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Review

Genomic-based discovery and validation of novel anti-filarial drugs

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Background: Lymphatic filariasis is a mosquito-transmitted disease, while onchocerciasis is transmitted by blackflies. There is no vaccine for these infections. New drugs are required for improvement of current therapies.
Objective: This article reviews recent advances in filarial parasite genomics and opportunities for new anti-filarial drug research.
Methods and results: Genomic approach to filarial parasites provides new prospects for target validation. Comparative genomics filters enable us to select filarial parasite-specific gene products of interest. Functional genomics filters allow the selection of gene products essential for pathogen survival. The validated targets could be prioritized and categorized by informatics methods and manual curation.
Conclusion: Lymphatic filariasis and onchocerciasis can be eliminated by either sterilizing or killing adult worms. It is most advantageous to target *Wolbachia* species for developing new drugs. Functional genomic approaches using microarrays, proteomics, and model organisms, have significantly expanded options for researchers. The genomic-based approach is promising for anti-filarial drug discovery in the future.

Keywords: Anti-filarial drugs, comparative genomics, functional genomics, lymphatic filariasis, onchocerciasis, *Onchocerca volvulus, Wolbachia*, *Wuchereria bancrofti*.

Lymphatic filariasis (elephantiasis) and onchocerciasis (river blindness) are important tropical diseases. Lymphatic filariasis is the second leading cause of long-term disability worldwide [1]. It is caused by mosquito-transmitted filarial parasites, including *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* that colonize the lymphatic system. *W. bancrofti* is present throughout tropical regions of Asia, Africa and Latin America. *B. malayi* is found in China, Indonesia, Malaysia, Korea, the Philippines, Vietnam and South-West India. *B. timori* is confined to a small area of Indonesia [2].

In Thailand, bancroftian filariasis is endemic at the Thai-Myanmar border, while brugian filariasis is endemic in Southern Thailand [3-7]. The prevalence of nocturnally subperiodic *W. bancrofti* (rural type, Thai strain) has been reduced to 0.57/100,000 population in Thailand [7], but the potential exists for re-emergence of bancroftian filariasis [3].

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Onchocerciasis is caused by the filarial nematode *Onchocerca volvulus*, which is transmitted by blackflies (genus *Simulium*). It is the second leading cause of infectious blindness worldwide.

It is very difficult to treat lymphatic filariasis and onchocerciasis. There is no vaccine for these infections. Current treatments such as diethylcarbamazine (DEC) and ivermectin, kill microfilariae, but not the adult worms. New drugs against adult worms are required for more effective treatment. Genomic approaches may provide new prospects for targeted therapy design.

We reviewed genomic information and functional genomic techniques to identify and validate novel targets for filarial parasites. In Part I, we describe basic information for anti-filarial drug candidates and control of filarial parasites as well as conventional approaches to potential anti-filarial drug discovery. In Part II, we discuss strategies for new anti-filarial drug discovery with reference to target product profile, criteria and integrated approach.

(Part I. Basic information for anti-filarial drug discovery)

Biology of filarial parasites

Lymphatic filariasis

Infection of lymphatic filarial parasites is initiated by infective third-stage larvae (L3) introduced by the bite of an infected mosquito. The L3 then migrate to the lymphatics and develop through two further molts to become adult worms. The adult worms are long-lived, and can survive for nearly a decade in the human host. They produce millions of microfilariae (**Fig. 1**) shedding them into the circulation. When ingested by a susceptible mosquito, the larvae develop for about 12 to 15 days in the mosquito to become L3. These are transmitted to a new host when an infected mosquito takes the next blood meal [8].

The majority of infections are asymptomatic microfilaraemics. All individuals with microfilaraemia have some degree of subclinical disease that includes microscopic haematuria and/or proteinuria, dilated and tortuous lymphatics. Males with *W. bancrofti* infection, often suffer from scrotal lymphangietasia (seen by ultrasound) [9]. Asymptomatic microfilaraemics have an inability to proliferate or produce interferon -gamma (IFN γ) in response to the antigen [10]. This lack of cellular responsiveness appears to be primarily directed at the parasite stage found in the blood circulation (microfilariae), a stage that represents (at the quantitative level) the major repository of parasite antigen. Whether this antigen-specific hypo-responsiveness is a cause or a result of the heavy intravascular parasite burden remains to be determined. What the immune system is doing during the development of this "non-inflammatory pathology" is to keep itself "down-regulated" through the production of contra-inflammatory immune molecules; specifically, the characteristic mediators of T helper 2 (Th2)-type responses (IL-4, IL-5, IL-10) and antibodies of the IgG4 (non-complement-fixing) subclass that serve as "blocking antibodies" [10].

There is another pathological process that is mediated by host inflammatory responses. The principal changes in chronic lymphatic filariasis result from lymphatic obstruction that is the result of immune responses to dead or dying worms in the lymphatics [11]. Patients with chronic pathology tend to show strong Th1-type responses (IFN-γ, IL-2). However, these same patients generally also had low levels of IL-10.

Onchocerciasis

Infection occurs when a blackfly introduces *O. volvulus* L3 into the host during a blood meal [12]. The female nematodes develop to adult worms and permanently incarcerate themselves in fibrous capsules, whereas male adults move freely throughout the skin and subcutaneous spaces. Each adult female worm sheds hundreds of thousands of microfilariae that migrate through the skin of the human host, with particular affinity for the eyes. When ingested by a blackfly, the microfilariae penetrate the blackfly gut and migrate to the thoracic flight muscles, where they develop to L3 and then find their way to the blackfly's feeding apparatus. They then enter another human host during the next blood meal to complete the life cycle. Microfilariae can persist in the human host for 3-5 years, in contrast to the adult female worm's life span, which is 2-15 years.

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Fig. 1 Microfilariae in peripheral blood (Giemsa stain). *Wuchereria bancrofti* (A) and *Brugia malayi* (B). (LFRU: Lymphatic Filariasis Research Unit).

Onchocerciasis most commonly presents itself as a diffuse papular dermatitis, often with intense pruritis [12]. The infected patients tend to demonstrate a strong Th1-type immune response. In patients with chronic disease, however, cutaneous manifestations may be differentiated from pruritic lichenification on one end to asymptomatic depigmentation on the other which they may resemble. Chronic papules and lichenification are associated with strong Th2 response, whereas depigmentation has been shown to correlate with a milder Th2 reactivity [13]. A subset of patients with chronic disease has papular disease that is similar in appearance to acute papular eruptions of other causes but it is Th2 predominant.

Retinol and retinoic acids accumulate in tissues after the death of microfilariae and may be partially responsible for skin and ocular symptoms [14]. Exposure to *Onchocerca* breakdown products induces a strong eosinophilic response as well. In contrast, the ocular pathology has been attributed to an immune reaction to *Wolbachia* antigens released as microfilariae undergoing natural attrition over time [15]. An angiogenic protein, produced by the adult female, is thought to contribute to the formation of the nodules.

Wolbachia

Filarial nematodes harbor intracellular bacteria of the genus *Wolbachia*. They are of the class *Alphaproteobacteria* belonging to the order *Rickettsiales* and family *Anaplasmataceae* [16].

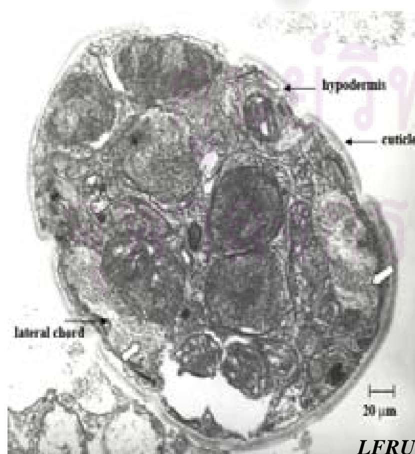

Fig. 2 Electron microscopy of a cross-section of a *Dirofilaria immitis* microfilaria show *Wolbachia* (⇨) in the lateral chords of the hypodermis. (X 500). (LFRU: Lymphatic Filariasis Research Unit).

These obligate intracellular gram-negative bacteria are widespread in arthropods and filarial nematodes, including *W. bancrofti*, *B. malayi,* and *O. volvulus*, the major human parasitic filarial nematodes, and *Dirofilaria immitis*, the pathogenic filarial nematode of dog heartworm disease [17]. *Wolbachia* are found in all developmental stages of filarial nematodes where they live restricted to the lateral hypodermal cords of filarial nematodes (**Fig. 2**) and in the reproductive organs of females. Evolutionary studies suggest long-term co-evolution and co-adaptation which is usually seen in mutualistic relationships.

Control of filarial parasites: chemotherapy

The programs to control human filarial infections have been based on chemotherapeutic interventions. The primary objectives were transmission control and, in onchocerciasis, also symptom alleviation [18]. Different strategies of chemotherapy have been applied.

Chemotherapeutic approaches to the control and treatment of filarial diseases have traditionally been difficult because radical curative agents are not available. Both lymphatic filariasis and onchocerciasis control strategies currently rely for operational purposes on drugs that mainly have microfilaricidal activity. Long-standing research to develop a macrofilaricide (killing adult worms) with qualities, compatible with public health use, has failed so far [18].

Drug treatment

Diethylcarbamazine (DEC) is an inhibitor of arachidonic acid metabolism and makes the microfilariae more susceptible to immune attack [19]. DEC has been used for treatment of lymphatic filariasis for more than 50 years. There is reasonable evidence from ultrasound and clinical observations that DEC kills some adult worms after a single dose [20]. Adverse effects of antifilarial drugs can be serious (though almost never fatal) and affect compliance. The most serious adverse reaction appears to be due to a host immunologic reaction to the dying worms [21]. These effects include fever, headache, malaise, muscle pain, and blood in urine. Local effects include localized pain, tender nodules, lymphadenitis (inflammation of the lymph nodes), and lymphangitis (inflammation of lymph vessels) [20].

Ivermectin has been used for the treatment and community control of onchocerciasis and, more recently, has been effective in community control

programs for lymphatic filariasis [22]. Ivermectin inactivates parasitic nematodes by binding to glutamate gated chloride channels (GABA mediated, present in nerve and muscle cells). This binding promotes increased membrane permeability to chloride ions, which causes hyperpolarization of the nerve or muscle cells, and results in neuro-muscular paralysis which may lead to death [23]. It can be used in many places, but is particularly important in areas where both onchocerciasis and lymphatic filariasis coexist. This is because DEC can cause eye damage if given to individuals with onchocerciasis. However, recent ultrasound studies suggest that adult worms are not killed by ivermectin, even at high doses over a period of six months [20].

Ivermectin and DEC both kill microfilariae, and DEC may have some temporary sterilizing effect or actually kill adult worms. One treatment with either drug can affect microfilarial levels for many months [24]. Reductions of 90 % from pre-treatment microfilarial levels have been observed after a single dose of DEC or ivermectin, even one year after treatment. The impact on transmission can be enhanced, if currently available antifilarial drugs demonstrate a killing or sterilizing effect on adult worms, in addition to their effect on microfilariae. There are concerns that over-reliance on a limited range of drugs may eventually cause resistance, although there is little direct evidence that this is currently a problem in filariasis [25].

Albendazole has been used widely to treat intestinal parasites since the late 1980s and may have a potential role in lymphatic filariasis control [24]. Albendazole has antiparasitic effects by binding tubulin and disrupting the assembly of tubulin dimers into microtubules [26]. Albendazole works by keeping the worm from absorbing sugar (glucose), so that the worm loses energy and dies. It is suggested that albendazole in repeated high doses has a killing or sterilizing effect on adults of *W. bancrofti* [27]. The drug does not have a role in morbidity management, which means it will not treat the symptoms in people already affected by filariasis [28]. However, at least one trial has considered the effectiveness of albendazole in reducing both disease progression and incidence of new symptoms (such as hydrocoele) [29].

To interrupt transmission of the lymphatic filarial infection, the entire “at risk” population must be treated for a period long enough to ensure that levels of microfilariae in the blood remain below those necessary to sustain transmission. For the yearly, single-dose, two-drug regimens being advocated (albendazole 400 mg plus DEC 6 mg/kg; or albendazole 400 mg plus ivermectin 200 μg/kg), this period has been estimated to be 4-6 years, corresponding to the reproductive life span of the parasite. For the treatment regimen based on the use of DEC-fortified salt, the period has been empirically determined to be 6-12 months of daily fortified salt intake [30]. However, none of these is effective in killing the long-lived adult worms.

The early chemotherapy of onchocerciasis was hampered by the fact that only two drugs were available and both had severe side-effects [31]. The first macrofilaricidal (adulticidal) drug, suramin, required weekly injections over 6-8 weeks to be fully effective. It frequently gave rise to severe adverse reactions, particularly dermal and renal complications. Given the need for weekly injections, cases had to be treated in a well equipped facility. Large-scale public-health use was not possible. Attempts to use low-dose regimes of suramin were fraught with problems, and use of this drug is now recommended only in special circumstances [32]. Although the other drug, DEC, is an effective microfilaricide, it provokes the immediate death of large numbers of microfilariae, resulting in frequent and sometimes severe allergic and immunological “Mazzotti” reactions. The potential adverse effects of DEC include the aggravation of existing eye lesions, and therefore the use of this drug is no longer recommended for onchocerciasis [33].

Currently, onchocerciasis is treated with single dose of 150 μg/kg of ivermectin, which can prevent further damage to the eyes [32]. Annual treatments reduce circulating microfilariae, thereby disrupting transmission of the infection, but ivermectin does not kill adult worms [23]. New drugs that affect new molecular targets are required to improve treatment and control by killing adult worms. Ideally, full target validation requires molecular genetic methods for assessing biological functions of target molecules.

Other approaches

Wolbachia as a chemotherapy target.

Filarial nematodes require *Wolbachia* for successful reproduction and development [34, 35]. Treatment of *Litomosoides sigmodontis*-infected rodents with tetracycline, an antibiotic with antirickettsial activity, results in inhibition of embryogenesis and infertility, inhibition of L3

development, and stunting of adult worm growth, coincident with the depletion of *Wolbachia* [34]. In addition to the rodent filariae, effects of tetracycline in blocking embryo development have been observed in two filarial nematodes, *B. pahangi* (a feline filaria) and *D. immitis* [35]. Notably, the study in cattle infected with the African bovine parasite *O. ochengi*, a nodule-dwelling filarial nematode closely related to *O. volvulus*, demonstrated the death of the adult filariae, following 6 months of oxytetracycline therapy [36]. The data suggest that tetracycline and their derivatives are likely to be a novel microfilaricidal drug target, as well as macrofilaricidal therapy against filarial infections.

So far, the results obtained from human trials have clearly revealed the superior efficacy of doxycycline to clear the adult worms and microfilaraemia [37, 38]. In the treatment of lymphatic filariasis, a 6-week regimen of doxycycline treatment was effective at reducing *Wolbachia* levels by >95 % [37], leading to sustained reductions in the microfilariae level. An 8-week regimen also showed substantial macrofilaricidal activity [38].

According to a recent study by Debrah and colleagues [39], a 6-week regimen of antifilarial treatment with doxycycline against *W. bancrofti*, in combination with standard antifilarial therapy (albendazole plus ivermectin), showed strong macrofilaricidal activity and reduction in plasma levels of lymphangiogenic factors (vascular endothelial growth factor (VEGF)-C and soluble VEGF receptor-3, and with an improvement of pathology in lymphatic filariasis patients. Another clinical study by Turner *et al* [40] demonstrated that a 3-week course of doxycycline plus albendazole and ivermectin is more effective in inducing a long-term amicrofilaraemia than is standard treatment alone. However, this regimen is insufficient to kill adult worms.

Current research is focused on the minimum length of time necessary to achieve macrofilaricidal efficacy comparable to that of 8- or 6-week courses of treatment with doxycycline and to attempt to reduce treatment length with alternative antibiotics targets to *Wolbachia* or with an antibiotic plus novel antifilarial drug combinations [40].

At present, we can obtain the full genome sequence of the *Wolbachia* from *B. malayi* (*w*Bm) which is included in bioinformatic searches for drug targets against filariae [41]. The *Wolbachia* metabolites which might be essential to the filarial nematode are assessed based on predictions from the *Wolbachia* genome sequences [42] (**Table 1**). It may open up the possibility to find novel antifilarial drugs for use in humans that might inhibit key biochemical pathways in the *Wolbachia*, leading to sterility or killing of the adult worms in shorter treatment time.

Vaccine candidates.

Individuals living in filariasis endemic areas require long-term chemotherapy in order to prevent re-infection and transmission. Such strategies are difficult to implement on a permanent basis at the population level. The development of an effective vaccine would benefit control of filariasis [43]. At the beginning**,** vaccine development in filariasis has focused on stage-specific microfilarial chitinase which has been

Table 1. Metabolic pathways *Wolbachia* from *Brugia malayi* that might supply essential products to the host *Brugia malayi* [42]**.**

***w*Bm metabolic pathway**	**Physiological importance**	**Possible tetracycline-induced phenotypes**
Riboflavin and flavin adenine dinucleotide biosynthesis	Essential coenzymes; biosynthesis genes are lacking from *B. malayi*	Failure to grow to maturity
Heme biosynthesis	Prosthetic group of cytochromes that catalyses the biosynthesis of steroid hormones	Delayed or abortive molting, delayed reproductive maturation
Nucleotide biosynthesis	Might supplement host nucleotide pool	Disruption of oogenesis and embryogenesis
Glutathione biosynthesis	An essential metabolite for protection against oxidative stress; might supplement host defenses	Failure to grow to maturity

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identified as a candidate antigen for a prototype transmission-blocking vaccine against lymphatic filariasis [44]. This enzyme is thought to be essential for the worm's development and involved in the exsheathment of microfilariae. The critical antigenic element at the carboxy terminus of chitinase has been identified. Vaccinating gerbils (*Meriones unguiculatus*) with a recombinant *Brugia* chitinase had little impact on microfilaraemia, whether administered before infection, or during pre-patent or patent infection [45]. This emphasizes the limitations of chitinase vaccine in an animal model.

Analysis of the *B. malayi* L3-expressed cDNA's in the EST database led to the identification of the highly expressed abundant larval transcripts, ALT-1 and ALT-2 [46]. Immunization with ALT-1 and ALT-2 conferred 64-76 % protection against a challenge infection in the gerbil model [46-48]. The level of protection indicates that ALT-1 and ALT-2 are promising candidates for further development. Further studies will determine whether a combination of all antigens could increase the rate of protection [48].

The prospect for the development of a vaccine against onchocerciasis is hindered by the fact that antigens of *O. volvulus* are complex and show extensive cross-reactivity with other filarial parasites of humans and animals. Challenges to development of the vaccine include research using immunology and molecular biology to develop a vaccine against *O. volvulus* [49]. Although immunisation with recombinant antigens such as chitinase, *O. volvulus* Abundant Larval Transcripts (*Ov*-ALT-1), *Av*-ABC (ATP-binding-cassette transporter), and Ubiquitin (*Av*-UBI) has been investigated [50], the most effective protection is still obtained with irradiated larvae [51]. More vaccine candidates for onchocerciasis are expected to evolve as the River Blindness Genome Project is nearing completion [52].

Emerging technologies in protein identification and DNA vaccination have accelerated the pace at which vaccine candidates are identified, characterized, and tested. Many promising strategies for vaccine delivery are emerging from research with other pathogens that await evaluation with filarial parasites [53].

Conventional approaches for anti-filarial drug discovery

Antinematode drug discovery has traditionally relied either on direct screening of compounds against whole target organisms, or on chemical modifications of existing anthelmintics [54]. These conventional approaches have resulted in relatively few classes of agents acting on a limited number of known biological targets. All drugs currently available for treatment of human filarial diseases were first developed for the animal health market.

In the modern animal health industry, antiparasitic drug discovery utilizes an integrated combination of direct *in vitro* compound screening on target parasites, screening of compounds on surrogate organisms predictive for efficacy against target parasites, and biochemical or cell-based screening against validated molecular targets important for parasite viability [54]. For filarial parasites of humans, these approaches are impractical owing to the limited availability of parasites, lack of long-term *in vitro* culture systems, and a paucity of validated molecular targets amenable to high-throughput assays.

Ideally, full target validation requires molecular genetic methods for assessing biological functions of target molecules. Until recently, such methods have not existed for filarial nematodes. Thus, despite the need for new anti-filarial drugs, they are unlikely to be developed without concerted effort. New approaches that help predict the most promising antifilarial drug targets are necessary to increase the likelihood that lead compounds identified in high throughput screens will proceed into clinical trials, thereby lowering the overall costs of new drug development.

(Part II. Strategies for new anti-filarial drug discovery)

Target product profiles

New drugs with strong macrofilaricidal activity are urgently needed to add to the current treatment regimen, and to ensure that the path towards reduction and elimination of filariasis continues. Desired characteristics for new anti-filarial drugs and a set of target selection and validation criteria have been documented by the WHO Scientific Working Group on RNA Interference (RNAi) as a means of identifying drug targets for filariasis and to provide general strategic guidance to filarial research [54]. A target product profile for a suitable new anti-filarial drug should possess the properties summarized in **Table 2**.

Table 2. Ideal product profile for a new anti-filarial drug [54].

1. Primary desired characteristics
Oral use
Microfilaricidal
Macrofilaricidal or cause permanent sterilization of adult female (and ideally male) worms, for *Onchocerca*, *Brugia* and *Wuchereria*
Lack of side effects directly following worm death (i.e. causes slow killing of worms)
Effective with one treatment per year for several years
Stable for two years under disease-endemic area conditions
Safe to administer without prescription or professional supervision
Safety profile comparable with albendazole and ivermectin
No evidence of cross-resistance to existing anthelmintics
2. Secondary desired characteristics
Oral use for all ages, including children as young as two years
Safe to use during pregnancy and lactation
Single dose
New mode of action
Molecularly defined mode of action (allows for improvements, resistance monitoring, *etc*.)
Efficacy and safety in combination with existing anthelminthics in use for filariasis
Reasonably affordable for large-scale distribution in endemic countries

Criteria for selection [54]

The candidate drug target genes can be examined for their suitability for further development. For example, what kind of proteins do they encode and are assays already available? Is the target likely to be amenable to inhibition by small molecules? Is the encoded protein a proven druggable target in nematodes or other organisms for which inhibitor classes are already known? What is the nature of the RNAi phenotype, given that a small number of non-wild type phenotypes are not detrimental to *Caenorhabditis elegans*? Is the protein present in other non-mammalian genomes, such as nematodes, insects, pathogenic fungi or bacteria? [55]. Criteria to the prioritization and decision path for validation of candidate targets are assessment of 'druggability' and 'assayability' of the candidate target molecules. The most important criteria for selecting appropriate drug targets for further investigation are summarized in **Table 3**.

New approach using genomics filtering

Assay development for molecular target-based compound discovery and subsequent high-throughput screening are major undertakings requiring considerable investments of time and money [54]. The identification of biologically validated targets amenable to miniaturized assays is thus of critical importance. Fortunately, recent scientific advances in genomics and molecular biology have opened up new opportunities for target validation for anti-filarial drug discovery.

Genomics filtering, composed of comparative and functional genomics filtering, is a rapid approach to identify and prioritize molecular targets for drug discovery [55]. For infectious disease applications, comparative genomics filters allow the selection of pathogen-specific gene products, whereas functional genomics filters, such as RNAi, allow the selection of gene products essential for pathogen survival.

Comparative genomic filtering

Typically, a major consideration in selecting candidate anti-filarial drug targets based on genomic data is how similar the parasite molecule in question is related to molecules (if any) in the mammalian host. By focusing the search on potential target genes that are not present as close homologues in humans and other mammals, it should be possible to identify therapeutic agents with reduced or no host cross-reactivity. Since complete mammalian and nematode, including *B. malayi*, genome sequences are available, comparative bioinformatics can quickly reveal how similar filarial parasite protein is related to those in the host organisms.

The Filarial Genome Project (FGP).

The Filarial Genome Project (FGP), initiated in 1994 with funds from the World Health Organization (WHO/TDR/UNDP/World Bank), was led by a

Table 3. Criteria for selecting suitable anti-filarial drug targets [54].

1. Selectivity and validation

(i) Either:

- Target is absent from mammals, or
- Target has molecular properties that distinguish it from related mammalian proteins, and/or
- Evidence exists that the target can be selectively chemically inhibited or agonized relative to other members of the same protein family

(ii) Evidence (RNAi, knockouts, inhibitors, *etc*.) that the target is essential for growth, survival or fertility

2. Druggability

- Priority is given to:
- Molecules with a small-molecule ligand-binding pocket (*e.g.* channels, receptors, transporters and enzymes)
- Molecules that have precedents (*i.e.* existing drugs or ligands)

3. Structure

- Amino acid sequence of the target is known
- Desirable, but not essential: crystal or NMR structure of related proteins is known or obtainable, preferably with bound cofactors, inhibitors or agonists/antagonists

4. Assayability

(i) Important features:

- Expression precedent available
- Existing biochemistry/enzymology
- Single subunit is desirable
- Specific readout that can be predicted, especially optical, that is compatible with high-throughput screening
- Active site chemistry available

(ii) Other desirable features include:

- Focused chemical library already available for the class of molecule
- Cell-based assays
- Assays with functional endpoints
- Assays with fewer steps (*e.g.* washes)

5. Potential for development of resistance

- Absence of isoforms of the target with varying susceptibility within a species
- Absence of biochemical 'bypass' reactions to circumvent function of the target

consortium of international laboratories to generate genomics resources and data for filariasis research [56]. *B. malayi* was initially chosen as a representative organism for analysis because of the availability of material from a variety of life cycle stages, its ability to be cultured *in vitro* and to be grown in a gerbil host, and its use for many immunological studies. The FGP is a multinational initiative to identify new drug targets in human parasitic filarial nematodes through expressed sequence tags (EST) sequencing, and identify new vaccine candidates in human parasitic filarial nematodes through EST sequencing.

The genome of the human filarial parasite, *B. malayi*, has been sequenced at nine fold redundancy using a whole-genome shotgun approach [56]. The *B. malayi* nuclear genome is estimated from the genomic data to be between 90-95 megabase (Mb) in size with approximately 11,515 genes [57]. The overall G + C content are 30.5 %. So far >26,000 ESTs, derived from >20 cDNA libraries representative of the significant *Brugia* life cycle stages, have been sequenced and submitted to dbEST (GenBank release 081503). In the course of sequencing the *Brugia* ESTs and the nuclear genome, data for two other genomes, mitochondria and *Wolbachia*, were also obtained [56].

Genomic filtering tools.

Comparative genomics involves the use of computer programs that can line up multiple genomes and look for regions of similarity among them [58]. Some of these sequence-similarity tools are accessible to the public over the Internet. One of the most widely used is Basic Local Alignment Search Tool (BLAST), which is available from the NCBI. The BLAST program compares nucleotide or protein sequences to sequence databases and calculates the statistical

significance of matches. For the parasites, there is a WU-Blast2, Washington University Basic Local Alignment Search Tool Version 2.0 which is specifically designed to blast sequences against parasite genomes.

Recent comparative analyses among nematodes show that >35 % of *B. malayi* genes have *C. elegans* matches [59]. The average pairwise identity of *B. malayi* proteins with orthologs from either *C. elegans* or *C. briggsae* is ~ 48 % [57]. Over 80 % of the predicted proteins in *C. elegans* have similarity to human sequences [60]. The functions of genes conserved among nematodes can then be tested using the experimentally tractable and well-characterized free-living nematode *C. elegans*, providing surrogate target validation with a stronger focus on filarial targets than has previously been possible.

Functional genomics filtering

The role of functional genomics in modern drug discovery is to prioritize drug targets [61]. The information generated from functional genomic tools will provide a crucial link between genomic analysis and drug discovery. In the field of functional genomics, the approaches that have been applied to drug discovery include RNA interference (RNAi), microarrays, proteomics, and model organisms.

RNA interference (RNAi).

To prioritize the identified nematode genes for their utility as targets, RNAi has been used to determine the phenotypic effects of transient transcript knock-down for each gene. RNAi is a technology to down-regulate the expression of a gene by the addition of gene-specific double-stranded RNA (dsRNA) [62]. In *C. elegans*, this RNAi mechanism can be triggered by injection of dsRNA into the adult worm body, by soaking any life cycle in dsRNA, or by feeding worms on bacteria expressing dsRNA.

While phenotypic consequences from RNAi might not precisely mimic drug effects on a target, as RNAi eliminates mRNA and drug blocks protein functions, the severity of defect by RNAi is a quickly obtainable indicator of target utility [55]. The development of RNAi provides a tool for probing biological functions of genes known only by DNA sequence [62]. Large-scale RNAi screens have greatly accelerated elucidation of genetic functions in *C. elegans* [63]. Phenotypes observed in *C. elegans* can generally be assumed to apply to other nematode species, including filarial parasites that have orthologs of the selected genes. The most attractive RNAi phenotype would be lethality, as a result of interference with a vital process. This would ideally apply to all developmental stages including long-lived adult worms that are generally less susceptible to currently available antifilarial drugs. Other attractive phenotypes would include sterility, or any block in worm development, that would also ultimately lead to death.

In the last few years, there have been several reports of successful RNAi in a number parasitic nematode species but this technique has not yet been adopted as a standard functional genomics approach as it has in *C. elegans*. Possible reasons for this are problems that have arisen with the efficiency, specificity and reproducibility of the current RNAi techniques in parasitic nematodes. There are several factors requiring further research to optimize RNAi in parasitic nematodes as follows [64]:

- The presence of fully functional RNAi pathways.
- Culture conditions to maintain the target parasite stage.
- Uptake of dsRNA by the parasite stage under investigation.
- Delivery of dsRNA to maximize uptake.
- Transmission of dsRNA to subsequent developmental stages in the life cycle.
- The site of expression of the target gene.
- The level of expression of the target gene.
- The capacity for spreading throughout the worm tissues.
- The capacity of RNAi-treated worms to retain infectivity to examine *in vivo* effects.

RNA interference in filarial parasites.

RNAi is already being used to investigate gene functions in *B. malayi* [65]. Three *B. malayi* genes, beta-tubulin, RNA polymerase II large subunit, and *B. malayi* microfilariae sheath protein could be targeted by soaking adult female *B. malayi* in dsRNA complementary to these transcripts in the optimized culture system. RT-PCRs indicated that all target transcript levels started to drop after between 14 and 17 h of soaking. After 24 h, worms soaked in dsRNA for beta-tubulin and the RNA polymerase II large subunit, were dead. Targeting the microfilarial sheath protein was not lethal to adult females in culture, but RNAi induced a marked reduction in microfilariae release and 50 % of the released microfilariae did not have fully elongated sheaths.

RNAi has also been used on the L3 of the human filarial nematode *O. volvulus* [66, 67]. Filarial serine protease inhibitors were targeted by soaking the L3 in dsRNA, resulting in the specific knock-down of the target transcript levels, and a loss of native proteins. The L3 showed a significant reduction in both molting and viability after 7 days of incubation. Similar phenotypic effects were obtained by targeting the cathepsin L- and cathepsin Z-like cysteine proteases. However, specific transcript levels for the latter were not examined.

The actin transcription was suppressed by soaking adult *L. sigmodontis*, a rodent filarial parasite, in different concentrations of actin dsRNA [68]. Soaking with actin dsRNA consistently reduced the actin transcript levels to <10 % of the controls. Treated worms showed paralysis between 48 and 72 hours after the start of the experiment and also released significantly less microfilariae.

The success of RNAi technique in *B. malayi*, *O. volvulus* and then later in *L. sigmodontis* were exciting to the field of filariasis as previously there was no genetic tools for filarial nematodes. However, the technique, as described for *B. malayi,* had a limitation. The dsRNA concentration used was extremely high, about 35 μM. The financial cost to perform experiments with this concentration of dsRNA would hinder effective RNAi studies in filariae [68]. Moreover, toxicity due to high dsRNA concentration resulted in off-target effects that have been shown in other systems [69]. The optimum concentration, as the concentration of specific dsRNA that knocks down specific mRNA transcription without reducing the transcription of non-specific genes, should be defined.

In addition, most studies have evaluated RNAi in parasitic nematodes using the soaking technique, with worms maintained in a medium containing the dsRNA [64]. However, filarial nematodes will not survive for more than a few days under these conditions, with the result that apparently lethal RNAi phenotypes can arise because the worm is already severely compromised. Improved culture methods are required to investigate the longer-term effects of RNAi.

Microarrays.

The accumulation of large amounts of sequence information has led to the development of high-throughput methodologies that can be applied at the genome scale to define patterns of gene expression [70]. The most widely applied method to date is the use of DNA microarrays. DNA microarray (also commonly known as gene or genome chip, DNA chip, or gene array) are a collection of nucleic acid targets attached to a solid surface, such as glass, plastic or a silicon chip forming an array for the purpose of expression profiling, monitoring expression levels for thousands of genes simultaneously, or for comparative genomic hybridization. Hybridization of fluorescently labeled probes made from nucleic acids in the test sample to these targets allows analysis of the relative concentrations of mRNA or DNA in the sample. Microarrays have opened the way for the parallel detection and analysis of the patterns of expression of thousands of genes (currently about 20,000-40,000) in a single experiment. In a microarray experiment, usually the genes of interest are either overexpressed (up-regulated) or under-expressed (transiently expressed) under the experimental conditions being studied [71].

The DNA microarray technique holds great promise as a means of studying stage-specific or tissue-specific gene expression in nematode parasites. Attractive target genes for development of antimicrobial drugs and vaccines could be potentially used in controlling vital functions for virulence, metabolism, cell-wall synthesis, and persistence [72]. An important use of microarrays is likely to be the search for cell-surface antigens that are expressed at a particular time in a pathogen's life cycle or infective process since these would be potential vaccine targets.

Recently, microarrays have been used to investigate changes in gene expression between *B. malayi* male and female worms, providing the first broad view of gender-regulated gene expression in *B. malayi* [73]. It is likely that many of these genes are involved in reproduction (gamete production, gamete function, and embryo development). However, much more work will be required to understand how the products of these genes function and interact, and to explore their potential as new drug targets. The microarrays for other filarial parasites have never been reported. The gene sequence data available for *W. bancrofti* and *O. volvulus* is necessary in order to include these genes in subsequent versions of the filarial microarrays [74].

Proteomics.

Proteomics is the collective term for all of the proteins produced from the instructions encoded by the genetic material in cells. Proteins determine the

biological phenotype of an organism and proteins are the primary targets for most therapeutic agents. Proteomics is used to determine differential protein expression, posttranslational modifications and alternative splicing and processed products [75].

Two key technologies are at the core of proteomics [75]. Firstly, two-dimensional gel electrophoresis (2-D electrophoresis or 2-DE) with powerful image analysis software and, secondly, mass spectrometry with database searching. Protein separation can be performed using 2-DE, which usually separates proteins first by isoelectric point and then by molecular weight. Once proteins are separated and quantified, they are identified. Individual spots are cut out of the gel and cleaved into peptides with proteolytic enzymes. These peptides can then be identified by mass spectrometry, specifically matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry.

It is possible to produce proteome maps from different points in the parasite life cycle to identify and quantify specific protein spots. Comparisons of protein patterns are aided by a variety of labeling techniques including the use of Cy-dyes, lectins or spot identification after immunoblot probing [76]. Proteins can often be quantified by the intensity of their stain.

Proteomics can help to define the function and expression profiles of all proteins encoded within a given genome. Proteomics is likely to have a major impact on drug discovery and validation. This type of analysis can be used to confirm that a novel drug does affect the target protein *in vivo* [77]. Drug treatment will usually cause the up-or down-regulation of particular biochemical pathways and definition of these will help to define the mode of drug actions. The same approach can be used to define mechanisms of resistance to existing drugs, a vitally important research area given the extent of drug resistance in parasite populations [75].

Few proteomic data of filarial parasites and the endosymbiotic *Wolbachia,* have been published. Characterization of antigens derived from *Wolbachia* was successful using the optimized isolation protocol for *Wolbachia* using, high-resolution 2-DE, and immunoblot analysis with specific antibodies [78]. These *Wolbachia* antigens could be candidates for further investigation of the role of *Wolbachia* in the pathogenesis of lymphatic filariasis [79]. The defined major proteins could be potential targets to *Wolbachia*.

Use of model organisms.

C. elegans, a free-living soil nematode, has become one of the typical model organisms for studying gene functions by genetic analyses since1974 [80]. Although *C. elegans* is a simple organism, it shares many of the essential biological characteristics with humans. *C. elegans* has a conserved mode of development, neuronal functions, cell death, behavior, and aging. *C. elegans* is only 1 mm long and is usually grown on small plates seeded with bacteria. All 959 somatic cells of its transparent body are visible with a microscope, and its average life span is only 2-3 weeks. Adding to its simple anatomy and rapid development, a small number of chromosomes (only 5 autosomes and a sex chromosome) made this system an ideal platform for genetic studies.

Since the completion of the genome project of *C. elegans* in 1998, functional genomic approaches have been applied to elucidate the gene and protein networks in this model organism [58]. To date, *C. elegans* remains the most extensively studied nematode, with databases describing functional analyses by RNAi, microarray transcriptional analyses, protein expression pattern data, protein interactions, and detailed genetic analyses of certain genes and pathways [81]. RNAi screenings in *C. elegans* have been performed to screen the whole genome for the genes whose mutations give rise to specific phenotypes of interest. All the RNAi phenotypes can be searched on a public database (*www.wormbase.org*).

The availability of the complete genome sequence of *C. elegans*, combined with molecular protocols that allow rapid progression from gene sequence to potential function, have paved the way for investigations into cellular and developmental processes. This in turn has facilitated the study of the corresponding processes in parasitic nematodes [82, 83]. A strategy for identifying and validating antifilarial drug targets using RNAi in *C. elegans* and *B. malayi* was developed [54]. Information from *C. elegans* was utilized for use in the primary informatics filter, because current RNAi techniques for *B. malayi* are too labor-intensive and costly to provide a practical initial screen, and results of genome-wide RNAi screens in *C. elegans* are already available [63].

Curation of validated anti-filarial drug targets

Subsequent to comparative and functional genomics filters, the validated targets are prioritized and categorized by informatics methods and manual

curation. By considering possible next steps for nematode-control discovery, the targets were classified in four strategic categories as follows [55].

Category I includes enzymes where molecular level details are known about the substrate and product that can be used to predict potential inhibitory compounds for *de novo* synthesis or for purchase from existing commercial collections. These enzymes are an area of particular interest. A similar strategy has been used successfully to jump-start antimalaria and anticoccidia drug discovery by concentrating on biochemical pathways recognizable from prokaryotes, fungi or chloroplasts. Examples include the mevalonate-independent pathway of isoprenoid biosynthesis and the shikimate pathway.

Category II includes proteins predicted to be secreted or transmembrane and expressed on accessible surfaces of the parasite, such as the intestine. The nematode intestine is an interesting target organ as its large lumen surface area is exposed to the environment and (in many cases) the nematode is actively ingesting molecules. It is a 'validated' target organ as several nematicidal small molecules, antibodies and proteins act via the intestine including benzimidazoles, and H11-specific antibodies.

Category III is conventional 'druggable' molecular targets. There are small-molecule ligand-binding pockets (e.g. channels, receptors, transporters and enzymes) with known functions.

Category IV is largely nematode-specific proteins where little is known about their functions.

Besides category-specific information, numerous other features can be used in prioritizing targets, including knowledge of stage and tissue of target expression in parasites (to insure that drug availability and requirement for the target will coincide) and the availability of full-length cDNAs of unambiguous target orthologs in parasites for biochemical studies [55].

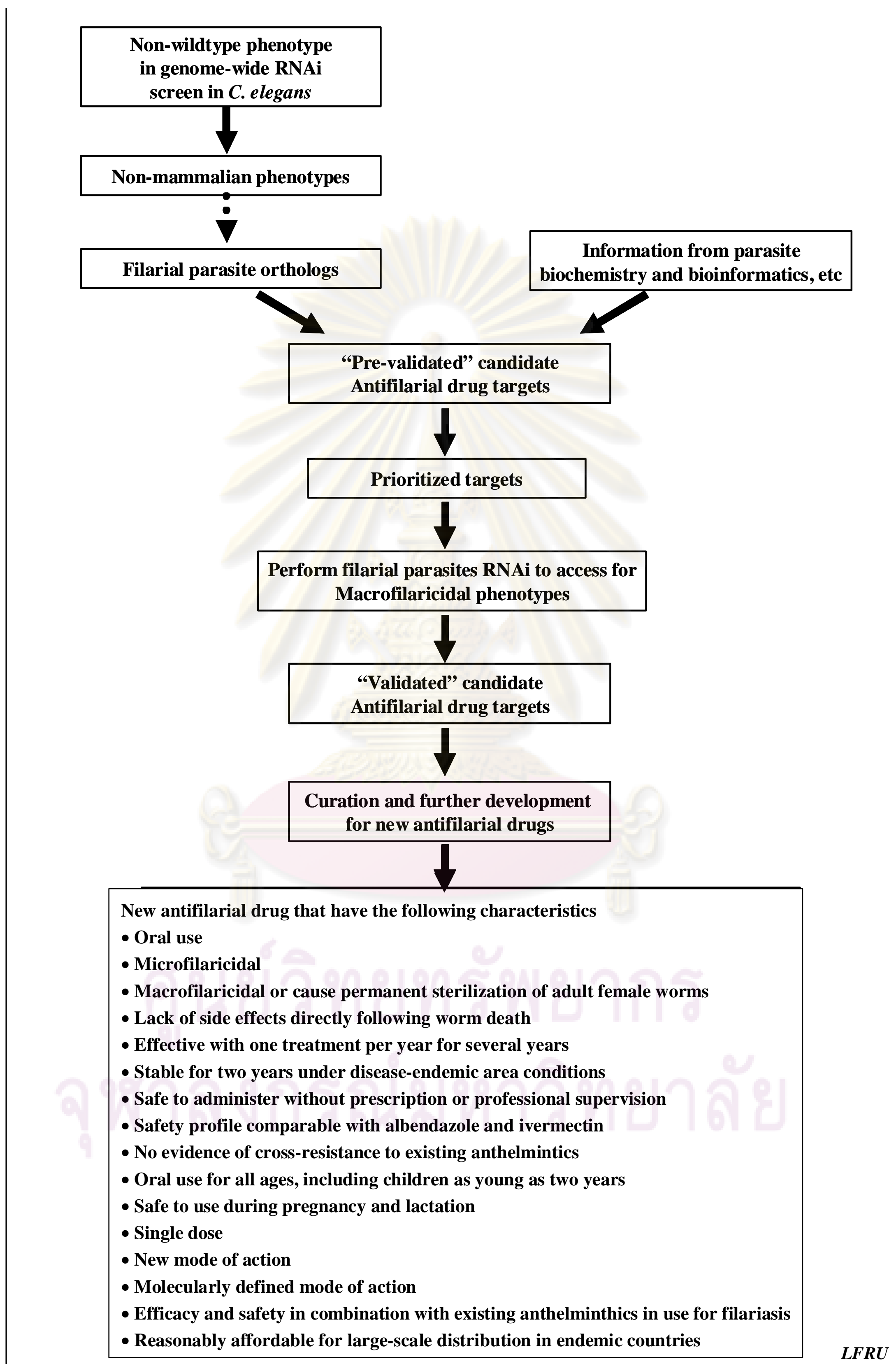
Integrated approach towards new anti-filarial drug targets

A coordinated RNAi- and genomics-based strategy can be envisaged to seek new candidate antifilarial drug targets [54]. This would take advantage of nematode and mammalian sequence information and incorporate RNAi gene function data from *C. elegans* to produce a set of non-mammalian worm genes, disruption of which affects filarial nematode biology (**Fig. 3**).

Briefly, all genes that give a non-wild type phenotype in genome-wide RNAi screens in *C. elegans* can be compared with the completed mammalian genomes at a pre-defined stringency to identify those without mammalian counterparts. This reduced set can then be compared with the filarial parasites, *B. malayi* and *O. volvulus*, sequence databases to identify subset orthologs in filarial parasites. The set includes examples of known classes of potential targets such as cofactor-independent phosphoglycerate mutase, chitin synthase, and fatty acid desaturase, as well as certain collagens and cuticulins [84], thereby demonstrating the utility of the approach.

Additional candidate targets would come from filarial biochemistry studies and bioinformatic studies of filarial genome sequences that identified genes not present in *C. elegans* but conserved in yeast, *Drosophila* or other systems where information on gene function was available. Such a set of genes can be considered to encode 'pre-validated' candidate drug targets. All candidate genes would be prioritized for functional assessment by RNAi in *B. malayi* by applying the criteria for selecting suitable antifilarial drug targets. Candidates that elicit suitable RNAi phenotypes in *B. malayi* would be considered 'validated' and would be further assessed and processed with the aim of proceeding to development of novel antifilarial drugs [54].

However, the bioinformatic pipeline depicted in **Fig. 3** could be modified by changing the order of the steps or by removing certain filters so as to yield different datasets [84]. For example, elimination of the 'non-mammalian' filter (dashed line in **Fig. 3**) would allow inclusion of potential drug targets that have orthologous proteins in mammals. It is established that chemistry can distinguish between homologs in some cases, divergent sequence space has the advantage of allowing greater freedom of exploration of chemistry without immediate cross-toxicity challenges and possibly of allowing the use of smaller molecules that are less expensive to synthesize. This approach is also warranted because many existing anthelmintics, such as ivermectin, exploit biochemical or physiological differences between parasite molecules and their corresponding host proteins [85].



(continued)

Fig. 3 A bioinformatic pipeline for identifying and validating antifilarial drug targets. RNA interference (RNAi) functional databases from *Caenorhabditis elegans* indicate which genes have important functions in this model nematode. The elimination of genes with mammalian counterparts leaves a set of non-mammalian genes of mostly essential function. Orthologs of *C. elegans* genes with suitable RNAi phenotypes would then be identified from the filarial parasite *Brugia malayi* genome and also, where sequence information is available, *Onchocerca volvulus*. Additional candidate targets would come from studies of filarial biochemistry and bioinformatic studies of filarial genome sequences that identified genes not present in *C. elegans* but conserved in yeast, *Drosophila* or other systems where information on gene function was available. Those genes can be considered as potential "pre-validated" drug targets worthy of further study. The target selection criteria outlined in **Table 3** would be applied to prioritize the most promising antifilarial drug targets. Functions of high-priority targets would be tested using RNAi in *B. malayi* and (where possible) *O. volvulus*. Candidate targets validated in the *B. malayi* RNAi screen would then proceed to intensive downstream investigation. Note: One possible alternative strategy is to remove the 'non-mammalian' filter (dashed line) to obtain a larger set of proteins that would include those with similarity to mammals. Molecular, biochemical or physiological differences between filarial parasites and mammalian proteins might provide opportunities for selective targeting of the nematode protein.

Conclusion

Either sterilization or killing of adult worms may accelerate success in eliminating lymphatic filariasis and onchocerciasis. It is most advantageous to target *Wolbachia* species for developing new drugs to kill adult worms. From the biological perspective, sterilization of adult worms is as useful as killing them.

Genomic filtering is a rapid approach for drug target prioritization, particularly relevant to filarial parasites. This approach to filarial parasites may lead to molecule identification strategies by generating a collection of ranked targets for a variety of downstream studies. However, screening of candidate parasite genes by RNAi will require considerable effort, because the RNAi methodology for filarial parasites is not currently high-throughput, and also each of candidate parasite genes must be cloned. Already-established functional genomic approaches (*e.g.* microarrays, proteomics and model organisms) have significantly expanded options that can be employed by researchers. The genomic-based approach is most promising for anti-filarial drug discovery in the future.

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