is an active area of research in our group.

As we discover potent PFT inhibitors that are cytotoxic to trypanosomatids in vitro, we will examine the best compounds for their ability to reduce parasitemia in trypanosomatid infected rodents. Fortunately, there is a wealth of information already available about the pharmacokinetic behavior of many of these CaaX mimetics (in connection with the anti-cancer studies of PFT inhibitors noted above), and thus we can choose compounds for animal studies that are known to be bioavailable to tumors and presumably to parasites.

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# On the mathematical modelling of metabolic pathways and its use in the identification of the most suitable drug target

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***Abstract:*** *Using glycolysis of bloodstream-form* Trypanosoma brucei *as an example, we show how mathematical models of parasite metabolism may provide useful information in the process of drug design. A computer model of trypanosome glycolysis was developed, based on experimentally determined kinetic data of the enzymes of the pathway. This model was used to obtain quantitative information about the control on the glycolytic flux as exerted by the individual enzymes. Moreover, it was calculated to what extent each enzyme should be inhibited to decrease the flux by 50%, thus providing an important part of the information required to decide which enzyme of the pathway is the most suitable drug target. It is also shown how such computer simulation could be used to make predictions with regard to the effectiveness of competitive versus noncompetitive or irreversible enzyme inhibitors as drugs, and the possible synergistic effects of inhibitor cocktails.*

## 1. Introduction

For the development of effective chemotherapeutic agents against infectious diseases the necessity to fulfil two criteria is generally accepted:[1,2] (a) The drug should interfere with an essential process in the metabolism of the parasite or with another step vital for maintaining its viability or propagation.

(b) The drug should be selective, i.e. not interfering with the corresponding or a different process in the host organism, at least not to the extent that important host functions are compromised. In addition, a third criterium seems important albeit not essential: the drug should preferably act on a target molecule that is not present in large excess. If the drug interacts with a step in a metabolic process, its effectiveness will be enhanced if the target enzyme will contribute to controlling the flux through the pathway because it is not present in excess.

In the current strategy for developing new antiparasitic drugs, the above mentioned criterium 1 ("target validation") is rationally met for instance by genetic manipulation, mimicking loss of function by therapeutic intervention.[3] The second, so-called selectivity criterium, involves the rational design or selection of inhibitors based on differences in enzyme structure.[2] However, the third criterium is rarely taken into account. In this chapter, we will argue that Metabolic Control Analysis may serve to address this issue, and may also contribute to enhance the drugs' selectivity.

## 2. Metabolic Control Analysis

Metabolic Control Analysis is a tool for quantitative analysis of the behaviour of metabolic pathways.[4-6] In this analysis, the control of an enzyme on a metabolic flux is quantitatively expressed by a flux-control coefficient, defined as the relative change of the flux J divided by the small, relative change of the enzyme activity that is responsible for this change in flux, at constant activities of all other enzymes. In mathematical terms:

$$C_i^J = \frac{dJ / J}{de_i / e_i} = \frac{d \ln J}{d \ln e_i}$$

Here J is the flux, $e_i$ is the activity of enzyme i. Graphically, the flux-control coefficients are the slopes to the curves in Figure 1, at 100 % activity. When an enzyme has a flux-control coefficient of 1, it is truly rate limiting (curve 1). If it has a flux-control coefficient of 0, it is not limiting at all (curve 3). Experimentally all values between 0 and 1 can be found (e.g. curve 2).[4-6] An important feature of flux-control coefficients is expressed by the summation theorem: in an ideal pathway the sum of the flux-control coefficients of all enzymes is 1.[4-6] Or, mathematically:

$$\sum_i C_i^J = 1$$

A consequence of the summation theorem is that if one enzyme has a high flux-control coefficient, then the others must have low control coefficients and vice versa. Also, the theorem replaces the notion that total control must reside in a single (rate-limiting) step. Although it can be confined to a single step, control is more often distributed among several, or even all enzymes in a pathway.

By offering methods to determine control, Metabolic Control Analysis can thus help to predict which enzymes of a pathway are the most appropriate targets for drugs. Preferably, one should use an inhibitor that affects the activity of an enzyme with a high flux-control coefficient. Moreover, one can increase the selectivity of a drug by choosing a target enzyme with a high control coefficient in the parasite and a low one in the host (called "differential control analysis"). The application of this strategy to identify optimal drug targets in a pathway will be illustrated vis-à-vis glycolysis in bloodstream-form *Trypanosoma brucei*.

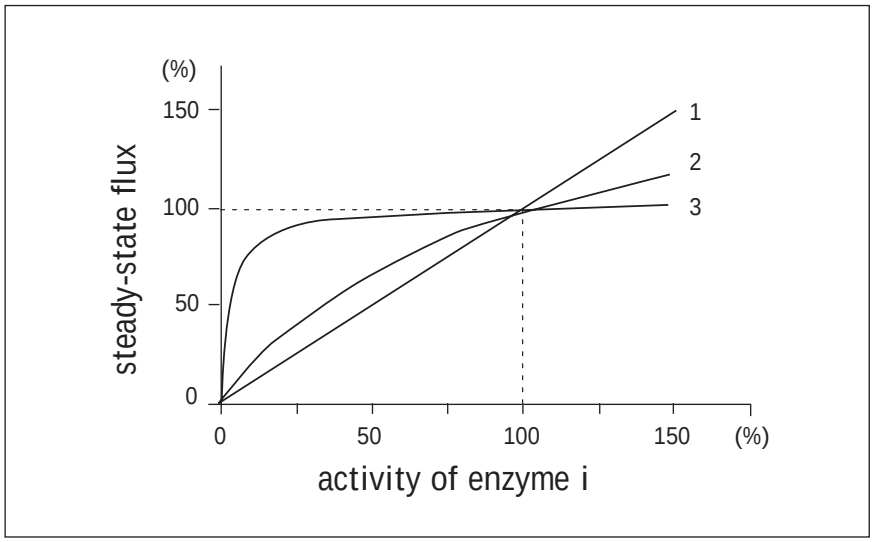


**Figure 1. Three possible correlations between the steady-state flux through a metabolic pathway and the activity of one of its enzymes. Flux and activity are expressed as a percentage of uninhibited flux and activity at the condition under which the flux-control coefficient is evaluated.**

## 3. Glycolysis in bloodstream-form *Trypanosoma brucei*

Glycolysis has been perceived as a potentially excellent target for the design of new antitrypanosome drugs because it is the only process that supplies ATP for African trypanosomes living in the mammalian bloodstream.[1,7,8] Indeed, it has been shown that starvation for glucose or incubation with inhibitors of various glycolytic enzymes leads to rapid death of the parasites.[9-13] However, none of these inhibitors is selective for parasite enzymes. The glycolytic pathway in bloodstream-form *T. brucei* is organized in a unique manner. The majority of the enzymes is sequestered in peroxisome-like organelles called glycosomes (Figure 2).[7,14] In other eukaryotic cells, glycolysis occurs usually in the cytosol. The glycosomes contain the seven enzymes converting glucose into 3-phosphoglycerate; the last three enzymes of the pathway reside in the cytosol. The pyruvate thus produced is excreted into the blood. The glycosomal membrane is hardly permeable to metabolites (15,16). The NADH produced by glyceraldehyde-3-phosphate dehydrogenase in glycolysis is reoxidized by molecular oxygen via a mitochondrial glycerol-3-phosphate oxidase to which the electrons are transferred through a glycosomal glycerol-3-phosphate dehydrogenase and a putative glycerol 3-phos-

phate:dihydroxyacetone phosphate transporter across the glycosomal membrane. As a result of this organization, the intraglycosomal milieu is in redox and ATP balance. The net synthesis of two molecules of ATP per molecule of glucose, during aerobiosis, occurs in the cytosol, in the reaction catalysed by pyruvate kinase.

The long evolutionary distance between trypanosomes and humans,[17] and the unusual organization of the pathway in the parasites[7,14] have endowed the trypanosome enzymes with distinct structural and kinetic properties that are being exploited in a structure and catalytic-mechanism based design of selective inhibitors.[1,18]

## 4. Glycolytic flux control in bloodstream-form *Trypanosoma brucei*

An initial control analysis has been performed for glycolysis in bloodstream-form *T. brucei*, using both a theoretical and experimental approach. Flux-control coefficients of the glycolytic enzymes have been determined. In order to identify the most promising drug targets, these have been compared with those of the corresponding enzymes in cells of the human host as known from the literature.

To get a first appreciation of the distribution of control in bloodstream-form trypanosomes, a detailed kinetic model of this pathway was constructed.[19] It contained kinetics for most of the parasite's glycolytic enzymes as determined previously.[7,14,20] Also the compartmentation of glycolysis was included in the model, with the glycosomal membrane considered impermeable to most metabolites. Circumstantial evidence supports the notion that the membrane is hardly permeable, except to those metabolites that need to cross the membrane to allow glycolysis to proceed.[15,16] It is presumed that the membrane contains specific transporters for the latter compounds (i.e. glucose, 3-phosphoglycerate, dihydroxyacetone phosphate, glycerol 3-phosphate, inorganic phosphate). For lack of kinetic information on these transport processes, it was assumed that these metabolites fully equilibrate across the membrane. Because this is an assumption, any flux control by the glycosomal metabolite transporters will have been missed in the analysis.

The model reproduced various experimental observations quite well, for instance the steady-state glycolytic flux and the steady-state metabolite concentrations as a function of the enzyme kinetic parameters and of the concentrations of metabolites in the host blood. However, it has not been possible yet to subject the model to stringent tests, especially because uncertainties concerning the glycosomal volume preclude accurate experimental determination of the concentrations of glycosomal metabolites.

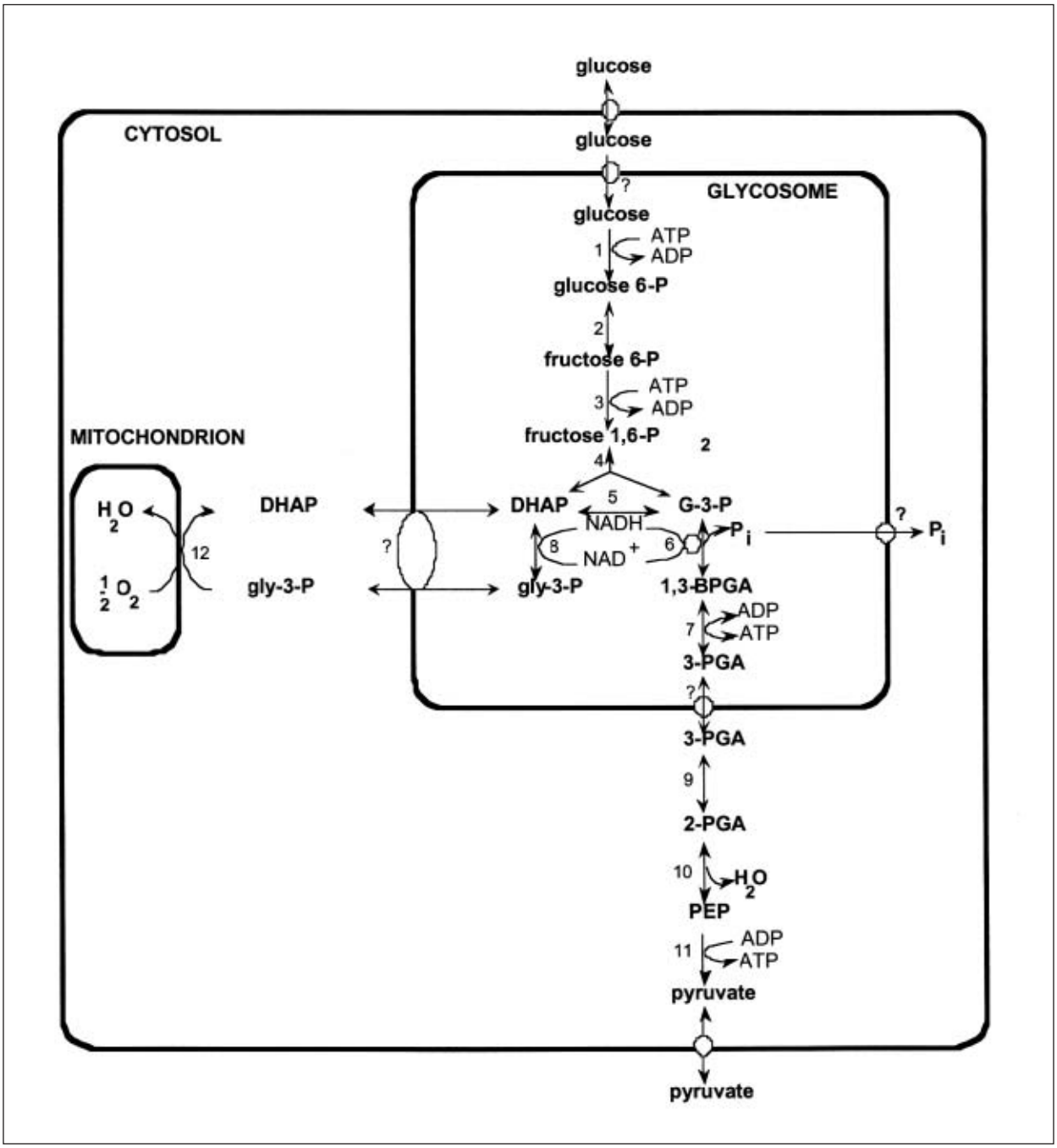


**Figure 2. The stoichiometric scheme of aerobic glycolysis in bloodstream-form *T. brucei*. 1, Hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, phosphoglycerate kinase; 8, glycerol-3-phosphate dehydrogenase; 9, phosphoglycerate mutase; 10, enolase; 11, pyruvate kinase; 12, glycerol-3-phosphate oxidase. Substrate and metabolite transporters in membranes are indicated by circles; the involvement of specific carrier molecules in metabolite transport across the glycosomal membrane has not yet been established unambiguously. Abbreviations: 1,3-BPGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate; Gly-3-P, glycerol 3-phosphate; PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.**

Since the model reproduced the glycolytic process of the trypanosome, it was used to calculate the flux-control coefficients of most glycolytic enzymes, by modulating the activity of each enzyme independently, according to the

above definition.[21] At 5 mM of extracellular glucose, i.e. the normal concentration in blood, most of the flux control appeared to be exerted by the transport of glucose into the cells. This result however, was rather sensitive to the values of the kinetic parameters of the glucose transporter. If the glucose transporter was made slightly more active than had been measured, it lost part of control in favour of a group of four enzymes: aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and glycerol-3-phosphate dehydrogenase. Hexokinase, phosphofructokinase and pyruvate kinase, which are often thought to be rate-limiting steps of glycolysis (and in some other organisms have been reported to have a high control coefficient - reviewed in [20]), did not assume any control in the trypanosome model unless they were inhibited strongly. From the modelling it was thus concluded that the glycolytic flux in trypanosomes is controlled either by the glucose transporter or by a group of four enzymes, or by all five molecular processes simultaneously.[21]

To complement the modelling studies and check the predictions, experimental control analysis is required. So far, detailed measurements of flux-control coefficients have only been performed for the plasma-membrane glucose transporter.[22] Its activity was modulated by the transport inhibitor phloretin. This led to an estimated flux-control coefficient of the transporter of between 0.3 and 0.5 at a normal blood glucose concentration (5 mM). At a low extracellular glucose concentration (0.4 mM), the transporter assumed all control, in accordance with the model prediction.[21] This result confirms that under physiological conditions glucose transport is one of the steps controlling the glycolytic flux in *T. brucei*, and that it shares control with other steps. From the modelling work, it was concluded that these other steps are aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and glycerol-3-phosphate dehydrogenase, and that the irreversible enzymes hexokinase, phosphofructokinase and pyruvate kinase exert hardly any control.

In order to determine which enzymes are the most suitable drug targets from the perspective of inhibitor sensitivity, it was also calculated to which extent each individual enzyme should be inhibited to obtain a 10% or 50% inhibition of the glycolytic flux.[21] It turned out that the hierarchy of effectiveness of the targets parallelled the hierarchy of flux-control coefficients. Glucose transport, the step with the highest flux-control coefficient, needed to be inhibited by only 51% to obtain a 50% flux inhibition. Aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and glycerol-3-phosphate dehydrogenase with lower, but substantial flux-control coefficients, were intermediate drug targets, requiring to be inhibited by 76 to 85% in order to inhibit the flux by 50%. Hexokinase, phosphofructokinase and pyruvate kinase, which did not participate in the control, had to be inhibited by 93 to 97% to obtain a 50% flux reduction.

The flux-control coefficients of the parasite's glycolytic enzymes were compared with those of the corresponding enzymes in cells of the host, as published. Here we note that decades of research into metabolic control notwithstanding, the information concerning flux control is virtually absent. This suggests that, for design of antiparasitic drugs, research into the flux con-

trol of the various host tissues is desirable. Schuster and Holzhütter[23] analyzed the effects of large-scale enzyme activity alterations resulting from mutations on the metabolism of human erythrocytes. They noticed that these cells were most susceptible to deficiencies of hexokinase, phosphofructokinase, enolase and pyruvate kinase. In contrast, a decrease of the activities of aldolase, glyceraldehyde-3-phosphate dehydrogenase or phosphoglycerate kinase to 5% of the normal level was predicted not to cause any clinical symptoms. These results are almost the opposite of what was found for *T. brucei*. Provided that they are backed up experimentally and are generizable to the other host tissues, these findings point to aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase as promising drug targets. The same applies to glycerol-3-phosphate dehydrogenase, which in the mammalian host does not play a major role in the reoxidation of glycolytically produced NADH, because of the presence of lactate dehydrogenase and the malate:aspartate shuttle transporting the NADH to the mitochondria where it can be oxidized by the respiratory chain. Hexokinase, phosphofructokinase and pyruvate kinase are less interesting targets from the perspective of Metabolic Control Analysis. Glucose transport was not included in the erythrocyte model, since glucose is believed to equilibrate readily across the erythrocyte plasma membrane. Implicitly, this should make the transporter an important target, were it not for the fact that in other mammalian cell types glucose transport may be an important controlling factor.[24]

## 5. Type of inhibitors and inhibitor cocktails

Metabolic Control Analysis can also be used to make predictions about the effectiveness of different types of inhibitor. Eisenthal and Cornish-Bowden[25] have argued that there be little prospect of killing trypanosomes by depressing their glycolytic flux. Their analysis focused on the effects of competitive inhibitors. Many inhibitors are competitive, since they are often designed as substrate analogues. The authors showed that competitive inhibitors do not always work in vivo, since they can easily be competed away by an increase of the concentration of the substrate with which they compete. Indeed this is a serious concern with important implications for drug design. However, as we discussed elsewhere,[26,27] and was subsequently confirmed by Cornish-Bowden and Eisenthal,[28,29] it is quite well possible to inhibit glycolysis by competitive inhibition if one selects the appropriate targets. The peculiar organization of the glycolytic pathway in trypanosomes has as a result that the coenzymes and metabolites within the glycosome are engaged in moiety conservation, i.e. the sum of all glycolytic intermediates inside the organelle and the sum of glycosomal NAD plus NADH is conserved. Therefore, inhibitors which compete with these coenzymes (or these glycolytic metabolites) should be effective. The concentrations of the competing coenzymes do not increase beyond the total concentration of the coenzyme couple, at least not as a result of the glycolytic activity itself. Of course, the inhibitor should be sufficiently potent. The disappointing conclusion reached by Eisenthal and

Cornish-Bowden[25] should also be attributed to the fact that these authors did not allow the inhibitor concentration to exceed the inhibition constant ([I]/Ki = 1). This is an unnecessary restriction, since several inhibitors are known to accumulate to high concentrations in the cell. Moreover, with competitive inhibitors active in the low nanomolar range it should be quite possible to achieve high [I]/Ki values and thus to inhibit the glycolytic flux. Indeed, Aronov et al.[30] succeeded in developing potent inhibitors specific for trypanosomatid glycosomal glyceraldehyde-3-phosphate dehydrogenase, which are competitive with NAD and appeared biologically active. The arguments described in this paragraph are less relevant for noncompetitive and irreversibly acting inhibitors which should be expected to arrest glycolisis, provided they are sufficiently potent. Of course, the flux control exerted by their target enzymes also contributes to the effectiveness of these inhibitors.
Often the administration of two drugs simultaneously could be beneficial. The use of drug combinations should lower the chance to select drug-resistant parasites. Moreover, the two drugs, when applied to inhibit two different steps of a single pathway, may act synergistically. However, using our computer model of glycolysis of *T. brucei*, we surprisingly noticed that such synergistic action does not always occur, particularly when using competitive inhibitors.[26] In some cases, the administration of inhibitors of two different enzymes (e.g hexokinase plus phosphoglycerate kinase) appeared less effective in inhibiting the glycolytic flux than when using an inhibitor for a single enzyme (phosphoglycerate kinase). The result could be attributed to some intricate metabolic interactions, as was readily discernible by the computer analysis. This demonstrated an additional function of mathematic modelling: to help understand the nonintuitive features of the complex cellular metabolism.
All results discussed above demonstrate that metabolic modelling is a useful tool in the process of drug design. It could provide important information with respect to the best target to choose in a metabolic pathway to optimize the effectiveness and selectivity of inhibitors, and it could be used to predict the effectiveness of a certain type of inhibitor and the synergistic efficacy of inhibitor combinations.

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# From tropical lianas to novel antiplasmodial agents: The naphthylisoquinoline alkaloids

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***Abstract :*** *Exemplarily for the naphthylisoquinoline alkaloids of* Triphyophyllum peltatum *(Dioncophyllaceae), a 'part-time carnivorous' tropical liana, our strategy for the detection of such natural products from plants is described, and the broad structure variety of this novel class of highly bioactive plant metabolites is presented. These alkaloids are not only interesting chemically (unique structural framework, axial chirality), but also pharmacologically: Due to the wide variety of activities - i.e. related to bilharzia, Chagas disease, sleeping sickness, leishmaniasis, river blindness, elephantiasis and, in particular, malaria - and given the chemical infrastructure of our group, our strategy to identify such new active compounds is that of a chemically oriented search and characterization, followed by an intensive investigation of the bioactivities of the pure isolated compounds. Remarkable is the antimalarial activity e.g. of dioncophylline C (2) both in vitro (*Plasmodium falciparum, P. chabaudi chabaudi, P. berghei *including exoerythrocytic forms) and in vivo (*P. berghei *in rodents). Attempts are presented to further improve the antimalarial activities by chemical synthesis, by quantitative structure-activity relationship (QSAR) investigations, and by further isolation work on related plants. The mode of action of these novel antiparasitic agents remains to be explored in collaboration with parasitologists and pharmacologists.*

Higher plants have to defend themselves 'chemically', by bioactive compounds developed in the course of evolution. The value of this unique phytochemical resource has early been recognized by the traditional medicine of many countries in the world and thus the knowledge of healers can provide important hints at novel potential agents useful to mankind.[1]

A botanically most peculiar plant and simultaneously rich source of novel antiparasitic compounds, is the tropical vine *Triphyophyllum peltatum*

(Dioncophyllaceae).[2] The Dioncophyllaceae constitutes a very small family, with only three species: *T. peltatum* (a 'part-time' carnivorous liana i.a. from Ivory Coast), *Habropetalum dawei* (from Sierra Leone), and *Dioncophyllum thollonii* (from Gabon). Closely related, but not carnivorous, are the Ancistrocladaceae, a still relatively small, monogeneric family (only genus: *Ancistrocladus*), with ca. 25 species in the palaeotropic rain forests of Africa and Asia. Some of these plants are used in folk medicine, i.e. for the treatment of malaria, elephantiasis, and other severe tropical diseases.[2-5] Such rewarding ethnobotanical information may sometimes be extracted from the literature, but normally it is available only by asking traditional healers and reliable ethnobotanical partners. The bioactivities reported are a strong motivation for us to investigate these plants phytochemically.

From several expeditions, material of *T. peltatum* became available to us on a multi-kg scale. For the isolation of the active compounds, our group will not pursue bioassay-guided strategies, since the required test systems cannot be established in our Institute of Organic Chemistry, but we have to follow chemical protocols. So, while our collaboration partners are testing bioactivities of the extracts and fractions that we have prepared, we will start doing our chemical isolation work already in parallel, and then provide pure isolated compounds to be retested at the end.

In this approach, *T. peltatum* proved to be a rich source of intriguing, unprecedented alkaloids:[2] Thus, dioncophylline A, the main alkaloid of this plant,[6,7] can be obtained from dried material by grinding, standard extraction (e.g., with dichloromethane and ammonia) and chromatography on silica gel or by High Speed Countercurrent Chromatography (HSCCC), a mild and substance-conserving method, so that finally dioncophylline A is obtained in a pure form.

For the identification of novel bioactive compounds, we have developed a broad spectrum of methods for the rapid and unambiguous attribution of the full stereostructures of such compounds (see Figure 1), comprising modern methods for the early detection in the living organisms by in vivo NMR[8] and FT-Raman,[9] as well as the identification of novel compounds by a unique and unprecedented 'triad' of HPLC coupled to mass spectrometry (LC/MS), to nuclear magnetic resonance (LC/NMR), and to circular dichroism (LC/CD), here for the first time in phytochemical analysis.[10] Methods for the isolation of the alkaloids under mild conditions (e.g. by HSCCC) are also important.[11]

The structures of the isolated compounds are elucidated both by chemical methods (e.g. by oxidative degradation), as well as by modern NMR investigations (e.g. long-range NOE), X-ray structure analyses, and CD investigations. The reliable knowledge of the full 3D-stereostructure is of critical importance if in the end one wants to design and synthetically realize even better agents. An ultimate confirmation of the absolute stereostructure of a new compound, e.g., of dioncophylline A, is provided by its chemical synthesis in the laboratory.[7] The particular value of such syntheses lies in the additional option of producing sufficient quantities for extended biotests, independent of delicate or inaccessible tropical plant material, and in addition, in then performing similar syntheses to structurally modified analogs, aiming at the preparation of possibly even better compounds (i.e., with higher activities and hopefully lower toxicities).

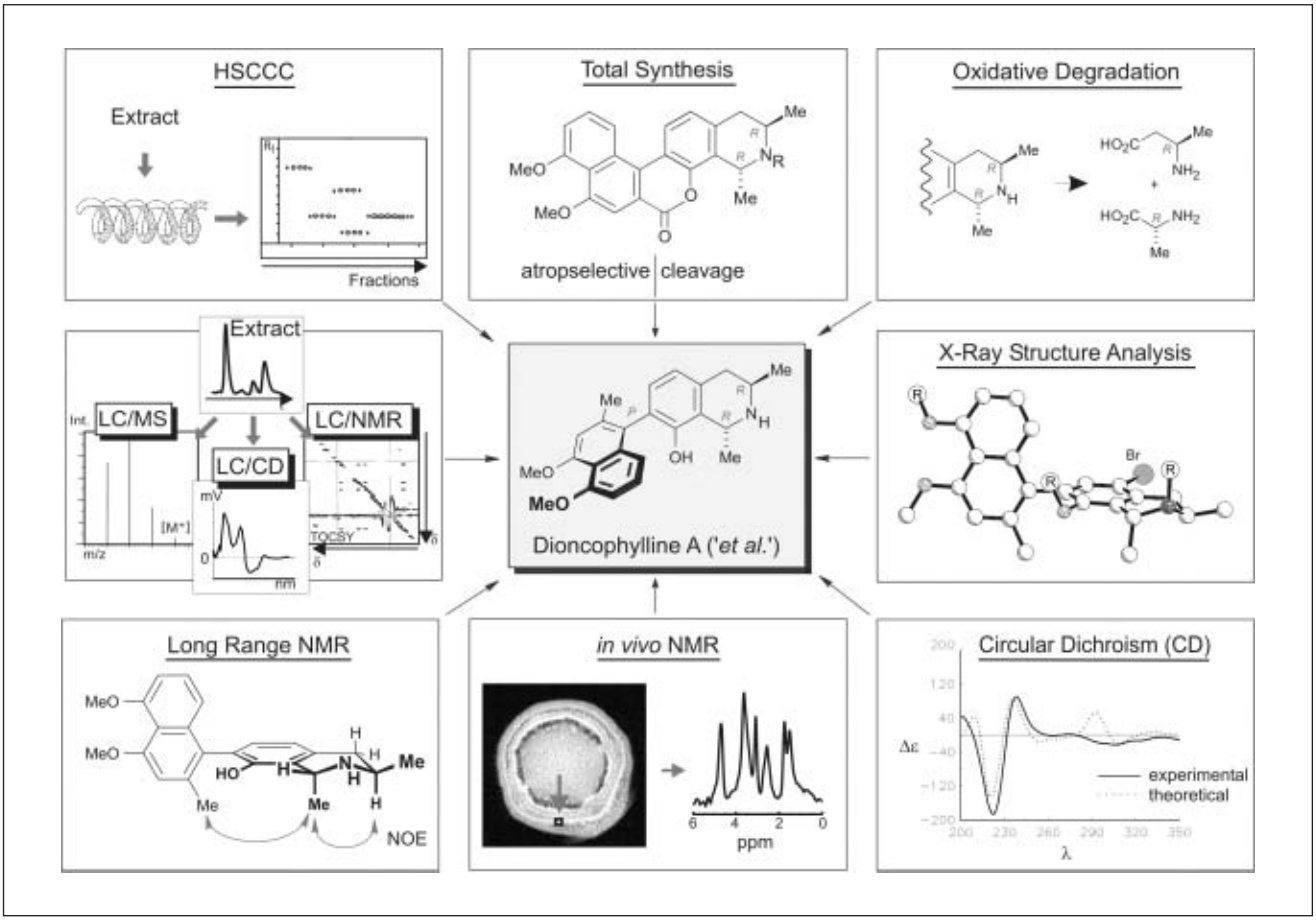


**Figure 1. Methods for the detection, isolation, and structural elucidation of naphthylisoquinoline alkaloids.**

All these methods establish dioncophylline A to have the full absolute stereostructure 1, consisting of a naphthalene and isoquinoline moiety, joined together by a rotationally hindered biaryl axis. *T. peltatum* is a highly productive plant: Besides dioncophylline A (1), it contains a series of nearly 20 related, but likewise new alkaloids, whose structures we have elucidated, again by applying our methodology – a broad series of compounds, all with the characteristic biaryl axis between the isoquinoline and the naphthalene parts, among them alkaloids like dioncophylline C (2, see Figure 2), whose 'vertical' axis is rotationally fixed, and dioncophylline B (8) which is rapidly rotating around the axis.[2]

No doubt these are chemically unique novel structures, but even more importantly, as already expected from the use of the plants in folk medicine, they display various interesting bioactivities. Now having the pure compounds in hands and stimulated by the activities already detected at the level of the crude extracts, we were in a position to investigate these activities in more depth. Together with our scientific partners, we found a large diversity of different bioactivities, among them fungicidal activity against plant-pathogenic fungi as well as antifeedant and growth-retarding activity against herbivore insects.[12] But most importantly, some of our alkaloids are strong agents

of potential relevance to severe widespread tropical diseases like bilharzia,[13] Chagas disease (e.g. IC50 of 1 against *Trypanosoma cruzi*: 0.70 µg/ml), sleeping sickness (IC50 against *T. brucei rhodesiense*: down to 0.19 µg/ml), leishmaniasis (IC50 against *Leishmania donovani*: down to 12.1 µg/ml), river blindness, elephantiasis, and, in particular, malaria – and some of the alkaloids are also active against larvae of the vectors *Anopheles stephensi*[14] and *Aedes aegypti*.[15] By a bioassay-guided search, it would have been difficult to find all these activities, given the largely divergent and manifold fields of indication.

While in particular the results on the antileishmanial and antitrypanosomal activities are 'brand new', the antimalarial activities have been discovered a few years earlier, due to the use of some of the plants against malaria in folk medicine. Stimulated by this key hint from ethnobotany, we found that already some of our extracts exhibit excellent activities against *Plasmodium falciparum* (down to IC50 = 0.014 µg/ml), also against chloroquine-resistant strains.[16-18] Among the pure isolated compounds, dioncophylline C (2) and dioncopeltine A (3) show the best IC50 values (cf. Figure 2).[19]

These good activities and the comparably low cytotoxicities, leading to selectivities of e.g. ca. 300 for dioncophylline C (2), made it rewarding to perform first in vivo experiments on the 'curative potential' of such alkaloids against malaria. Indeed, OF-1 mice infected with *Plasmodium berghei* erythrocytic forms, and then treated with 2, showed a parasitaemia of zero from day 4 on, and even after months the animals were still alive and without any symptoms.[20] Even for single doses, we find good inhibitory effects in vivo (e.g., 97% inhibition of parasitemia by p.o. application of 30 mg/kg of 2). Dioncopeltine A (3) and the related alkaloid korupensamine A (9) are also capable of prolonging the survival time of the mice significantly, although, in contrast to dioncophylline C (2), they cannot cure them.

Another most remarkable fact is that naphthylisoquinoline alkaloids do not only act against the blood forms of *Plasmodium spp.*, but also against the exoerythrocytic forms[21,22] – a most promising additional perspective for these novel antimalarial agents. For synchronized forms of *P. chabaudi chabaudi*, stage specific activities have been found.[23]

What can one do to further improve these novel natural lead structures? Our group has developed chemical methods for synthesizing and modifying such compounds,[24] so we have prepared a series of structural analogs, empirically revealing some structural features required for good activity. As an example, some synthetic naphthylisoquinoline dimers[25,26] such as jozimine A (4), were found to exhibit unexpectedly higher antiplasmodial activity as compared to the monomeric parent compound (here dioncophylline A (1), by a factor of 20.[27] This unnatural dimer is one of the three most active naphthylisoquinolines now (see Figure 2), after dioncophylline C[2] and dioncopeltine A (3)!

The value of our synthetic work in this field becomes even more evident when looking at the structures of the as yet most active naphthylisoquinolines: All of the 'top 12' of these active compounds (see Figure 2) have already been synthesized in our lab. No less than five of these twelve active compounds are unnatural and would not have been detected without our synthetic work. In order to select rewarding synthetic target molecules even more efficient-

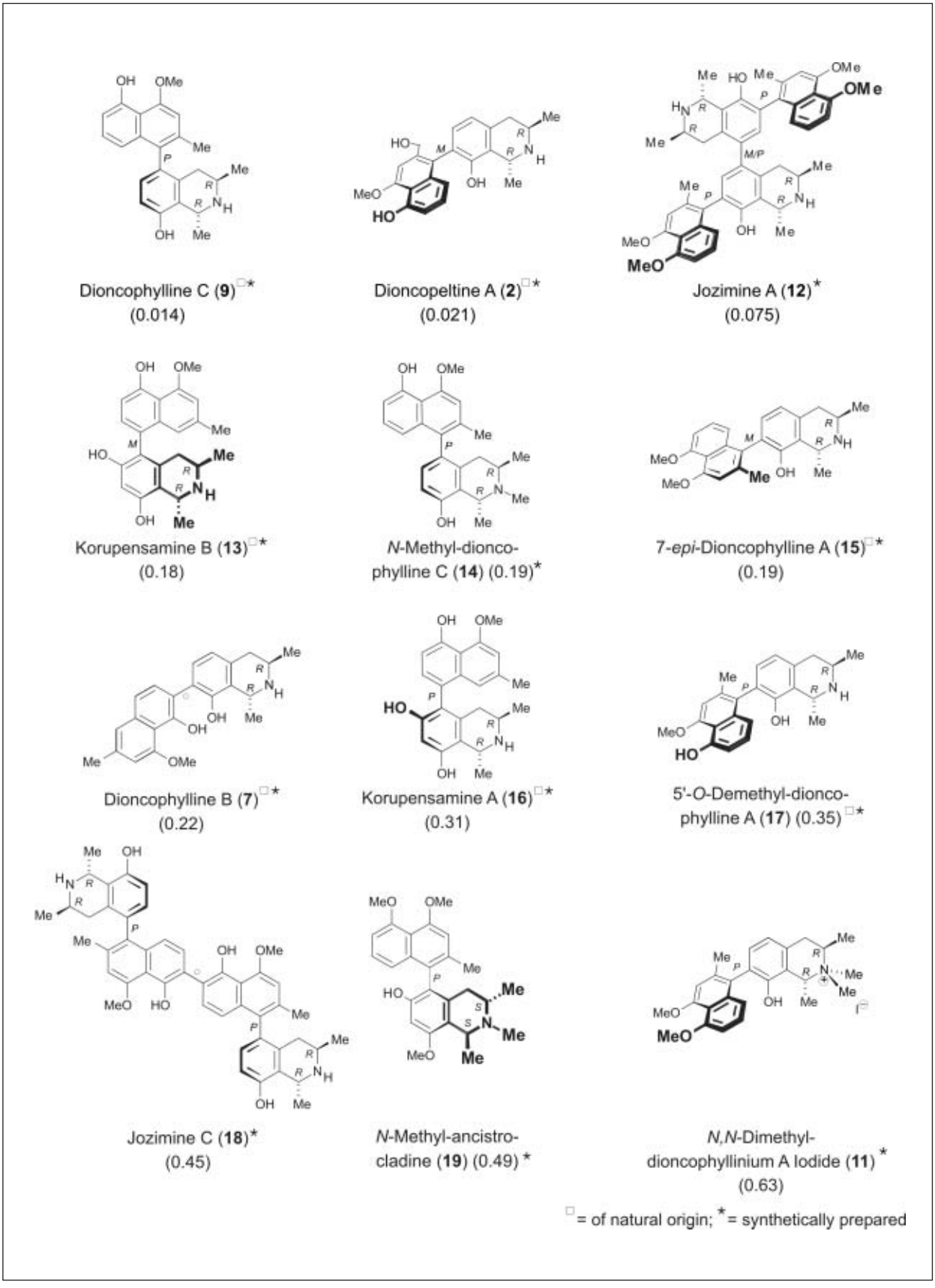


**Figure 2. Chemical structures of the 'top 12' most active naphthylisoquinoline alkaloids against *P. falciparum* in vitro ( IC50 values in µg/ml )**

ly in the future, we have just started predicting structures with better activities by computational methods, by quantitative structure-activity relationship (QSAR) investigations using the Comparative Molecular Field Analysis (CoMFA) technique,[28] which allows us to predict the activities of yet unsynthesized structures, even though the molecular target and the mode of action are not yet known (2). Based on a training set of initially 34 compounds, we statistically compare the activities with the chemical structures, thus deducing the joint structural requirements for high activities. Using this first QSAR approach on naphthylisoquinolines, we have found a very good correlation between calculated and experimental IC50 values, leading to an excellent correlation factor (q2 = 0.76), so that we have now started predicting more active compounds to be synthesized and tested.

Another approach to look for new structural analogs, is to investigate botanically related plants, i.e., the other – only two! – very rare further species of the Dioncophyllaceae family: *Habropetalum dawei* from Sierra Leone and Liberia, and *Dioncophyllum thollonii* from Gabon. These two 'phylogenetic neighbours' of *T. peltatum* produce large amounts of naphthoquinones and related natural products. Nonetheless, by applying our most efficient novel 'triad' LC-MS / LC-NMR / LC-CD – we found naphthylisoquinolines in small quantities in these species – some of them known, but mainly new alkaloids (10). Given their low concentrations, we would not have found them just by a bioassay-guided screening, only by our sensitive chemical analysis. We will now provide enough material for bio-testing, by extended isolation and by chemical synthesis.

Besides the extremely small Dioncophyllaceae family, the Ancistrocladaceae (Africa and Asia) also produce a broad variety of naphthylisoquinolines (3). Altogether, nearly 80 alkaloids of this type have so far been discovered, including anti-HIV active dimers, named michellamines.[29]

Future work will focus on the search for even better structural analogs, by isolation from plants and by molecular modeling assisted synthesis, and on a thorough investigation of the pharmacological potential of these compounds, including the mode of action. This is an important and urgent task, to which we as natural product chemists can contribute by analytical, synthetic, and computational chemistry, assisted by our engaged parasitological and pharmacological collaboration partners!

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# The molecular basis of *Plasmodium falciparum* chloroquine resistance

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***Abstract :*** *The emergence and spread of chloroquine resistant* Plasmodium falciparum *has seriously compromised malaria treatment and control. Chloroquine resistance (CQR) is associated with reduced accumulation of chloroquine (CQ) in the parasite digestive food vacuole, where the drug forms toxic complexes with haem molecules produced from host haemoglobin. Recent genetic and epidemiological studies reveal a central role for a vacuolar transmembrane protein, PfCRT, in CQR. Adaptive changes in a* P. falciparum *P-glycoprotein encoded by pfmdr1 may modulate CQR levels in vitro, although they are not responsible for CQR and their effect on clinical resistance is unclear. Changes in pfcrt and pfmdr1 may affect parasite susceptibility to other haem-binding antimalarials including quinine and mefloquine. Here we review the role of these genes in drug resistance. A better understanding of the molecular basis of CQR should improve diagnostic tools and stimulate new initiatives for drugs that can act in combination with or replace CQ.*

## 1. Chloroquine and its mode of action

CQ binds to haematin, a μ-oxo dimeric form of the haem moieties that result from proteolytic processing of haemoglobin inside the digestive vacuole (DV) of the intraerythrocytic parasite.[1-5] CQ-haematin complexes in the DV are toxic and are thought to interfere with the normal polymerization of haem into inert malaria pigment (haemozoin).[2,6-8] CQ effects on haem processing

may lead to parasite death through processes that include the production of reactive oxygen intermediates and peroxidation of lipids and membranes.[5,9,10]

## 2. Impact of chloroquine resistance (CQR)

Because of its efficacy, low cost, low toxicity and safety, CQ was for many decades the leading malaria treatment.[11] However, CQ became increasingly ineffective as CQ-resistant (CQR) strains spread across malaria-endemic areas.[12] CQR strains were first reported in the late 1950's in Southeast Asia and in South America, a decade after the initial deployment of CQ on a massive, global scale.[13] *P. falciparum* CQR strains were first reported in Africa in the late 1970s, plausibly from an Asian source.[13,14] Greenberg et al.[15] documented a pronounced increase of malaria-related pediatric morbidity with CQR in Zaire, and studies in Senegal have shown that the arrival of CQR strains were followed by significant increases in malaria mortality and morbidity.[16] CQR strains of *P. falciparum* are firmly implanted in almost all malaria-endemic regions today.[12,13,17] The impact of CQR has not only reduced the usefulness of CQ, but it has also brought an increasing need to rely on the more expensive and more toxic current alternative antimalarials. These alternatives are few and their use is increasingly compromised by the appearance of multidrug-resistant *P. falciparum* strains. For example, in Cambodia and Thailand, malaria parasite strains exist that are resistant to all available antimalarials except the artemisinin compounds.[17] Understanding the molecular basis of CQR should enable the development of tools to diagnose CQR malaria and stimulate new pharmacological initiatives to develop compounds that can replace or be used as adjunctive agents to restore CQ efficacy.

## 3. Physiology and biochemistry of the CQR mechanism

CQR parasites are characterized by their reduced accumulation of CQ and by the ability of diverse agents including verapamil (VP) and various antihistaminics and tricyclics to reduce *P. falciparum* resistance to CQ, i.e. to chemosensitize the CQR parasite to CQ action.[18-21] This "resistance reversal" effect is not detected in chloroquine-sensitive (CQS) parasites.

Leading proposals for the biochemical basis of CQR include:

1) increased efflux of the drug from the DV, possibly resulting from changes in the transport properties or a drug pump at the DV membrane;

2) altered drug partitioning resulting from a physiological change at the plasma membrane, or at the parasitophorous vacuole membrane that separates the parasite from the host erythrocyte;

3) reduction in CQ access to its heme receptor, leading to a change in CQ-receptor binding; or

4) accelerated detoxification of CQ-heme complexes by a glutathione-mediated mechanism (reviewed in[22,23]).

Candidate molecules that have been evoked in these proposals include: a P-glycoprotein multidrug resistance-like protein (Pgh1), drug transporters of

other classes, a chloride channel regulator that affects pH intracellular gradients, a sodium hydrogen exchanger, a factor that alters the apparent affinity of CQ for its receptor, a regulator of hemozoin formation, and glutathione reductase or a related molecule.[19,24-32]

## 4. Is CQR a multigenic trait that requires a P-glycoprotein-like molecule encoded by pfmdr1?

The slow genesis and spread of CQR from a limited number of foci suggests a complex and exceedingly rare event (or set of events) in its acquisition by *P. falciparum*. Some proposals have postulated a requirement for mutations in two or more genes.[3,33,34] One gene that has been intensively investigated in such proposals is the multidrug-resistance-like (mdr-like) gene pfmdr1.[35-39] This gene was isolated in response to the finding that VP partially reverses *P. falciparum* CQR.[19] VP is thought to reverse multidrug resistance in tumour cell lines by inhibiting the drug transport properties of P-glycoproteins encoded by mammalian mdr genes.[40-44]

pfmdr1 was identified by screens for *P. falciparum* genes with homology to the mammalian mdr genes.[35,36,45] The P-glycoprotein-like molecule, Pgh1, encoded by pfmdr1, is known to exhibit point mutations and changes in gene copy number.[35,36,46] Mutations that have been proposed to be associated with CQR are present in the 7G8 (Brazil) line (Y184F, S1034C, N1042D, D1246Y) and the K1 (Thailand) line (N86Y). A survey of the literature shows that 7G8 or K1 point mutations have been associated with CQR in approximately half of the reported field studies, with the other half showing no association.[47-57] Some experimental data suggest that Pgh1 overexpression, through increased pfmdr1 copy number, can accompany increased susceptibility of CQR lines to CQ along with a decreased susceptibility to mefloquine, quinine and halofantrine.[58,59] In recent experiments on the role of pfmdr1 point mutations in CQR, Reed et al.[60] used an allelic exchange strategy to replace the three C-terminal "mutant-type" Pgh1 mutations (S1034C, N1042D and D1246Y) in the CQR 7G8 line with the "wild-type" sequence. These cloned transgenic parasites displayed a 2-fold decrease in the CQ IC50 value, however their CQ+VP IC50 values remained unchanged. No change in CQ response occurred upon introduction of these 3 point mutations into the wild-type allele of the CQS D10 line. These allelic exchange data suggest that pfmdr1 mutations can have some influence on the degree of resistance (as measured in vitro) in lines already resistant to CQ, although they do not confer CQR to sensitive lines.

## 5. Evidence from a genetic cross that CQR is the result of mutations in a single gene, pfcrt

To investigate the basis of CQR in *P. falciparum*, a genetic cross was performed between a CQR clone (Dd2, Indochina) and a CQS clone (HB3, Honduras).[45] Analysis of the progeny revealed segregation of the VP-

reversible CQR phenotype in a manner consistent with inheritance of a single gene. This gene did not map with pfmdr1 located on chromosome five. Instead, the CQR determinant was localized to a 36 kb segment of chromosome seven.[14,61] Initial sequence analysis of this segment revealed polymorphisms between the two parental lines (Dd2 and HB3) in two genes, cg2 and cg1. Preliminary linkage studies found a very strong degree of association between the presence of a single cg2 haplotype and CQR in Southeast Asian and African isolates. These data, combined with evidence for the cg2 gene product at the parasite/erythrocyte interface and in the DV, suggested a hypothesis that CQR could involve the specific set of cg2 mutations found in the CQR parent Dd2.[14,62] However, this initial investigation also revealed one line, Sudan 106/1, which carried the cg2 sequence of Dd2 and yet was clearly CQS. Using allelic exchange, we subsequently demonstrated that cg2 mutations did not account for CQR.[63] These data led to a renewed examination of the 36 kb segment and discovery of a highly interrupted gene, pfcrt.[64] Sequence analysis of pfcrt in parents and progeny of the genetic cross revealed the presence of 8 point mutations (encoding M74I, N75E, K76T, A220S, Q271E, N326S, I356T and R371I) that distinguished the CQR from the CQS clones. The gene product PfCRT is a 49 kDa protein of 10 transmembrane domains that localizes to the DV membrane.[65] Amino acid changes cluster within or near the junctions of the transmembrane domains.[64]

Our initial population surveys revealed that seven of the eight PfCRT point mutations identified in Dd2 were present in every one of 14 independent CQR Asian or African (Old World) lines. The 8th PfCRT mutation (I356T) was found in half of the CQR lines. None of the analyzed lines was found to have an intermediate CQ phenotype.[14] Of the 15 CQS lines tested, 14 contained the canonical pfcrt sequence in the CQS parent HB3. The CQS line that did not contain this sequence, 106/1, proved particularly informative. As noted above, this line carries the cg2 sequence of Dd2 and earlier provided a clear exception to the association of this cg2 sequence with CQR.[14] The 106/1 pfcrt sequence, however, was found to have an important difference when compared the Dd2 106/1 sequence: codon 76 encoded lysine (K) instead of the threonine (T) residue found in Dd2, although elsewhere in the 106/1 sequence all mutations were identical to those in Dd2. Further analysis showed that all CQS lines analyzed, independent of origin, possessed the PfCRT K76 residue, whereas the T76 residue was found in all CQR lines. The presence of the K76T mutation is always accompanied by additional mutations in PfCRT, suggesting the possibility that a single K76T mutation may not be viable on its own but can only occur in the presence of additional accommodative mutations that preserve a critical native function of the molecule.

Compelling evidence that the CQS phenotype in 106/1 derives from the presence of the PfCRT K76 residue comes from two independent laboratory studies in which CQR mutant parasites were in vitro selected from 106/1 parasites. The selected lines were found to have undergone a novel mutation (K76I) at this amino acid position 76.[64,65] CQR field isolates, however, have universally shown the K76T mutation, as well as the A220S mutation, with strains outside of Asia and Africa displaying distinct sets of mutations else-

where in the PfCRT sequence. Such distinct mutation patterns (haplotypes) are consistent with the separate origins of CQR in these regions.[13] The South American patterns include the 7G8 (Brazil) form (C72S, K76T, A220S, N326D, I356L), the Ecu1110 (Ecuador) form (K76T, A220S, N326D, I356L) and the Jav (Colombia) form (N75E, K76T, H97Q, A220S, R371T).[64] The consistent presence in New World and Old World CQR isolates of a mutation at position 76 that involves a charge may be an important clue to the molecular mechanism of CQR.

## 6. Investigations of pfcrt mutations by episomal transformation and allelic exchange

To confirm the role of pfcrt in CQR, we episomally transformed three CQS lines (GC03, 106/1 and 3D7) with plasmids expressing pfcrt sequences from Dd2 or the mutant line 34-1/E (which respectively encode the K76T and K76I mutations), under the regulatory control of 5' hrp3 and 3' hrp2 UTR elements.[64] Episomally transformed parasite lines were selected that could grow at CQ concentrations of up to 80 nM, while nontransformed control lines were killed at = 35 nM CQ. In comparison, the reference CQR lines Dd2 and FCB propagated at 150 nM CQ. Elevated CQ IC90 levels in the episomally transformed lines were accompanied by acquisition of VP-reversibility, a characteristic of CQR parasites. Further experiments demonstrated that CQS lines transformed with plasmids that used an independent selectable marker (human dihydrofolate reductase) and that expressed pfcrt from a separate promoter (calmodulin) also acquired a moderate, VP-reversible shift in CQ IC90 values.[64] These data provided evidence that mutant pfcrt alleles could confer a degree of VP-reversible CQR. However, the co-expression of introduced mutant (CQR) and native non-mutant (CQS) PfCRT in the same transformed parasite prevented a definitive interpretation. An additional complexity also arose from the use of heterologous promoters, which can lead to different timing and levels of gene expression. Recently, we have overcome these limitations by allelic exchange of the full set of pfcrt mutations from CQR parasites into the pfcrt locus of a CQS line, while retaining endogenous regulatory elements. Results provide conclusive genetically-controlled data that pfcrt mutations can confer VP-reversible CQR (A. Sidhu and D. Fidock, in preparation).

## 7. Epidemiological and clinical evidence pointing to pfcrt as the key determinant of CQR in *P. falciparum*

Release of the pfcrt sequence to the World Health Organization shortly after its discovery in late-1998 enabled malaria research groups to quickly incorporate screening for the PfCRT K76T mutation into epidemiological investigations of drug resistance markers. For this purpose, PCR-based assays are typically performed on dried filter paper blood spots (see **http://www.medschool.umaryland.edu/CVD/nejm2001djimde.htm** for methodological

details). The first field report, based on a study performed in Mali and published in 2001 by Djimdé et al.,[66] found the PfCRT K76T mutation in 100% of the cases of CQ treatment failure, versus a baseline mutation frequency of 41% in samples obtained at the onset of treatment. A lesser, albeit significant, degree of selection was observed for Pgh1 mutations (86% versus baseline frequency of 50%). The presence of the PfCRT K76T mutation was more strongly associated with CQ treatment failure (odds ratio, 18.8) than was the presence of the Pgh1 N86Y mutation (odds ratio, 3.2) or the presence of both mutations (odds ratio, 9.8). Research investigations in Laos, Papua New Guinea, Brazil and several African countries likewise found a very tight association between CQ treatment failure and the marker PfCRT K76T mutation in areas where this mutation had not attained 100%.[67-73] In one of these studies, this association was strengthened when independent polymorphic markers (in this case msp-2) were used to discriminate between recrudescence and reinfection.[69] The clinical data are consistent with the finding of an excellent degree of association between the PfCRT K76T mutation and the in vitro CQR phenotype.[70,71,74-77] The reports demonstrate a universal selection of the K76T mutation under the effect of high level CQ pressure, accounting for the sweeps of CQR through endemic regions in recent years, from perhaps four or more separate foci.[78] These studies further show that the bloodstream presence of parasites carrying the K76T mutation does not always mean that they cannot be cleared after CQ treatment.[66-68,79] Patients who are infected with such parasites and have sufficient pre-existing immunity (premunition) against malaria can often derive benefit from CQ and even resolve their infection. This benefit is apparent in the data from Djimdé et al.,[66] who found that the ability of CQ-treated children to clear CQR parasites correlates with age-related protection against malaria in Mali. Clearance of CQR strains after CQ treatment has also been recognized in studies that have compared rates of in vitro resistance and rates of clinical failure (reviewed in [78]). Besides premunition, variations in CQ pharmacokinetics and metabolism may affect apparent therapeutic responses, as whole blood levels of CQ and its major monodesethylchloroquine metabolite can differ significantly among individuals.[80]

The key role of age-related acquisition of anti-parasitic immunity in clearance of CQR infections has recently been quantified in a new way in a second report by Djimdé et al.[81] These authors found similar numerical relationships between CQ treatment failure rates and prevalence of PfCRT K76T mutation in three separate endemic settings with different parasite transmission rates (spanning an estimated range of 4 to 41 infectious bites per person per month). These relationships were similar after adjusting for age-related protection. Predictions of CQ failure rates in endemic regions may thus be possible for different age groups on the basis of molecular surveys. Such predictions may be most useful in areas where CQR is not yet highly prevalent. In regions of highly prevalent CQR strains where CQ is no longer often used, mutation surveys can be used to monitor for declining rates of resistance that might permit renewed use of CQ under appropriate circumstances. These molecular assays promise to be of significant benefit in monitoring CQR by building upon the results that can be provided using the more labour- and personnel-intensive WHO-standardized 14- or 28-day CQ efficacy surveys.

## 8. PfCRT and Pgh1 may play an important role in vacuolar physiology

PfCRT and Pgh1 are both located on the membrane of the DV, wherein CQ accumulates and generates toxicity against the parasite.[38,65] What are the native functions of these proteins? Plausibly, PfCRT and Pgh1 are involved in critical aspects of DV physiology. Paul Roepe and colleagues (Georgetown University, Washington DC) have found an association between PfCRT point mutations and kinetics of acridine orange (AO) uptake, which may reflect intracellular pH changes.[64,65,77,82] While the intracellular localization of AO in parasites is a matter of ongoing debate.[83] increased AO uptake has been repeatedly found to correlate tightly with CQR and VP reversal. Continuing genetic and physiological experiments are needed to confirm whether PfCRT and Pgh1 directly transport CQ, to determine their natural substrates and physiological functions, and to understand how these functions are altered by point mutations.

## 9. Summary

Recent genetic, drug assay and clinical data provide conclusive evidence that mutations in the *P. falciparum* pfcrt gene are central to CQR. An association of pfcrt and pfmdr1 mutations in some studies may relate to fitness adaptations in the parasite in response to the physiological changes resulting from pfcrt mutations. Recent evidence confirms that the combination of pre-existing immunity and CQ can often resolve malaria in individuals infected with CQR parasites. Variation in the pharmacokinetics and metabolism of CQ may also influence the outcome of such infections. Further studies into the mechanism of the CQR phenotype and a better understanding of the host and parasites factors that determine treatment outcome are needed. A sustained, multidisciplinary approach to tackling this problem presents an important and rational path to improving current means of malaria treatment and control.

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# In vitro and in vivo models for the identification and evaluation of drugs active against Trypanosoma and Leishmania

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***Abstract :*** *In vitro assays and animal models have been developed to determine the activity of compounds or drug formulations against* Leishmania spp.*,* Trypanosoma cruzi *and* Trypanosoma brucei spp*. These models are used in drug screening programmes and proved to be effective in the identification and evaluation of novel drugs for the treatment of leishmaniasis and trypanosomiasis. However, drugs can show highly variable activities in both in vitro assays and animal models depending upon the parasite stage, strain or species and the culture conditions used in in vitro assays, as well as the rodent host strains or species used in animal studies. It is therefore essential to carefully define the parameters of in vitro and in vivo assays and to ensure that they are validated with standard drugs before they are used in drug screens.*

## 1. Introduction

The current chemotherapy for the treatment of human African trypanosomiasis (sleeping sickness, HAT) and South American trypanosomiasis (Chagas disease) is inadequate. Melarsoprol remains the only drug available for the treatment of late-stage disease; supplies of the alternative therapy, eflornithine, are limited.[1] Benznidazole is now the only drug manufactured for the treatment of Chagas disease, although some stocks of nifurtimox are still available. Both benznidazole and nifurtimox are only effective against the acute phase or early chronic phase of the disease and there is variation in the sensitivity of *Trypanosoma cruzi* strains.[2] The situation for leishmaniasis is slightly better as other antimicrobials (amphotericin B and its lipid formulations, paromomycin) do, and the anticancer drug miltefosine and the

lepidine WR6026 might, provide an adjunct to the recommended pentavalent antimonial drugs.[3]

The search for new drugs has stimulated biochemical and molecular research that has led to the identification and characterization of a large number of potential drug targets in the Leishmania and Trypanosoma parasites that cause these diseases.[4,5] This proliferation of drug targets together with a renewed interest in rational drug design, synthetic chemistry with combinatorial libraries and the isolation of plant products offers hope that new antileishmanial and antitrypanosomal drugs will be identified and developed.[6] In this process in vitro and in vivo screens have a critical role to play in the identification and evaluation of new drugs. This review will focus on both the requirements of drug assays and animal models as well as aspects of the biology of these pathogens that are essential in any consideration of the potential of a new compound.

## 2. The Organisms

Although the organisms that cause leishmaniasis, Chagas disease and HAT are classified together as trypanosomatids and share many distinctive and unique characteristics, for example the kinetoplast, glycosomes, the enzyme trypanothione reductase, and an extensive microtubular skeleton, phylogenetic trees based upon molecular parameters show a divergence in this group of organisms.[7] This divergence is reflected in differences in the biochemistry of leishmanias and trypanosomes accompanied by large differences in drug sensitivities. The drugs currently used for the treatment of leishmaniasis, Chagas disease and HAT, and those on trial or in experimental study, are almost exclusive to each disease (see Table 1).

**Table 1.** ***Current Drugs for Leishmaniasis and Trypanosomiasis***

**Leishmaniasis (Visceral and Cutaneous)**
pentavalent antimonials – Pentostam (sodium stibogluconate), Glucantime (meglumine antimoniate)
amphotericin B, AmBisome (liposomal formulation)
pentamidine
paromomycin (aminosidine) – parenteral and topical formulations

**South American Trypanosomiasis (Chagas Disease)**
benznidazole – acute and early chronic stages
nifurtimox – no longer manufactured

**Human African Trypanosomiasis (Sleeping Sickness)**
melarsoprol – late-stage
eflornithine – late-stage *T.b. gambiense* infection only
pentamidine – haemolymphatic stage
suramin – haemolymphatic stage

There are always exceptions, for example pentamidine that is used for both HAT and leishmaniasis and nifurtimox that has been used for both Chagas disease and HAT.

In addition to this difference at the "disease" level, it has become clear over the past two decades that there is considerable variation is the sensitivity of species and strains to both established and experimental drugs:

(a) HAT: The causative organism of West African trypanosomiasis *T. brucei gambiense* is sensitive to the polyamine biosynthesis inhibitor eflornithine(DFMO), whereas *T. b. rhodesiense* is relatively insensitive to this drug.[8,9]

(b) Chagas disease: Strains of *T. cruzi* have a well defined and extremely wide variation in their sensitivity to the standard drugs benznidazole and nifurtimox both in in vitro and animal models.[10,11]

(c) Leishmaniasis: There are up to 15 different species of Leishmania that can cause disease in humans and there is an intrinsic variation in the sensitivity of strains and species to both the standard pentavalent antimonials[12,13] as well as paromomycin[14] and the sterol biosynthesis inhibitor ketoconazole.[15]

These factors underline the importance of any decision about which target species/strains to use in a screening strategy. For leishmaniasis the fact that visceral leishmaniasis (VL) is potentially fatal whereas cutaneous disease is disfiguring but self-curing ensures that a new therapy for VL is the major goal for drug development; therefore primary screens use *L. donovani*. At the secondary level, other species that cause VL, *L.infantum* or *L. chagasi*, and those that cause CL for which models are readily available, *L. major*, *L. mexicana* and *L. panamensis*, can be introduced. However, for HAT and for Chagas disease the strategy is not clear. The parasite that causes West African sleeping sickness, *T.b.gambiense* is less amenable to in vitro screening and animal models than *T.b. rhodesiense*. *T.b. rhodesiense* is therefore normally used in primary screens and *T.b.gambiense* in secondary screens (see below). A further complication is that the CNS models of infection in mice use *T. b. brucei* strains, i.e. parasites that are not infective to humans. There is a similar difficulty with *T. cruzi* strains. Those strains that are useful in primary in vitro and acute animal tests are not the same as those best suited for producing chronic infections in rodent models. In addition, a laboratory that is engaged in the evaluation of a new drug for Chagas disease needs to have models of a number of strains of varying sensitivity to the standard drugs.

## 3. *In vitro* assays

In an earlier review,[16] requirements for an in vitro assay, designed to indicate the intrinsic activity of antiparasitic drugs and to be predictive of in vivo activity were suggested. These included using:

- mammalian stage of the parasite
- dividing population (though not in all cases, see *T. cruzi* below)
- quantifiable and reproducible measures of drug activity
- activity of standard drugs in concentrations achievable in serum/tissues

Increasing consideration in assay design is focussed on features that make

the assay adaptable to medium-throughput screening:
- small amounts of compound are required (< 1mg) from the chemist
- quick throughput (automation, time to completion, number of compounds/test) and reporting
- low cost of tests (time of staff, consumables)

Other factors that make an in vitro assay attractive include it's ability to be used in studies on:
- variation of drug sensitivity using recent isolates, different species/strains, resistant strains
- effects of immune or metabolic components.

For Leishmania and Trypanosoma, in vitro assays with both advantages and disadvantages are available:

*3.1. Leishmania assays*

Promastigotes: This extracellular stage is easy to culture and drug activity is easy to determine. However, there are significant differences between promastigotes and amastigotes in biochemistry and sensitivity to standard and experimental drugs.[12,13] Promastigotes should be limited to use as cytotoxicity indicators in bioassay-guided fractionation of plant products.

Macrophage-amastigote models, primary isolated cells: Tissue macrophages are the host cells for Leishmania amastigotes. The most widely used models for testing drugs against *L. donovani*, *L. infantum*, *L.major*, *L. tropica*, *L. mexicana* and *L. panamensis* have been either murine peritoneal macrophages or human-monocyte tranformed macrophages. These models are able to show species/strain variation in drug sensitivity.[12,14] In these differentiated non-dividing macrophages the rate of amastigote division in host cells and drug activity can be clearly determined. Drug activity is measured by either the percentage of infected cells or number of amastigotes/macrophage.[17] Currently, these parameters are determined by time-consuming microscopical counting. There has to be a renewed attempt to automate this system by isotopic, colorimetric or fluorometric methods including the use of transfected Leishmania. One problem with these macrophage models is the slow rate of division of *L. donovani* and *L. infantum* amastigotes.

Monocytic cell lines: Mouse (J774) and human (THP-1, U937, HL-60) monocytic cell lines have been used in drug assays (18). Although the use of dividing cell lines overcomes the difficulty of isolating and establishing primary cultures, problems of (a) determining the effects on an intracellular infection when drugs can also effect the rate of host cell division, and (b) the decrease in number of amastigotes/host cell in untreated controls over a 3-5 day test period, makes these models less than ideal.

Axenic amastigotes: The development of axenic amastigote cultures offers new opportunities for in vitro assays. Protocols have been described for *L. mexicana*[19] and *L. donovani* and *L. infantum*[20,21] There remains some concern over (a) whether these are "real" amastigotes, although biochemical and immunological parameters have confirmed amastigote characteristics in some systems, (b) the high concentration of serum that is required and (c) the differences in sensitivity of axenic *L. donovani* amastigotes to intracellular amastigotes. This latter point can be explained by differences in uptake of some types of drugs by macrophages and their accumulation in the phagolysosome.

Other factors that can effect drug activity include the level of macrophage of infection, higher infection having lower drug activity,[17] the period of drug exposure, the concentration of foetal calf serum (Croft, unpublished), and temperature (cutaneous species are incubated at 34°C and visceral species at 37°C).

*3.2. Trypanosoma cruzi assays*

All three stages of the *T. cruzi* life cycle have been used in in vitro assays.

Epimastigotes: This is the insect stage, easy to culture and for use in drug assays, but with different biochemistry and drug sensitivities to mammalian stages. Epimastigotes can be a useful cytotoxicity indicator in bioassay-guided fractionation of plant products.

Amastigotes: Amastigotes divide in macrophages, muscle, nerve and many other cell types in the mammalian host; this stage is the major drug target. Consequently, a range of macrophage, myoblast (e.g. L-6 rat skeletal muscle) and fibroblast (e.g. Vero, MRC-5) cell lines have been used in assays. Irradiation of dividing host cell populations prior to infection was shown to overcome the problems associated when determining anti-*T. cruzi* activity in assays with both dividing host cells and parasites.[22] Whatever the cell type, the amastigotes transform to trypomastigotes after 3-5 days of infection (depending on the *T. cruzi* strain) and the length of drug assay has to be set accordingly. Host cell type can also influence drug activity significantly; although only a 2-3 fold difference in anti-*T. cruzi* activity was observed between infected macrophages and Vero cells in the activity of benznidazole this increased to a 60-fold difference for amphotericin B, a drug more readily accumulated by macrophages.[23] Drug activity against amastigotes can be determined by time consuming microscopical counting of stained cell preparations or more rapidly, but less accurately, by the number of trypomastigotes in the host cell overlay tha have differentiated from viable amastigotes. A recent assay based on *T. cruzi* transfected with *E. coli* b-galactosidase has enabled a quantification of intracellular parasites in a colorimetric assay following the addition of the substrate chlorophenol red b-galactopyranoside.[24] Established infections in L6 myoblasts can be drug treated for 96 hours and the drug activity determined on an ELISA reader.

Trypomastigotes: This non-dividing invasive bloodstream form has been considered a problem in relation to blood transfusion. A rapid parasiticidal alternative to gentian violet active at 4°C for inclusion in stored blood is still being sought. An assay using trypomastigotes isolated from the blood of a rodent are incubated with drug for 24 to 48 hours at 4°C and the number of surviving parasites counted.[25]

*3.3. Trypanosoma brucei spp. assays*

Procyclic trypomastigote: This insect stage is easy to grow in culture at 26°C, but differences in the metabolism (e.g. glucose metabolism) make it inappropriate for drug assays.

Bloodstream form trypomastigotes: This form of the parasite has been used increasingly in drug assays since the first adaptations of some strains to axenic growth[26,27] During an exponential period of growth over 3 days trypo-

mastigotes are exposed to drugs after which viability can be assessed by a variety of available metabolic markers; these include Alamar Blue[28] which has either photometric or fluorometric endpoints, or the less reliable tetrazolium salt MTT[29] which uses photometric determination. Other fluorochromes have been used as has the colour shift in pH indicator of phenol red following change in culture medium pH with viable trypanosomes (reviewed in [30]). However, *T. b. gambiense* is the target organism and as strains are also be fully adapted to axenic growth in Baltz medium and they should be used in primary screens in preference to *T.b. rhodesiense*. The difference in sensitivity of the subspecies to drugs, for example eflornithine (DFMO) which is active against *T.b. gambiense* and much less active against *T.b. rhodesiense* makes the case for this more compelling. The example of eflornithine illustrates another point; pentamidine and melarsoprol, the standard drugs normally used in these assays, are exquisitely active against the *T. brucei spp.* in vitro with ED50 values in the nM range and in comparison eflornithine might appear relatively inactive and be missed in the screen. In the identification of "hits" and "leads" in in vitro screens any knowledge of novel chemical structure or novel bichemical target should be taken into consideration.

## 4. *In vivo* assays

Animal models enable drug activity to be determined in relation to absorption (route of administration), distribution (different sites of infection), metabolism (pro-drugs, immunomodulators) and excretion. They also give an early indication of the toxicity of a new drug. Models for trypanosomiasis were amongst the first to be introduced in the first decade of the twentieth century and were used by Ehrlich in early studies on arsenicals. For leishmaniasis and trypanosomiasis a range of rodent models are available. Most of the models use mice (advantage: at 18-20g/mouse a lower amount of compound is required), which are available as SPF and inbred strains (advantage: reproducible results which enable the use of only 5 animals/group), which are susceptible - resistant to a number of strains and species (advantage: acute and chronic, non-cure and self-cure models are available). The obvious disadvantage of all these models are the differences in the physiology of humans and mice (metabolic rate, permeability of gastro-intestinal tract to drugs) and pathology of the disease are different in humans and mice.
For most diseases the aim using the animal model is to find a drug that can be administered orally, be effective in a short course (< 10 days) and have no indication of toxicity at the top doses tested (100mg/kg).

### *4.1. Leishmaniasis*

Visceral leishmaniasis:
Inbred strains of mice are widely used in studies on visceral leishmaniasis with susceptible, resistant and intermediate strains being available. The BALB/c mouse is commonly used strain in chemotherapy tests and provides an illustration of the advantages and problems faced. The mouse strain is widely available, can be used at 18-20g, gives highly reproducible levels of

infection when an amastigote inoculum is administered i.v., is susceptible to a wide range of strains and the test is completed in 2 weeks. However, the timing of both dosing and necropsy is important as the liver infection develops rapidly over the first 3 weeks of infection, then reaches a plateaux before slowly declining. In contrast the spleen infection develops slowly and reaches a maximum at 6-7 weeks after infection. Therefore, a test in week two after infection examines the activity of the drug against the liver infection but not the spleen infection. The infection in each mouse strain needs to be characterised for each parasite strain used to ensure that drugs are tested appropriately. Athymic and scid mice provide a model for treatment of VL in immunosuppressed cases. The hamster also provides a good model for VL and provides a more synchronous infection in the liver and spleen that can develop into a chronic non-cure infection more similar to human VL. However, the disadvantages of weight (50-60 g), the absence of an easy route for i.v. inoculation (the intracardiac route is used) and the poor availablity of inbred SPF animals make this model less predictable. A problem in all the models is the determination of drug activity. This is dependent upon necropsy, the preparation of liver (and/or spleen) smears and microscopy to determine the level of infection in untreated and treated animals; this is time consuming (31).

Cutaneous leishmaniasis:

Inbred strains of mice with defined susceptibility/resistance, cure and non-cure to *L. major*, *L. mexicana* and *L. panamensis* are available. Again the assays have to be designed with care to ensure effective use of these models to determine drug activity and the host-parasite action is of importance. The BALB/c mouse - *L. major* model has been used widely in drug studies but it is an exceptionally rigorous non-cure model in which only the most active drugs have any efficacy and without absolute cure relapse of the infection occurs. In this model the standard antimonial drugs are ineffective[32] Other mouse models (CBA, C57/Bl) that self-cure, like most humans, should be used for studies on lead compounds. Lesions on the back of mice are also amenable for testing topical formulations. The determination of drug efficacy by measuring changes in lesion size (two/three dimensions) during and after the course of treatment is straightforward. However, as much of the lesion is composed of inflammatory cells and the amastigotes are in the dermal layer of the skin, lesion measurement can be considered a crude and indirect measurement of antiparasitic activity. Culturing parasites from biopsies provides an alternative measurement of activity but poses other problems (sampling, contamination).[33] The absence of animal models for *L. tropica*, *L. aethiopica* and *L. braziliensis* has been a limitation for chemotherapy studies on these important species; the *L. braziliensis* hamster model deserves some examination in this respect.

*4.2. Chagas disease*

A number of widely used strains of *T. cruzi*, for example Tulahuen, Y and Peru, can produce an acute infection in mice. In preliminary tests the suppressive activity of a drug is easily determined against an acute infection by monitoring blood parasitaemia and survival. The determination of cure is more difficult as recent studies on mice using PCR and other markers have

shown how widely parasites disseminate within the mouse model.[34,35] Traditionally, xenodiagnosis (using a triatomine bug, the parasite vector), haemoculture and serological assays were considered sensitive enough to assess the curative dose of a novel drug; PCR and/or RT-PCR should be considered essential tools for this job. In curative studies it is also essential is to establish that a lead compound is active against a panel of strains of *T. cruzi* with known sensitivities to the standard drugs (see above) preferably derived from clinical isolates from different regions of South and Central America.

The absence of a drug for the cure of the chronic infections of Chagas disease presents another challenge for chemotherapy. The chronic infection is characterised by a high level of inflammation, fibrosis and very low numbers of tissue parasites. Whether the host response is due to autoimmunity or is induced by residual parasites is much debated (36). However, assuming that the chronic disease results from an infection, there are two questions: (a) how to determine drug activity against sparse, widely distributed parasites, and (b) the relevance of using the chemotherapeutic approach alone in such a disease state.

*4.3. Human African Trypanosomiasis*

Acute *T. brucei*/*T. rhodesiense* model:

For the first or haematolymphatic stage of sleeping sickness various strains and clones of *T.b. brucei* or *T.b. rhodesiense* are used in inbred mice. For safety reasons *T.b. brucei* is preferred over *T.b. rhodesiense*. A trypanosome population that kills the control animals in 6-8 days is ideal since drug efficacy is judged on disappearance of parasitaemia and survival time. Mice are infected intraperitoneally and treatment initiated 24 hours later. One day or 4-day treatment protocols using intraperitoneal application are standard procedures. Mouse tail blood is examined twice/weekly. If the mice stay aparasitaemic for 30 days after treatment they are considered cured. Standard drugs (melarsoprol, diminazene aceturate, pentamidine suramin) as well as experimental drugs cure this acute model.[37]

Acute *T.b. gambiense* model:

This subspecies is more difficult to propagate in laboratory animals than either *T.b. brucei* or *T.b. rhodesiense*. *T.b. gambiense* does not grow in inbred mice but only in nurseling rats, the vole *Microtus montanus* or the multimammate rat *Mastomys natalensis*. The last species is easy to breed and to handle. Immunosuppression by cyclophosphamide (200mg/kg i.p. one day before infection) or dexamethasone (5mg/l in the drinking water ad libidum) increases the parasitaemia which still remains 100-fold below parasitaemias reached by *T.b. brucei*. Treatment should be initiated when all animals are parasitaemic. Control animals usually do not die within the first two months, therefore, treated animals have to be monitored for parasites for at least 3 months before they are to be considered cured.[37]

Chronic (CNS) model:

A CNS mouse model of infection for the second or late-stage of sleeping sickness has been validated.[39] Inbred mice are infected with a low virulence *T.b. brucei* strain (e.g. GVR35) and infection allowed to progress to day 21 post-infection when a CNS infection is established. Treatment lasts for

7-10 consecutive days. A control group is treated once on day 21 with Berenil (diminazene aceturate) 40mg/kg. These mice relapse around day 40 post-infection due to parasites invading the bloodstream from the CNS. Treated animals have to be monitored for parasitaemia for 6 months. The standard drug is melarsoprol which cures this model with 7 x 10mg/kg applied on day 21 to 27 (39). This model is very stringent for experimental compounds. For melarsoprol, which easily cures acute infections, a 10-times higher dose is required to achieve cure of the CNS model (Brun, unpublished observation).

## 5. Conclusions

Although it is possible by varying the conditions of testing to significantly alter the activity of a drug in both in vitro and in vivo assays, reproducible results are produced by clearly defining the parameters of the assay as discussed above. The parameters are defined by the purpose of the test. In all assays the inclusion of a standard drug is essential as it gives a marker that should be consistent between tests and indicate factors that could invalidate a particular test. All tests need to be repeated and when a lead compound has been established it is advisable to have it tested in another laboratory to confirm the claimed high level of activity. At the same time as antiparasitic efficacies are being determined in in vitro and in in vivo it is essential that the toxicity of lead compounds to mammalian cell lines and rodents is determined as the aim is to identify a compound with selective activity and a large therapeutic index.

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# Animal models in antimalarial drug development

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***Abstract :*** *Several in vivo models exist for testing potential new compounds against malaria. New compounds are usually initially analysed in vivo using rodent models to test efficacy against rodent malaria parasites. Further development of a drug includes efficacy testing against the human malaria parasites* P. falciparum *and* P. vivax *using non-human primate models, or against closely related natural primate malaria parasites. The current possibilities of some of these animal models and future developments including benefits from the malaria genome project will be highlighted.*

## 1. Introduction

The emergence of resistance to the most commonly used drugs against the two most prevalent human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*, underlines the importance of the development of new drugs active against these parasites. In addition, only one compound, primaquine, is known to be active against the dormant liver stages of *P. vivax*, the hypnozoites.[1, 2] The indications that resistance against primaquine is already developing in *P. vivax*[3,4] illustrates the need for development of new hypnozoitocidal drugs for accurate treatment of *P. vivax* infections.

Initial testing of activity of new compounds is usually performed in vitro against blood stages of *P. falciparum*, since these stages of this parasite can easily be cultured in vitro.[5] Until recently, it was not possible to maintain long-term cultures of *P. vivax* blood-stage parasites, and the recent publication of in vitro cultivation of *P. vivax* blood-stage parasites using human reticulocytes[6] demonstrates the complexity of routinely culturing this human malaria parasite for the purpose of drug testing. Although in vitro assays pro-

vide the first evidence that a compound has antimalarial activity, bioavailability of the compound is an essential element that can only be assessed in vivo. Animal models thus play a critical role at several stages in the long developmental pathway from identifying novel compounds active against malaria parasites to clinical testing of these compounds.[7]

## 2. Rodent models

Initial in vivo efficacy testing of new promising compounds is usually performed in mice using the rodent malarias *P. berghei*, *P. vinkei*, *P. chabaudi*, or *P. yoelii*. Historically, these models have been widely used to study antimalarial drugs,[8,9] in part because of the ease of use of these models. Many different mouse strains can be used as a host for the rodent malarias and generally a rapid parasite development (the parasites have a 24 hour lifecycle in red blood cells) resulting in a fulminating parasitemia is observed after an infection with blood-stage parasites. The fulminating parasitemia is very beneficial for assessing the curative effect of new compounds. In addition, avirulent strains of *P. yoelii* and *P. chabaudi* have been developed that result in a resolving parasitemia in mice which might be useful for specific purposes. Avirulent *P. yoelii* and *P. berghei* have a preference for reticulocytes for invasion, while the other rodent malarias, including the virulent *P. yoelii*, prefer mature red cells for invasion and these differences may have important consequences for studying drug efficacy. Enzymes in reticulocytes may be absent from mature red blood cells and this may interfere with drug entry and/or stability in the cells. Therefore, conclusions must be carefully drawn when one or the other rodent malaria is used for initial in vivo drug efficacy testing.

A major difference between rodent and human or primate malaria biology that complicates in vivo studies on drug efficacy is the preerythrocytic development of the parasites. Rodent parasites have a fast pre-erythrocytic stage development (48-68h) as compared to human or primate parasites (132-192h),[9] and this can obviously have a marked effect on prophylactic drug efficacy. The active component of a prophylactic drug that would have a bioavailability of 50h could effectively eliminate rodent malaria pre-erythrocytic stages, while it could only have growth retardation effects on the young developing human or primate preerythrocytic stages. In addition, none of the rodent malarias produce hypnozoites meaning that they do not provide a really suitable model for *P. vivax*.

The above mentioned biological differences between rodent and human malaria parasites complicate extrapolation of results obtained in rodent malaria models. In addition, rodent hosts are quite different from human hosts which complicates extrapolation of results even further. Rodents have a high serum detoxifying activity, specifically high levels of esterase which might interfere with bioavailability of active compounds. In general, rodent metabolism and immune responses are quite different from humans. Since chemotherapy is an interplay between bioavailability of the active compound and the host immune system (which has to eliminate the parasite when the drug is less effective than 100%), animal models closer related to, and more predictive for humans are needed when rodent models give promising results.

## 3. Primate models

*3.1. Human malaria parasites in non-human primates*

The human malaria parasites *P. falciparum* and *P. vivax*, apart from developing in their natural host, only develop in a few New World monkey species (owl monkeys or Aotus species and squirrel monkeys or Saimiri species) and in chimpanzees. Chimpanzees have not been used routinely for malaria drug studies and will not be discussed here. New World monkeys are phylogenetically distant from humans (compared to Old World monkeys, see below), and form an artificial animal model. Although many studies involving antimalarial drugs have been performed in New World monkeys,[10-12] working with *P. vivax* in any of these models is complicated (reviewed in [13]) because i) parasites have to be adapted to the primate subspecies that is used in the study, which can be a tedious procedure, ii) due to reticulocyte restriction of *P. vivax* only resolving, slow growing, relatively low parasitemias are obtained which especially complicates studies on drug efficacy where high fulminating parasitemias are needed, iii) primates usually have to be splenectomised to yield higher levels of blood-stage parasitemia, iv) relatively low transmission rates do not favour these models for studying prophylactic drugs, and v) relapsing malaria has never been observed, excluding studies on hypnozoitocidal drugs. Alternatives for in vivo drug studies on *P. vivax* will be discussed below (section ii), and the remaining part of this section will focus on *P. falciparum* in New World monkeys.

To obtain high, fulminating parasitemia of *P. falciparum* in Saimiri monkeys, splenectomy is usually necessary, especially when the monkeys have been infected before with the same parasite strain.[14] The parasite strain needs to be adapted to growth in the Saimiri subspecies of which there are many, which can be a tedious process.[13] In view of the interplay between drug and the host immune system to fully cure blood stage parasitemia, the use of splenectomised monkeys is not ideal. Saimiris poorly support the development of gametocytes[13] and therefore transmission studies for testing prophylactic drugs are not readily performed in this model. *P. falciparum* in Aotus monkeys forms a better model for in vivo drug efficacy testing against different stages of the parasites life cycle.

For studies with *P. falciparum,* Aotus monkeys from Colombia are most commonly used, although Aotus from other origin have also been used.[13,15,16] Adaptation of *P. falciparum* strains can be tedious, but many strains are available that have been adapted successfully.[13,17-19] The primates do support gametocyte development, but transmission rates are low rendering this model less suitable for prophylactic drug studies. Blood-stage parasitemia is variable and depends highly on the adaptation to a particular type of Aotus and on the level of monkey passage, so care must be taken on the strain used for a particular study. Despite the scarcity of Aotus monkeys available for research, this model is highly valuable for in vivo drug research on *P. falciparum*.

*3.2. Primate malaria parasites in non-human primates*

As briefly discussed above, alternatives for in vivo *P. vivax* drug studies might be more informative and easier to use than *P. vivax* in New World monkeys. In addition, in the case of hypnozoitocidal drugs, alternative models supporting hypnozoite development are needed to be able to assess drug efficacy. Phylogenetically *P. vivax* groups with a number of parasites that naturally infect macaque monkeys.[20] Here we will discuss *P. knowlesi* that has been used historically for drug research, and *P. cynomolgi* that we have recently been using to test new drugs. Both parasite species infect the rhesus monkey (*Macaca mulatta*) through sporozoite as well as blood-stage induced infections, and are also infectious to man.

Rhesus monkeys are phylogenetically more closely related to man than New World monkey species and are immunologically better characterised. In addition, this primate species, the natural host for a number of malaria parasite species, is readily available for experimentation and is frequently used for preclinical pharmacokinetical studies.[21,22] Historically *P. knowlesi* has been frequently used for malaria drug studies. With its 24h asexual blood stage life cycle, *P. knowlesi* gives a rapid fulminating blood stage parasitemia in rhesus monkeys which is beneficial for assessing drug efficacy. The whole life-cycle of the parasite is available in vivo[23] and also in vitro ([24] and our own unpublished results) allowing efficacy testing of drugs against all stages of development, initially in vitro[25] but also in the natural host that is closely related to humans. *P. knowlesi* does not form hypnozoites precluding research to hypnozoitocidal drugs.

*P. cynomolgi* forms an appropriate model of *P. vivax*,[26] also because many biological characteristics (e.g. duration of blood stage maturation, recurrent type of blood stage infection, preference for reticulocyte invasion, hypnozoite formation) are shared.[23] Hypnozoite formation in rhesus hepatocytes makes this animal model particularly useful for developing new hypnozoitocidal drugs. Because the parasite is less severely restricted to reticulocytes than *P. vivax*, peak parasitemias in rhesus monkeys range between 1 and 10%, thereby providing more easy access to parasite material for experimentation and an easier assessment of drug efficacy. We have been using this model for testing new drugs against blood-stage infections of *P. vivax*-type parasites. Initially, to assess susceptibility of *P. vivax* and *P. cynomolgi* to the drugs under investigation, ex vivo drug assays were exploited.[27] Blood-stage parasites were derived from infected Aotus monkeys (different *P. vivax* strains) or rhesus monkeys (*P. cynomolgi* M strain) at young ring stage of development. Erythrocytes were washed in RPMI and plated in 96-well culture plates at 2% hematocrit and a final parasitemia ranging from 0.5 to 1%. Triplicate wells contained serial 3-fold drug dilutions with chloroquine as a control. Parasites were cultured until the trophozoite stage of development for 28h. 3H-hypoxanthine (1 mCi per well) was then added and culturing was continued for another 18h. Parasites at the schizont stage of development (˜6 nuclei) were harvested and incorporation of radio-label as a measure for surviving parasites was determined. The results indicated that there was variability in IC50 for certain compounds between the different *P. vivax* strains tested, and that *P. cynomolgi* had a comparable sensitivity to the compounds (our unpublished results).

We then tested one of the compounds in vivo in *P. cynomolgi* infected rhesus monkeys using protocols that had been established previously in *P. falciparum* infected Aotus monkeys. A control group of 5 rhesus monkeys developed a peak parasitemia of 5-10% 10-11 days post-infection with *P. cynomolgi* blood-stage parasites. The infection resolved after 15 days and a recrudescence with parasitemias ranging from 0.2-2.5% was apparent two days later. This pattern of recurrent infection is highly reproducible in rhesus monkeys, facilitating in vivo studies with *P. cynomolgi.* In the experimental group drug treatment was started at a parasitemia of 0.1-0.2%, and within 3 days blood stage parasites were not detectable anymore. In addition, no recrudescence was observed in a follow up period of 28 days. This experiment clearly demonstrates that valuable information on in vivo drug activity against *P. vivax*-type blood stage parasites can be obtained using the *P. cynomolgi* rhesus macaque model. This model in addition can be used to evaluate prophylactic drugs, as well as hypnozoitocidal drugs, since sporozoite infection of rhesus macaques can easily be established and the parasite is prone to produce hypnozoites.[23]

## 4. Conclusions

In the near future, data from the malaria genome project will result in the identification of new drug targets. The recent development of transfection technology for malaria parasites, especially for the *P. vivax*-type parasites *P. knowlesi* and *P. cynomolgi*[28, 29] which provide very informative models for in vivo drug testing, will assist in identification and validation of potential new drug targets. In addition, using this technology, problems like development of drug resistance can be investigated more directly by transfection of in vitro mutagenised target genes. In vitro studies using *P. falciparum* cultures provide an efficient first screen for newly identified targets and corresponding compounds. Promising compounds then have to be tested in vivo, since bioavailability is a crucial aspect of drug activity. Rodent malarias form efficient models for initial in vivo screening to preselect the most promising compounds. Finally, appropriate non-human primate models, i.e. *P. falciparum* in Aotus monkeys and *P. cynomolgi* as a *P. vivax*-type parasite in rhesus monkeys, provide invaluable information on bioavailability and efficacy of new drugs against different stages of the life-cycle of the human malarias, prior to clinical testing of new compounds.

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# In vitro and in vivo drug sensitivity tests for malaria

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***Abstract:*** *One of the applications of parasite cultivation is the in vitro drug sensitivity assay, which is a major tool for the screening of potential antimalarial drugs. We use the isotopic microtest as a routine procedure. The other in vitro assays that we have evaluated (lactate dehydrogenase-based assay and assay based on chloroquine efflux) do not seem to be suitable for in vitro drug screening. The results of the microtest are expressed as IC50, which corresponds to the concentration that inhibits 50% of the parasite growth in comparison with drug-free controls. To evaluate the activity of new compounds, we used either reference clones or fresh clinical isolates from patients. Assays performed against a sufficient number of isolates can be used to determine the level of in vitro activity of a new compound, to compare this activity with that of compounds belonging to either the same chemical class or other classes, and to evaluate the potential for in vitro cross-resistance. The in vitro assay may also be performed to analyze drug-drug interactions of different drug combinations. Unfortunately, in vitro assays need to be standardized as various factors influence the IC50 values.*

## 1. Introduction

The development of the technique for in vitro culture of *Plasmodium falciparum* by Trager and Jensen has led to a better understanding of malaria biology.[1] One of its applications is the in vitro drug sensitivity assay which, like in the case of antibiotic sensitivity assay, can determine sensitivity/resistance of the parasites to antimalarial drugs. However, unlike antibiotic assays, the in vitro drug sensitivity test for antimalarial drugs is not yet standard-

ized. Two in vitro tests have been widely used. The microtest, which was developed by Rieckmann and adopted by the World Health Organization (WHO) for epidemiologic monitoring of drug-resistant malaria, requires a parasite count under the microscope to measure the capacity of antimalarial drugs to inhibit parasite maturation to the schizont stage.[2] The results are expressed as the minimal inhibitory concentration. The second test, the isotopic microtest developed by Desjardins,[3] has been used in most research laboratories that work on antimalarial drugs. This assay measures the inhibition of the incorporation of radiolabeled DNA precursor into the parasites. The isotopic assay is less time-consuming than Rieckmann's assay but requires a well-equipped laboratory (sterile hood, cell harvester, liquid scintillation counter, etc.). The isotopic "semi-microtest" is a variant of the isotopic microtest, which differs in the volume and quantity of reagents, and is no longer used due to economic reasons.[4]

What are the applications of the in vitro assays today? In vitro assays for antimalarial drugs continue to play an important role in the following research fields: screening of novel compounds, analysis of in vitro cross-resistance, effects of drug combinations, determination of the phenotype of reference clones and strains, and epidemiologic description of drug resistance. After a brief description of the isotopic in vitro that we use routinely, the different applications of in vitro assays will be presented.

## 2. Isotopic microtest

The detailed description of the assay has been published in our article.[5] In this paper, we will focus on several factors that may confound the interpretation of results.

### *2.1. Sources of parasites*

Depending on the applications, several sources of malaria parasites are available to perform the assays. The parasites may be classified into three groups: clones, strains (or lines), and isolates. All of these parasites have been originally obtained from infected patients.

A clone, by definition, is genetically homogeneous. It is obtained by either micromanipulation or limiting dilution. About 10 reference clones are available in research laboratories in the world. The parasite stocks are cryopreserved at the University of Edinburgh. The use of clones is important to ensure reproducible assays over time.

The strains are parasites that are adapted to in vitro conditions but are not necessarily homogeneous in genetic terms. It may be assumed that the continuous culture may select subpopulations of parasites over time, leading to the homogeneous constitution of the parasites. However, it is also possible that different aliquots of the original strain may diverge independently in different laboratories.

An isolate refers to a set of parasite populations obtained from an infected person at a given time. The isolates represent the parasites existing in their natural state. Several studies have shown that a given isolate often consists

of several subpopulations of genetically distinct parasites. The isolates are most useful for studies that aim to define the epidemiology of drug resistance and to analyze the relationship between the phenotype (drug sensitivity) and genotype (DNA sequence of established or candidate resistance genes). The isolates used for these studies should be obtained from patients who have not self-medicated with antimalarial drugs since the presence of drugs may modify the assay results.

*2.2. Test compounds*

The preparation of solutions of test compounds and coating the culture plates are important steps for the assay. There is no standardized protocol for these procedures, which may be the reason underlying wide differences in the results observed in different laboratories. The most commonly used solvents are distilled water, ethanol, methanol, and DMSO. Most of these solutions may be stocked at 4°C for at least 3 months, with the exception of DMSO. To avoid the problems related with solubility, test compounds should generally be distributed into culture plates just before the parasites are added. For some compounds, the solutions may be distributed and air-dried, and the pre-coated plates may be stocked for several weeks to months, depending on the shelf-life of the test compounds.

*2.3. In vitro assay*

Fresh isolates or clones maintained in continuous culture (asynchronous or synchronized) may be used for in vitro assays. The parasitemia and hematocrit are two parameters that may influence assay results. For example, the inoculum effect refers to an inadequate inhibition of parasite growth due to an excessive number of parasites as compared with the drug concentration. The initial parasitemia should be less than 0.5 to 0.8% for a hematocrit of 1.5%. These parameters may be adjusted to lower values if the incubation time exceeds 48 hours.

The standardization of in vitro assays should also take into consideration other parameters that vary in different laboratories. The varying concentrations of folic acid or para-aminobenzoic acid in the culture medium have considerable effects on the activity of antifolate drugs. A prolonged incubation time lowers the 50% inhibitory concentration for drugs with either rapid or slow action. Serum substitutes or homologous sera during the acute phase of malaria infection do not seem to provide optimal serum sources although they are used in some assays.[6,7] As for the radiolabeled precursor, tritium-labeled hypoxanthine is most commonly used. Other precursors, such as adenosine and thymidine, do not seem to be well adapted for in vitro assays.

*2.4. Data interpretation*

Assay results are generally expressed as the 50% inhibitory concentration (IC50), which corresponds to the drug concentration at which 50% of parasite growth is inhibited. In addition to IC50, some authors calculate IC90 and IC99. Several computer softwares that fit linear or non-linear regression curves are available in the market.

*2.5. Other assays*

Several alternative assays have been developed. Gluzman et al. have developed an assay system that requires only a few hours to obtain the result.[8] The principle of a rapid in vitro test is based on two observations. First, chloroquine-resistant parasites actively expel chloroquine, while chloroquine-sensitive parasites accumulate the drug.[9] Second, the addition of verapamil inhibits chloroquine efflux in chloroquine-resistant parasites, but not in chloroquine-sensitive parasites.[10] Unfortunately, in our experience, the results of this rapid in vitro assay are discordant with the in vivo and isotopic in vitro tests.[11]

Recently, Makler et al. have proposed a novel in vitro assay based on an enzymatic reaction that specifically detects the presence of malarial lactate dehydrogenase (LDH).[12] The IC50s derived from this colorimetric test are highly correlated with those obtained by isotopic microtest. However, the initial version of the colorimetric test which does not use anti-LDH monoclonal antibodies is not very sensitive for an initial parasitemia < 1%.[13] The colorimetric test has the advantage of being non-radioactive. It needs to be improved to increase its sensitivity to detect LDH.

A minor modification in culture media (a mixture of RPMI 1640 and Waymouth media) allows a short-term in vitro culture of other human malaria species for drug sensitivity assay. Initially developed for *P. vivax* by Brockelman et al., this simple technique was adapted to perform isotopic in vitro assays for *P. ovale* and *P. malariae* in our laboratory.[14,15] Although the assays do not have a high success rate (about 40%), the results are comparable with those of drug-sensitive *P. falciparum*.

## 3. Applications of in vitro assays

*3.1. Screening*

According to the "Guidelines for antimalarial drug screening," the first step in the screening process is the evaluation of the in vitro antimalarial activity of candidate compounds. Drug screening is indeed the most important application of in vitro assays. Compared with the in vivo rodent malaria model, in vitro assays have the following advantages: the use of the same malarial species that infects man, the possibility to test several compounds simultaneously and at high concentrations, lower cost, rapidity, and exclusion of host-related factors. The limits of in vitro screening include the lack of information on drug metabolism, pharmacokinetics, and drug toxicity. When in vitro assays are performed against a sufficient number of clinical isolates (> 30), the level of activity of the test compounds can be evaluated and compared with that of either compounds belonging to the same chemical class or compounds from different chemical classes.[16]

*3.2. Cross-resistance*

An assessment of cross-resistance with other drugs is one of the important steps in the development of novel compounds. In vitro assays can be performed to determine the activity of several compounds simultaneously using

the same isolate of *P. falciparum*. Potential cross-resistance may be evaluated by performing in vitro assays against several dozens of isolates for several compounds. In vitro cross-resistance is measured by calculating the degree of correlation between the IC50s of two compounds. The existence of in vitro cross-resistance may suggest the potential risk of in vivo cross-resistance, especially if resistance to one of the drugs has attained a high level in an endemic area. Cross-resistance between compounds belonging to the same chemical class has been observed frequently (chloroquine/amodiaquine, mefloquine/halofantrine, pyrimethamine/cycloguanil).[17] The mechanism underlying in vitro cross-resistance between amino alcohols and artemisinin derivatives has not been understood.[18]

*3.3. Drug combinations*

The extension of resistance to practically all available antimalarial drugs necessitates a novel approach to antimalarial chemotherapy, notably the use of drug combinations. In vitro assays can evaluate whether a given combination is synergistic, additive, or antagonistic. In vitro studies have thus shown the synergistic combination between atovaquone and proguanil and confirmed the synergistic interaction between pyrimethamine and sulfadoxine which was initially observed in animal models.[19] In vitro assays have also been used to study the capacity of certain drugs belonging to various chemical classes (calcium blockers, tricyclic antidepressants, antihistaminics, penfluridol) to modulate resistance to chloroquine and mefloquine.[20] These in vitro approaches have been applied to understand the mechanism of action of antimalarial drugs and may be of great interest if modulation of drug resistance can be applied in vivo.[21]

*3.4. Epidemiology of drug resistance*

Although in vitro tests do not, and can not, replace in vivo tests to define and guide the national antimalarial drug policy, in vitro drug sensitivity assays play an important role in describing the epidemiology of resistance to classical drugs. In vitro assays are thus important tools to establish the baseline data on drug sensitivity in a given endemic area, especially before introducing new drugs in the area, and to monitor the evolution of drug sensitivity over time. The representative isolates that are adapted to establish the epidemiology of drug resistance are those that are obtained from patients who have not self-medicated in a well-defined study site where, if possible, previous in vivo response data are available.

## 4. Correlation between in vitro and in vivo responses

When in vitro and in vivo tests of resistance are performed in parallel, in vitro assays provide complementary data. In vitro test can notably detect the presence of resistant isolate in a patient who clears parasitemia due to acquired immune system. In vitro assay can also detect the presence of sensitive isolate in a patient who responds with a therapeutic failure associated with poor drug absorption or reinfection, and not due to drug resistance.

Thus, in order to document true drug resistance, it is necessary to perform both in vitro and in vivo tests, measure the drug level in the plasma, and compare the profiles of polymorphic genetic loci of the pre-treatment and recrudescent isolates. When the confounding host and parasite factors are excluded, in vivo test performed preferably in non-immune local population (usually aged < 5 years old) and in vitro test yield concordant results, and it will be possible to determine the in vitro threshold level of resistance.[22]

## 5. Conclusion

In vitro assay is an important tool for the study of new antimalarial drugs. It clearly has several advantages over the in vivo rodent model of malaria. In vitro assay, which is a phenotypic marker for parasite resistance, also provides complementary data with regards to the in vivo test, which evaluates the therapeutic efficacy of a drug. Despite these advantages of in vitro assays, these tests need to be standardized to allow a direct comparison of results between different laboratories and also require further improvement so that they can be widely used in the field.

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# Multidrug therapy to delay antimalarial drug resistance

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***Abstract :*** *Resistance is the prime determinant of the life-span of antimalarial drugs. Therefore, protecting these drugs against resistance is of the highest priority. This paper describes the impact of current and impending drug resistance on antimalarial drug R&D as well as measures that could be taken to develop effective and durable treatments. The development of strategies to delay the occurrence of resistance involves the study of appropriate multidrug regimens for mutual protection, as well as the study of the determinants of drug policy, deployment, and prescribing.*

## 1. Introduction

The stated goal of the symposium and the ensuing publication is to provide researchers with a practical guide to developing new antiparasitic drugs. One important, yet often understated, notion is that drug research does not end at drug registration. On the contrary, there should be a seamless line between drug R&D and deployment and they should not be regarded as mutually exclusive. The pharmaceutical industry, in particular, has long since accepted this concept. In prioritising R&D projects, drug companies use both marketing intelligence and an analysis of research opportunities. They also provide the research needed to support and optimise the use of drugs after registration to prolong their life-span. Whilst the objectives of the industry do not necessarily coincide with public health needs, the principles outlined above should apply to both.

Protecting current and newly introduced antimalarial drugs against resistance is of the highest priority. This paper addresses the issue of optimising the

life-span of antimalarial drugs by considering how antimalarial compounds are both developed and deployed.

Drug-resistant *Plasmodium falciparum* now occurs in virtually all malaria endemic areas.[1,11] All commonly used drugs are affected to various degrees except the artemisinin-type compounds. Data from Africa indicate that drug-resistant falciparum malaria results in increased morbidity and mortality.[1] Reduced drug effectiveness means that these drugs must be replaced with new ones. However, the supply of new drugs is limited and their use may be limited by cross-resistance with those currently used. Policy makers are confronted with the dual challenges of deciding when to change their first line drug and which new drug to use.[2]

Chloroquine has been the mainstay of therapy for 40 years but resistance is now widespread. As a result, sulfadoxine-pyrimethamine (S/P) has replaced chloroquine as first line treatment for malaria in some African countries. However, parasitological resistance to S/P is emerging and spreading rapidly in those countries which have made the change.[3,4] Available alternatives to S/P are over 10 times more expensive, and would be unaffordable for most African Ministries of Health in the volume required to treat all cases of outpatient malaria. As S/P loses its effectiveness, morbidity and mortality from malaria will inevitably rise.

One option is to combine currently available and future antimalarial drugs in such a way as to provide mutual protection against the development of drug resistance. This approach is discussed below.

## 2. Factors involved in the emergence of spread of parasite resistance

Understanding the underlying factors in the emergence and spread of drug resistant malaria is essential – many factors exist.[5] These are chiefly:

- Operational factors influencing drug policy decisions, drug deployment, prescription, use/misuse
- Epidemiological: the effects of malaria transmission intensity on drug pressure and the development of immunity
- Pharmacological: drug pharmacokinetic (PK) and pharmacodynamic (PD) characteristics, mechanism of drug action and resistance, transmission-reducing properties.

## 3. R&D, regulations, policies and drug resistance

The current approach to drug R&D and policy may play a role in the emergence of resistance. Antimalarial drugs are usually developed alone, deployed as monotherapy, and exploited well beyond the point at which they have lost efficacy. Traditionally, the use of multidrug therapy for the treatment of malaria has not been considered as a practical option. This thinking accounts for difficulties at various steps of the R&D-deployment continuum:

- It is commonly believed that registering a drug for single-agent use is far simpler and easier than registering it for combined chemotherapy. This is cer-

tainly true but fails to appreciate what is being done routinely e.g. in the field of tuberculosis where antituberculous drugs are developed and registered as single drugs but only for use in combination with other antituberculous drugs.
- Increasingly demanding drug regulations that do not necessarily take into account the needs of developing countries, play a role too.
- Policy makers consider two drugs to be more expensive, and, therefore, unaffordable. This does not take into account the direct/indirect costs of a failing first line drug on individuals, communities, and health services.

## 4. Drug PK, PD characteristics, malaria transmission & drug resistance

Emergence of resistance to drugs which act on the asexual life cycle of malaria is considered inevitable. Mutations in the parasite genes that confer drug resistance occur spontaneously in nature. Their spread depends primarily on the ability of mutated parasites to survive drug treatment, as well as on transmission intensity. Drug pressure selects for those mutations that confer maximal survival advantage of the parasite whilst least disturbing parasite functions. The pace at which resistance develops differs with different classes of antimalarial drugs and transmission intensity.[6]
- The mechanism of drug action/resistance and drug residence time in man are important determinants of drug resistance. Resistance is more likely to develop to drugs with slow onset of action, long half-lives, and to which resistance is conferred by point-mutations. Examples are the currently available antifolate drugs (pyrimethamine, sulfadoxine) and atovaquone. Resistance to quinoline-type compounds (e.g. chloroquine) is multigenic and has taken much longer to develop and spread.[7]
- Several mathematical models have been proposed to explain the dynamics of the emergence and spread of resistance, often with divergent results. A key issue is how to weigh transmission intensity with the resulting clone multiplicity. It is conceivable that in areas of lower transmission, fewer clones are exposed to higher drug pressure. Thus, resistance should develop more readily in areas of low transmission. Studies are needed to develop and validate models, to generate more data to be used in these models, and to see how these models can be applied to drug policy.

## 5. Multidrug therapy

The theoretical basis for combining drugs with unrelated mechanisms of action to delay and contain resistance in bacterial and viral infections is well known (e.g. tuberculosis, HIV). It is also cost-effective. In tuberculosis, multidrug therapy has long been based on two principal concepts:
- Simultaneous resistance to a combination of unrelated drugs is the product of the probability of resistance to the individual drugs in the combination
- Concomitant use of drugs based primarily on the drug PK/PD characteristics and not merely on in vitro synergy

Multidrug chemotherapy of malaria is not an entirely new concept and was advocated more than 20 years ago by Peters (8). Drug associations are currently in use but most of these are based on drugs with similar mechanisms of action and should not be regarded as multidrug therapy. The antifolate drugs act on different enzymes in the folic acid metabolic pathway e.g. pyrimethamine acts on DHFR (dihydrofolate reductase), and sulphadoxine, sulfalene, dapsone act on DHPS (dihydropteroate synthetase). Atovaquone (an inhibitor of mitochondrial electron transport and pyrimidine synthesis) is combined with proguanil (a DHFR inhibitor) but the nature of such synergy is unclear.

A fixed combination of pyrimethamine, sulphadoxine and mefloquine was introduced in Thailand in the 1980s in the belief that mefloquine would be protected. However, resistance to antifolates was already well established and as a result this combination failed because the net effect was to introduce mefloquine monotherapy. This offers an important insight into the timing of the mass deployment of multidrug therapy. Which drug combinations and when they should be used must be carefully determined.

Since the early 1990s, a combination of artesunate and mefloquine has been used on the Thai-Burmese border, an area of multidrug resistant falciparum malaria. The rationale for including an artemisinin derivative is based on the two PD properties of reducing the parasite biomass and gametocyte carriage.[9] The artemisinin derivatives reduce *P. falciparum* biomass by 100 000-fold per asexual life cycle, compared to 100–1000 fold for other antimalarial drugs. This parasiticidal effect results is a small residuum of parasites (maximum of 105 parasites) for the companion drug to kill, hence reducing selective pressure for the emergence of resistant parasites. The artemisinins also reduce gametocyte carriage which lessens the probability of transmission of resistant parasites.

Despite the very short residence time of the artemisinin compounds, they can be administered once daily. However, they should be given for a minimum of 7 days if used as monotherapy – a major disadvantage for patient compliance. By contrast, 3-day regimens of artesunate are highly efficacious when combined with other drugs such as mefloquine.[10] The introduction of artesunate (4mg/kg/day for three days) plus mefloquine (25mg/kg in two divided doses on day one and two) on the Thai-Burmese border, where mefloquine resistance averaged 30%, has resulted in a treatment efficacy of 98%, thus restoring the usefulness of mefloquine, and reducing the transmission of *P. falciparum*.[11]

More recently, a fixed combination of artemether and lumefantrine (Coartem) has received regulatory approval. It remains to be seen if this product will be affordable by malaria endemic countries.

The basic principles underlying the use of combinations are two fold: (i) the use of a rapidly acting drug e.g. an artemisinin compound to reduce parasite biomass, (ii) the availabilty of a suitable companion, schizonticidal drug. Currently, TDR is sponsoring a series of large, randomised, double-blinded, placebo-controlled studies to assess the safety and efficacy of combinations of artesunate with one of the currently used antimalarial drugs in various epidemiological settings in Africa and other endemic areas. Future longitu-

dinal population-based studies will be conducted to assess whether the use of these combinations will delay antimalarial drug resistance, whilst translating the results of this research into policies.
Artesunate is being used as a 'proof-of-principle'. It is the most researched of all the oral artemisinin derivatives and is rapidly converted to dihydroartemisinin. The results are expected to apply to dihydroartemisinin itself and other artemisinins such as artelinic acid, and artemether. The choice of the companion drug (chloroquine, S/P, amodiaquine, or mefloquine) depends on local resistance patterns, cost and availability. Other options for future combinations include chlorproguanil/dapsone and pyronaridine.

## 6. The outlook

Several aspects need to be considered for the successful implementation of measures to optimise drug treatment and prolong the lifespan of existing and future drugs.
Formulation: initial studies are being conducted with combinations of individual drugs. Subsequently non-fixed dose packaging (e.g. blister packs) could be used when the drugs are deployed. Ideally, fixed-dose combinations should be used, though these will need additional pharmaceutical work. Additional issues need to be addressed:
- PK interactions between either fixed or non-fixed combinations may occur e.g. increased toxicity, reduced absorption. The simultaneous availability of drugs manufactured under GMP.
- If a fixed-dose combination is used, its development requires longer R&D time lines, thus increasing R&D costs and ultimate cost of treatment
Regulatory status : a fixed combination is considered as a new chemical entity and requires submission for regulatory approval. For non-fixed combinations, there are no universally accepted rules for registration; they are usually dealt with on a case-by-case basis.
Costs are probably the most important element in policy decision. Current first line drugs in Africa (chloroquine, S/P) cost approximately 15-20 cents per treatment.The next available option is mefloquine which usually costs US$ 2 or more, an unaffordable option for many malaria endemic countries. The addition of artesunate (or other artemisinin) to a first line drug will certainly increase the cost to approximately 55 cents. A 3-day course of Vietnamese-manufactured artesunate (4mg/kg/day) for a child weighing < 30kg costs ca 35 cents, thus setting the total cost of treatment at about 50-55 cents.
An evaluation of the cost effectiveness of introducing combinations should include other costs: (a) the direct costs to patient and health sector e.g. cost of re-treating failures, treatment of severe malaria and its complications, personnel costs, etc, (b) losses e.g. schooldays, income, working days, death, and (c) the long-term benefits rather than the perceived short-term savings of continuing with an inexpensive first line drug.

## 7. Conclusions

Several lines of research are being followed in an attempt to circumvent resistance. They range from basic science e.g. understand the mechanisms of drug resistance, looking for new drug targets unrelated to the existing ones, to the research and development of novel chemical entities with novel mechanisms of action. However, the occurrence of resistance is a matter of time and additional measures should be taken. Research to develop safe drug regimens to delay the emergence of resistance, and research into drug use in order to create an environment where the rational use of drugs will not favour drug pressure. Multidrug regimens using artesunate can provide mutual protection of the combination. The principle has finally been accepted that antimalarial drugs should be deployed in combinations.[12] Studies are underway to assess the effects of adding an artesunate to first line drugs in current use.

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# Screening methodologies for chemotherapeutic targets. High-throughput screening as an essential complement to the genomic approach. Advanced technology to assist high-throughput screening

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***Abstract :*** *Therapeutic options for treating life-threatening parasitic diseases are extremely limited. The pharmaceutical industry has been more profilic in the treatment of diseases of developed countries than in diseases that kill the majority of people in the developing world. Of the 1223 new chemical entities that were registered by Western health authorities between 1975-1996, only 11 were specifically indicated for tropical diseases. This gap in the commercialisation of new drugs for tropical diseases can be explained for various reasons: one important driver for investment decisions is return on investment and developing countries markets are not profitable in this sense; the research and development process is a costly and risky business; the current system of patents is a disincentive favouring counterfeiting; the level of regulatory requirements favours wealthy markets. Pharmaceutical firms also recognise a social welfare mission for them as depository of the know-how in drug discovery. It is the role of the whole of society to take appropriate steps to find new avenues when the rules of the free-market economy act adversely.*

## 1. Introduction

Statistics on drug discovery in the pharmaceutical industry reveal that less than 20% of research projects reach clinical trial, and only 10% of compounds in development achieve registration. Three reasons can be pointed out mostly contributing to this high attrition rate: poor selection of targets, poor selection of drug candidates and lack of strategic fit to establish the

medical value of the product. Genetics and technology are leading drug discovery to a new age in which one of the keys to succeed will be how to manage the flood of information generated. Thousands of potential therapeutic targets will be proposed as a result of human genetics, interrogation of genomics databases, cellular and molecular biology, pharmacology and biochemistry. However, some of them may not be really relevant to the disease selected, and others may be intractable for screening since either they are not amenable to be assayed or no compounds of acceptable properties are found that can interfere with their biological activity. Once a target has been properly selected, a long and hard process starts up. Multiple hurdles will have to be sequentially overcome until a drug candidate is identified (Figure 1). Screening methodologies are the tool for the discovery of chemical entities that encompass suitable features to be selected as candidates for clinical development. This lecture will review some of the technological aspects of screening in the pharmaceutical industry, and in particular within antimicrobial agents. But we will try to go beyond and give an insight on the information management of the process, or in other words, how to use the information that science and technology provide in order to make decisions about pushing a molecule through each milestone of the drug discovery process. Rather than solving a mathematical equation, screening provides information and alternatives around multiple dilemmas that imply, 'go/no go', decision points for compounds, and allocation of resources. Each individual and organisation will have to wisely use their own criteria and tools in order to increase the ratio of success in the selection of drug candidates.

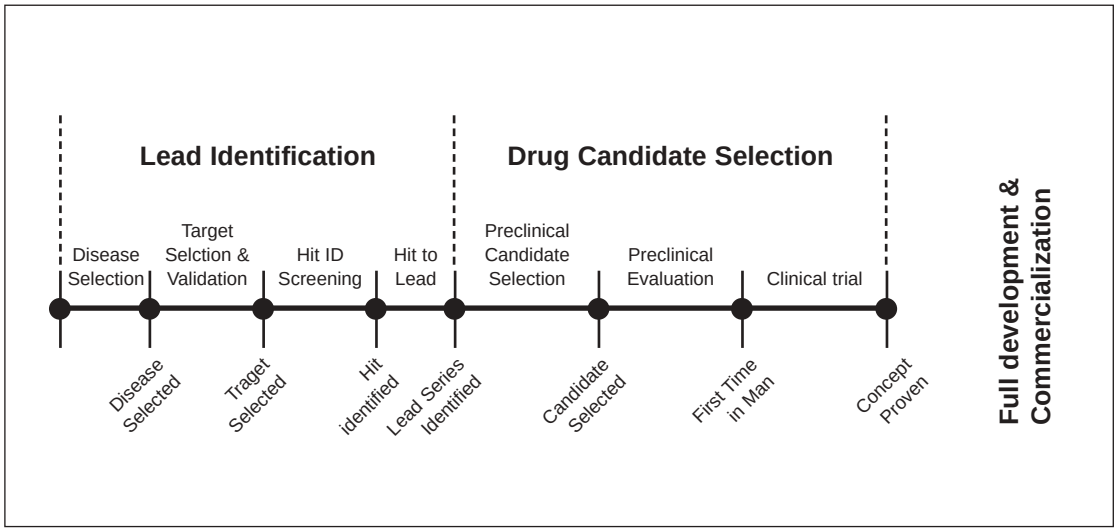


**Figure 1. Milestonesin in Drug Discovery Process**

## 2. How to select and validate antimicrobial targets for screening

The number of microbial genome sequences available is drastically increasing. Some of them have already been completed, released and analysed. Although some of the genome sequencing projects also includes the system-

atic functional analysis of genes, the vast number of potential targets available makes it almost impossible to achieve it in a comprehensive fashion. Thus it is advisable to use prior-isation criteria that sort the target candidates according to their value as a tool to be used for the identification of new chemotherapeutic agents. Once the number of targets has been reduced to a reasonable number, further experimental analysis may be individually conducted for each of them.

There are several criteria that can be considered when assessing the value of a gene as an antimicrobial target. Since the ultimate goal of the process is a safe drug capable of killing the microorganism, we should demand that the product of the gene is essential for the life-style of the bug in the infecting stage. Moreover, we could distinguish between just stopping the growth and cell killing. If so, genes that produce an irreversibly lethal phenotype when interrupted or turned off should be more valuable. Generation of gene knockouts is a very conclusive approach to assess target essentiality. However, there is also a room for other genetic tools (e.g. tuning of gene expression), biochemistry (e.g. comparative metabolic studies), and pharmacology (e.g. the existence of specific enzyme inhibitors with therapeutic activity). Specificity and selectivity refer to the possibility of finding effectors that exclusively hit the target. In particular, it is specially relevant that a chemical compound is able to discriminate the microbial target from the human counterpart. Hence, it should be positively scored those targets that either are absent in the human host or, albeit existing, they possess significant dissimilarities over the human homologues. As for drug discovery, a target will turn to be useless if it cannot be exploited in a bioassay that allows the identification of effecting molecules. If the product profile we want to achieve demands a broad spectrum of action, the occurrence of the target in the microorganisms of interest should also be assessed. Tractability reflects the practicability of developing an assay suitable for screening, and the likelihood of finding drug candidates acting through that particular target within the chemical diversity available that will be screened. The knowledge of the function and 3D-structure of the protein open a way to rationally designed compounds.

Bioinformatics tools can be developed that assist in performing this assessment automatically with a huge number of potential target genes.[1]

## 3. How to develop a high-throughput screening (HTS) assay

### *3.1. Concept and design*

As discussed above, to establish a clear link between the molecular target and the infectious disease is crucial. However, one can wonder whether there is a need for research into molecular targets when the real target of any chemotherapeutic agent must be the microorganism itself. Moreover, is it better to develop a target-based assay on live cells or on cell-free systems?[2,3] Figure 2 compares the most relevant characteristics of each type of assay. Targeted assays, either cellular or cell-free, have the advantages of allowing (i) a customisation of the screening programmes in order to favour the nov-

elty of positives, and (ii) a quicker approach to the molecular basis of the mode of action that eventually aids a rational design of new compounds. The accessibility of the target to the compound is less hampered in the cell-free assays. Thus they are, in principle, more sensitive to pick up traces of active compounds in complex mixtures (e.g. natural extracts or pools from combinatorial chemistry) or flickers of activity of novel chemotypes that can subsequently be optimised. Moreover, they allow the identification of specific inhibitors in complex mixtures where undesirable toxics are also present, and it is the only approach to HTS for non-culturable microorganisms. However, it is recommendable that primary hits are assayed in a secondary screen for their cellular antimicrobial activity in a whole-cell test. Very potent and selective compounds that are active in a cell-free assay but do not exhibit cellular activity might never end up as an acceptable antimicrobial agent, since lack of uptake and intracellular instability are issues tough to tackle. Cellular assays, either targeted or unspecific, may be in principle a more realistic scenario. They allow the identification of pro-agents and avoid the issue of supply of biological reagent, which may turn out to be one of the limitations of the cell-free assays. Whenever molecular biology tools are available to genetically manipulate the microorganism, targeted cellular assays are worth considering. For instance, strains in which the microbial gene has been replaced by the human one can be differentially tested for their susceptibility to compounds that distinguish both strains. Reporter systems may allow the detection of compounds interfering in a particular metabolic or signal transduction pathway, and synthetically lethal microorganisms can be engineered in order to identify compounds rescuing microbial growth.

**Figure 2. Comparison of screening assays for antimicrobials**

| | Targeted Cell-free | Assay cellular | Growth inhibition |
|---|---|---|---|
| **HURDLES** | | | |
| Target interactions | ✔ | ✔ | ✔ |
| Uptake | **No** | ✔ | ✔ |
| Microbial stability | **No** | ✔ | ✔ |
| **HITS** | | | |
| Novelty | ✔ | ✔ | **?** |
| Number | **Low** | **Very low** | **High** |
| Mode of action | ✔ | ✔ | **?** |
| Antimicrobial activity | **?** | **Likely** | ✔ |

*3.2. Requirements*

It is estimated that more than 400 000 diverse entities would have to be screened for finding out low-molecular weight (i.e. less than 600Da) hits of unknown target.[4] Thus, a HTS campaign based on diversity should be able to test hundreds of thousands of samples. This fact translates into important requirements that an assay has to fulfil in order to be accommodated to 'best

practice in HTS'. The overall cost of the campaign may become a serious limitation. As a rough indication, cost per assay point should not exceed 50 cents. Access and supply of reagents should be easy. The bioassay itself has to be robust (i.e. little dependence upon experimental conditions such as buffers, salts, solvents, temperature, shaking, etc.), reproducible (i.e. independent of operator and experiment) and use standard consumables and equipment (e.g. microtiter plates). The assay procedure should be amenable to automation, provide a measurable readout of unambiguous yes/no answer for the identification of positives, sensitivity to compounds of interest (sample concentration in the assay may not exceed micromolar units), and straightforward ('mix and measure' is a better option than separation steps).

*3.3. Bioassay technology : format and detection*

Although the most extended format for HTS involves assays in solution in microtiter well plates, other alternatives exist to achieve high density arrays. For instance, solid supports such as membranes, films and agar lawns have proved to be suitable to run cellular assays. This kind of format may avoid the technical issues associated to dispensation of nanoliter volumes, since the samples can be applied by replicating tools or blotting. Moreover, it allows screening of a range of concentrations around the application spot, which may be interesting for some cellular assays of differential output. However, the assay readout has to be done visually or by image analysis because most of standard microplate readers are not feasible for this format. Another limitation is the lack of physical walls separating each assay point that may complicate the readout when the final reporter molecule is able to diffuse very efficiently.

As indicated above, the assay procedure should try to avoid separation steps since they will probably become the bottleneck of the assay performance. In cases where extraction, filtration or in-plate binding cannot be abolished, it is advisable to develop the assay using devices amenable to automation, such as microplate washers, filter plates and plates for solid phase extraction. Nevertheless, the current trend is the development of 'mix and measure' assays, i. e. the reagents are added and mixed at one go, the reaction is allowed to develop and finally the signal is read. There is no need for extra addition of stopping or developing reagents, neither separation of reactants and products. It is worth mentioning two technologies available that enable this kind of assays, i.e. scintillation proximity[5] and homogenous time-resolved fluorescence (HTRF).[6] Both technologies are being profusely used and the list of applications is vast (e.g. enzyme assays for transferases and kinases, radioimmunoassays, receptor binding, cell viability, protein:protein interactions, DNA hybridisation, etc.)

Scintillation proximity is based on the principle that radioactivy from beta-emitters of low energy (e.g. tritium) is very efficiently quenched by water molecules in aqueous solutions. Thus, only those radiolabelled ligands in the proximity of a scintillant will be able to transfer their energy and convert it into light emission that is measured by scintillation counting. The scintillant can be embedded in beads (e.g. SPA by AmershamPharmacia) or in the bottom of microwell plates (e.g. Cytostar by AmershamPharmacia and

FlashPlates by New England Nuclear) coated with receptors. The assay has to be designed in a way that the product of the reaction can be specifically recognised by the receptor coating the scintillant, whereas the substrate or reactant will remain free in solution. Commonly exploited interactions are biotin-streptavidin and antigen-antibody. Since there are commercially available plates with a scintillating base compatible with growing monolayers of adherent cells, this technology can also be used for assays involving cell culture (e.g. cytotoxicity assays).

The principle exploited in HTRF is the time-resolved fluorescence of lanthanide chelates, such as europium cryptate. This kind of fluorescence emission is featured by the large wavelength shift of the excitation and emission spectra, the sharp emission peak and the long decay times. By flashing a sample with short light pulses and carefully timing excitation and data acquisition it is possible to achieve outstandingly high sensitivity and low background measurements. Moreover, the fluorescence from different lanthanides can be easily discriminated, so the technique allows resolution of multiple labelling. HTRF is a proximity assay based on the fluorescence energy transfer from a lantanide chelate to an allophycocianin protein. In contrast to scintillation proximity, HTRF is a non-radioactive technique, albeit this also implies that the labelling is less versatile and may be detrimental to the functionality of the molecule in the bioassay.

In addition to radioactivity and time-resolved fluorescence, other alternative detection technologies suitable for 'mix and measure' assays are prompt fluorescence intensity, fluorescence energy transfer (FRET), fluorescence polarisation and surface plasmon resonance (SPR) on biosensors. Assay miniaturisation is demanding very sensitive detection methodologies and instrumentation that are able to measure multiple high-density arrays. Current trends in HTS are towards the development of detection systems of fluorescence and luminescence by imaging analysis (e.g. FLIPR from Molecular Devices, ViewLux from Perkin Elmer, LEADseeker from Amersham Pharmacia, etc.).[7]

## 4. How to run a HTS campaign

### *4.1. Samples*

One of the key dilemmas in HTS is sample selection. As indicated above, a random approach for low-molecular weight hits against an unknown target should process hundreds of thousands of samples. This is a costly and resource-demanding strategy with a high attrition rate. However, it should be followed whenever access to novel chemotypes is being pursued. By using chemoinformatic algorithms, the universe of chemical diversity can be reduced to sets that theoretically will contain a representation of each chemotype. When some knowledge is available on the three-dimensional structure of the binding site or chemical entities have been previously reported that interact with the target, it is feasible to run a bespoke approach. Essentially, this consists of computer-aided selection of hit candidates. The latter approach will usually provide a higher success rate, as ratio of hits identified per total number of samples screened, but it will be unlikely to identify novel chemical classes.[8-11]

Natural products represent a rich source of biologically active compounds. On the other hand, combinatorial chemistry methodologies are a very efficient way to access to chemical diversity. It has been statistically shown that there are distinct differences in the structural properties of both compound sources,[12] so it would be worthwhile considering the inclusion of the two types of samples in each screening campaign. In spite of this, it is worth mentioning the important gap existing between these two methodologies in terms of infrastructure and skills required for sample supply and downstream process of hit identification. Although nature may be considered a source of unpredictable chemical novelty, natural products are in general more reluctant to optimisation by medicinal chemistry and require longer elapsed time for start and follow-up.

*4.2. Miniaturisation and automation*

Recently there is being a strong trend of miniaturisation and automation in HTS.[13,14] Although the increase of throughput is one of the aims behind this technological challenge, it is not the only reason. Miniaturisation also allows a reduction in the cost of labware, reagents and samples. The amount of compound from solid phase chemistry used in combinatorial synthesis may be limited by the loading capacity of the beads. By miniaturising the assays it is possible to increase the sample concentration tested off-bead and the number of screens run with each sample. Automation provides higher reproducibility, reliability and productivity. With the implementation of these new technologies, the bottleneck in HTS is being displaced from assay performance to sample handling and data management. It is important that, before adopting an automated solution, other aspects upstream and downstream of the assay are analysed in order to identify limiting steps and design a coherent work-flow through the system. For instance, sample tracking, weighting and dissolving of samples, cherry-picking of positives, integration and scheduling software packages, end-user customised workstations, data analysis and reduction, database posting and query, etc.

*4.3. Multiplexing*

The screening of multiple targets in parallel offers a series of advantages over the one-by-one approach. Firstly, it allows a best priorisation of hits, since these are ranked across targets and not only within an individual screen. Also, targets belonging to a particular class can be typed and classified according to their response to compounds. Pooling of targets should be considered if technically feasible, albeit pitfalls may exist relating to target competition for compounds and to the compatibility of multiple readouts. Pooling of samples is an alternative for increasing screening throughput, though possible compound interactions and solubility limiting effective sample concentration are issues to bear in mind.

## 5. How screening methodologies can aid drug candidate selection

Since HTS is intrinsically a reductionist approach, we should never forget that the ultimate goal is disease treatment in man, and that the new chemical entities identified must work in this context, not only against an in vitro target or isolated cells. HTS finds leads, not drugs. Once a lead is identified, the optimisation phase may deal with hundreds of closely related analogues possibly synthesised by combinatorial chemistry. The appropriate selection of drug candidates will result from the combined efforts of chemistry, metabolism, pharmacokinetics and preclinical studies that maximise the chance of successfully going to the clinic. Factors impacting drug candidate selection beyond potency and selectivity against a target are hydrophobicity, solubility, chemical stability, molecular size, charge, penetration through epithelial barriers, liver and kidney clearance, metabolism, protein binding, genotoxicity, cytotoxicity, enzyme induction, chemical scalability and tractability, cost of goods, chirality, pharmacy and formulation, etc. Traditionally, drug candidates have been selected following the model of a single compound being sequentially evaluated over several years. Decisions about whether or not a compound should be forwarded to next step were done without having gained an insight into most of these features. Currently, screening methodologies are being applied to develop surrogate markers that predict the toxicological and pharmacological profiles of the leads. Hundreds of compounds combinatorially synthesised may be evaluated in parallel using rapid, high-throughput predictive assays, over several months. At the end of the day, what is pursued is the improvement of the ratio of molecules that ultimately succeed as a medicine to bring value to healthcare.

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