RNA interference: recent trends and its application in controlling Neglected Tropical Diseases (NTDs)

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ABSTRACT

Degradation of homologous mRNA in some cells or organisms is possible through the introduction of double-stranded RNA (dsRNA), via a process called RNA interference (RNAi). The dsRNAs are processed into short interfering RNAs (siRNAs) which consequently bind to the RNA-induced silencing complex (RISC), and causes degradation of target mRNAs. RNAi has been widely used to study gene modifications and functions hence has the possibility to control disease pathogens or vectors. This promising potential led researchers to discovery gene control mechanisms in tropical diseases, by manipulating genes of pathogens and vectors, protozoans, animal parasitic helminthes and disease-transmitting vectors, such as insects. Many pathogens and vectors cause severe parasitic diseases in tropical regions and it is challenging to control them once the host has been invaded intracellular. The aim of this work is to show how RNA interference can be used as treatment candidate for controlling some neglected tropical disease as it is highly effective in impeding parasitic development and their proliferation within their host.

INTRODUCTION

Hundreds of organisms have had their genomes sequenced and this holds the potential of revealing the gene functions in most of these organisms. The idea of genome sequencing has aided researchers to progress more comprehensive methods to understanding the biology of living organisms (Liolios et al., 2006). However, functions of over half of the genes in sequenced species remained unknown, needing annotation to fully appreciate the biological functions of those genes. RNA interference (RNAi) has been extensively used as a molecular tool by which target transcripts can be cut, resulting in the reduction of mRNAs for protein expression (Novina and Sharp, 2004). Even though other genetic modifications such as Germ line transformation modify the genotypes of target organisms, introduction of double-stranded RNA (dsRNA) changes only the phenotypes of organisms without modifying the genotypes (Kang and Hong, 2008). The dsRNA reduces the transcripts of specific mRNA, instead of deleting or inserting a gene like genetic modifications. The RNAi method has been
regularly used for analyzing gene function and
has the potential to be a tool for disease therapy
by interfering with vector competence or
pathogen development (Shuey et al., 2002).
This indication has been a focal point for
controlling tropical diseases in recent times.
The designation “Tropical Diseases” has been
part of the medical vocabulary since the 19th
century. On the “Tropical Diseases” Web page
of World Health Organization (WHO), a list
of eight diseases that occurred exclusively in the
tropics and for all practical purposes, the term
“Tropical Diseases” refers to infectious
diseases that proliferate in hot and humid
weather conditions. Some of these diseases are
caused by protozoa, which include; malaria,
leishmaniasis, Chagas’ disease and sleeping
sickness. Others are caused by worms,
including; Schistosomiasis, Onchocerciasis
and lymphatic Filariasis. One is viral,
identified as dengue fever. The eight WHO
Tropical Diseases are spread to humans by
various means, but always a vector is involved
and is generally a haematophagous insect.
Schistosomiasis has no vector, but rather
intermediary hosts – snails – that discharge in
water the infectious forms for humans
(Camargo, 2008). Most of these tropical
diseases are neglected, including six other
parasitic and bacterial diseases were
recognized as being neglected but are found to
be among some of the most common diseases
that cause infections in the projected that.
2.7 billion people who live on less than $2 per day.
These diseases occur principally in rural areas
and in some poor urban settings of low-income
countries in sub-Saharan Africa, Asia, and
Latin America (Camargo, 2008).

These thirteen (13) parasitic and
bacterial infections are documented as
“Neglected Tropical Diseases” and they
comprise three soil-transmitted helminthic
infections (ascariasis, hookworm infection, and
trichuriasis), lymphatic filariasis,
onchocerciasis, dracunculiasis,
schistosomiasis, Chagas’ disease, human
African trypanosomiasis, leishmaniasis, Buruli
ulcer, leprosy, and trachoma (Molyneux et al.,
2005; Hotz et al., 2006). An extended list
could include dengue fever, the
treponematoses,
leptospirosis,
strongyloidiasis, foodborne trematodiases,
neurocysticercosis, and scabies, (Hotz et al.,
2006) as well as other tropical infections. The
devastating effects of these tropical diseases
had called for much importance on their
control. Although monitoring measures
involving environmental sanitation,
observation, early diagnosis and treatment and
vector control (Fevre et al., 2006; Yamagata
and Nakagawa, 2006; Alvar et al., 2006). RNAi
seems encouraging in silencing gene
expression in some of these parasitic pathogens
such as protozoans and helminthes, as well as
disease vectors by exactly targeting mRNA
interference. Investigation of gene functions in
 pathogens of infectious diseases and their
vectors is significant for research in drug
development, and the silencing effects may be
directly employed to regulate parasite
transmission and development (Kang and Hong,
2008). Nevertheless, there are certain
practical obstacles when using RNAi in
tropical disease pathogens. RNAi technology
could have been eliminated in certain parasitic
protozoans, hence the existence of RNAi
pathway should be established before
utilization (Ullu et al., 2004). While using
RNAi, the necessities for an ideal delivery
method should be recognized to generate
optimal RNAi effects. The dsRNA-generating
vector systems can be employed to explain the
issue of transient RNAi effects. It is also used
in the treatment of several forms of diseases
such as cancer, heritable disease etc. Due to
difficulty to treat genetic diseases and also
control animal and plant pest in recent times,
the mechanism of RNA interference is
employed to target specific pathogen or
disease. The use of RNAi is an example of
several biotechnological tools which can be
used to manipulate the genes of organism in
order to solve problems faced by man (Kargbo
et al., 2020). The aim of this work is to show
how RNA interference can be used as treatment
candidate for controlling some neglected
tropical disease as it is highly effective in
impeding parasitic development and their
proliferation within their host.
RNAi IN THE CONTROL OF TROPICAL DISEASES BY PARASITIC HELMINTHS

The control of tropical diseases initiated by parasitic helminths has engaged the contributions of RNAi. Silencing characteristic of RNAi to helminths genes may kill the worms directly or interfere with important functions necessary for their development. It can also be used as a tool to study gene functions in helminths, just as it has been used in other species. Moreover, genes recognized by RNAi can be useful as target genes for drug development or vaccine candidates, allowing utilization of RNAi for therapeutic purposes (Kang and Hong, 2008). To date, only 10 species (8 in nematodes and 2 in trematodes) have been studied for RNAi effects in animal parasitic helminths (Geldhof et al., 2007). This is somewhat surprising considering the fact that the nematode, Caenorhabditis elegans was the first organism in which dsRNAs were proven to be an RNAi inducing factor and it has served as a model organism to describe the RNAi mechanism (Kang and Hong, 2008). Below are some of the species of parasitic helminths in which RNAi has been studied.

Nematodes

Nippostrongylus brasiliensis

Nippostrongylus brasiliensis, is a rat intestinal parasitic nematode, this parasite is an important animal model since it shares an analogous life cycle with the human hookworms, Necator americanus and Ancylostoma duodenale. When 1,799bp-long dsRNAs targeting full length of acetylcholinesterases (AchEs) cDNA at the concentration of 1 mg / ml dsRNA were employed, the target gene was blocked nearly by 80% on the first day, but then the transcripts returned to normal levels in 4 days later. However, by targeting AchEs with 240bp of dsRNAs, AchEs were blocked by more than 90% and the effects lasted for 6 days, signifying that the short dsRNA were effective in suppressing the target gene expression (Hussien et al., 2002). This experiment provided the first confirmation of a successful RNAi effect in parasitic helminthes. Therefore, control of helminths via RNAi appears to be feasible once a suitable target gene is recognized (Kang and Hong, 2008).

Brugia malayi

RNAi effects in Brugia malayi showed a more promising control method while targeting housekeeping genes (β-tubulin and RNA polymerase II large subunit) (Aboobaker and Blaxter, 2003). This study showed that 300bp long dsRNA was very effective enough to result in the death of the filarial worm. The authors also utilized RNAi to target another gene, microfilaria sheath protein 1/ mF22, but this was not lethal to the worms although microfilariae release was abridged and half of the released microfilariae did not have fully elongated sheathes. B. malayi is an important lymphatic filarial nematode and it is challenging to block their transmission by mosquitoes. Therefore, the lethal effect from RNAi was substantial as it proved to be a latent control system for B. malayi. Although earlier studies have reported a high concentration of dsRNA was necessary to knock down target genes in various helminths (Hussein et al., 2002; Aboobaker and Blaxter, 2003; Cheng et al., 2005). Pfarr et al., (2006) claimed that low concentration of dsRNA was enough to definitely knock down a target gene in Litomosoides sigmodontis, a rodent filaria. All concentrations ranging from 0.035 to 35 Mof dsRNA comparably reduced actin gene transcripts in adult worms by more than 90%. They also measured induction of hsp60 gene to find out any stressful reaction on dsRNA injection. These concentrations of Mof dsRNAs reduced the target with the least difference and no hsp60 induction was observed, but high concentrations (17.5 and 35M) resulted in a significant increase in hsp60 transcripts, indicating that a stress level in the filarial worm by high dsRNA dosage. Their study however, recommends that low concentrations are enough to reduce transcript levels steadily whereas high concentrations of dsRNA may be stressful to the filarial worms. Thus, titration of appropriate concentration of dsRNAs would be required prior to RNAi experiments (Kang and Hong, 2008).
**Onchocerca volvulus**

Functional RNAi-knockdown was described in L3 larvae of *Onchocerca volvulus*. Lustigman et al. (2004) targeted cathepsin L and cathepsin Z-like cysteine proteases that showed a vital role in L3 to L4 larvae for molting. Soaking the third-stage larvae (L3) in a dsRNA solution reduced the molting rate by 92% for cathepsin L and 86% for cathepsin Z-like cysteine proteases. Gene silencing of these cathepsin transcripts hindered the molting process by 1-3 days, this shows a significant reduction in the viability of the L3 larvae in *O. volvulus* (Ford et al., 2005). Gene silencing of inorganic pyro phosphatase of the parasitic round worm, *Ascaris suum*, prevented molting from L3 to L4 by 31% (Islam et al., 2005). Although the inhibition rate was inferior than that of *O. volvulus*, discovery of functional gene silencing by RNAi in *Ascaris* worms was significant and this can be further exploited as a model system to study RNAi in human ascariasis (Kang and Hong, 2008). Issa et al., (2005) recommended that siRNA and electroporation are more efficient molecules and a delivery method, respectively, to induce gene silencing by RNAi in the sheep gastrointestinal parasite, *Trichostrongylus colubriformis*. The authors tested three different RNAi delivery methods; feeding of *Escherichia coli* expressing dsRNA, soaking of siRNA or dsRNA, and electroporation of siRNA or dsRNA. Ubiquitin and tropomyosin were used as target genes since their DNA sequences are well conserved and readily available. Ubiquitin transcripts were not reduced by the *E. coli* feeding method, but tropomyosin was suppressed. siRNA in both electroporation and soaking resulted in a significant reduction for both target genes (Kang and Hong, 2008). Their study showed that it is likely to have electroporation which will result in more reliable gene silencing, and the 22bp siRNA will be more effective than long dsRNA in reducing expression of both target genes.

**Haemonchus contortus**

A diversity of RNAi conditions were tested to optimize RNAi effects in *Haemonchus contortus*, a barberpole worm. Various life stages (L1-L4 and adult), 11 genes (β-tubulin, sec-23, Ca²⁺ binding protein, HSP70, vacuolar ATPase, cathepsin L, paramyosin, Cu-Zn superoxide dismutase, intermediate filament, type IV collage and GATA transcription factor) and 3 different RNAi delivery methods (feeding, soaking and electroporation) were tested in this organism to investigate RNAi (Geldhof et al., 2006; Kotze and Bagnall, 2006). Two -tubulin genes that were targeted by RNAi affected 3 life stages (L3, L4 and adult) and reduced target gene transcripts by the soaking method, still reduced motility and viability were only shown in the L3 stage (Kotze and Bagnall, 2006). Geldhof et al., (2006) tested RNAi effects on 11 different genes of the L1-L3 stages by 3 different delivery methods in *H. contortus*. The feeding method was not effective in reducing target gene transcripts, confirming previous data in *Trichostrongylius* (Issa et al., 2005). Only two transcripts (β-tubulin and sec-23) out of 11 genes in the L3 stage were significantly reduced by soaking in dsRNA. Fascinatingly, no phenotypic change was observed in the L3 larvae soaked in siRNA, and some control siRNA was even toxic to the L1 / L2 larvae (Kang and Hong, 2008). Electroporation was effective in reducing target gene transcripts in L1 larvae, as transcript levels of β-tubulin and superoxide dismutase were significantly decreased. However, larval death was observed in the L1/L2 stage by electroporation even in the presence of control dsRNA, signifying either electroporation is not a stable delivery method to this stage or current electroporation protocols for this stage are not optimal for analyzing RNAi effects (Kang and Hong, 2008). Similarly, eight genes were tested in the L1 and L3 larval stages of *Ostertagia ostertagi*, a cattle parasitic nematode, by electroporation and soaking delivery methods. Substantial reduction of transcripts was detected for five target genes (tropomyosin, β-tubulin, ATPase, superoxide dismutase and a polyprotein allergen) in L3 larvae, but dsRNAs of a transthyretin-like protein, a 17 kDa ES protein and ubiquitin did not reduce the target gene transcript levels (Kang and Hong, 2008). Electroporation was less effective, as only two
genes (tropomyosin and β-tubulin) were effectively silenced, and these RNAi effects were not even reproducible (Visser et al., 2006). This non-reproducible result indicated the RNAi delivery method will require further optimization to realize consistent results (Kang and Hong, 2008).

**Trematodes**

Most RNAi studies in trematodes mainly focused on *Schistosoma spp*, blood flukes, which cause hematemesis or liver failure, and may result in mortality.

**Schistosoma spp.**

*Schistosoma* worms causes a disease known as Schistosomiasis. It is one of the disease transmitted by freshwater snails. It affects about 200 million people in the world and about 600 people are at risk of infection in Africa, Latin America and Asia (Ntonifor and Ajayi, 2007). The first report of RNAi in *Schistosoma* targeted the cathepsin B gene (Skelly et al., 2003), an enzyme previously proposed to be responsible for degradation of host hemoglobin to digestible peptides (Brindley et al., 1997). Schistosomes were soaked in dsRNA targeting cathepsin B and cultured for 6 days. The authors confirmed by RT-PCR that parasites soaked in the dsRNAs showed reduced target gene expression. Subsequent studies recommended electroporation as an alternative way to introduce dsRNA, and reduction of transcript levels of cathepsin B in schistosomula was established. Later RNAi research showed that cathepsin B is needed for parasite growth, and not crucial for hemoglobin digestion (Correnti, et al., 2005). A much recent research was also conducted to optimize the application of RNAi to Genomic drug target validation in Schistosomes (Guidi, et al., 2015). The authors reported that there is no vaccine against the parasitoid, and the only potential cure relies on the use of the one available drug, praziquantel. This drug has been confirmed to be safe and effective against adult worms in a single dose administration (Zongo et al., 2016). However, the growing threat of emerging resistance makes the search for novel drugs a compelling priority. They reported that *S. mansoni* possesses a quite huge and complex genome so new *in silico* strategies to select genes which might represent possible novel drug target candidates and could be an important approach to drug development.

In their study, an initial list of 24 target candidates was compiled based on the identification of putative essential genes in *Schistosoma orthologous of C. elegans* essential genes. Knockdown of Calmodulin (Smp_026560.2) (Sm-Calm), topped the list, which produced a phenotype characterized by waves of contraction in adult worms but no phenotype in schistosomula. Knockdown of a typical Protein Kinase C (Smp_096310) (Sm-aPKC) resulted in the loss of viability in both schistosomula and adults. This had shifted their attention to other kinase genes that were identified in the above list and through whole organism screening of known kinase inhibitor sets followed by chemogenicomic evaluation. RNAi knockdown of these kinase genes failed to affect adult worm viability but, like Sm-aPKC, knockdown of Polo-like kinase 1, Sm-PLK1 (Smp_009600) and p38-MAPK, Sm-MAPK p38 (Smp_133020) resulted in an increased mortality of schistosomula after 2-3 weeks, an effect additional marked in the presence of human red blood cells (hRBC). For Sm-PLK-1 the same effects were seen with the precise inhibitor, BI2536, which also affected viable egg production in adult worms. For Sm-PLK-1 and Sm-aPKC the *in vitro* effects were reflected in lower recoveries *in vivo*. They concluded that the use of RNAi combined with culture and hRBC is a reliable method for evaluating genes significance for larval development. However, in view of the slow manifestation of the effects of Sm-aPKC knockdown in adults and the lack of effects of Sm-PLK-1 and Sm-MAPK p38 on adult viability, they recommended that the kinases may not represent suitable drug targets. RNAi was used to test another gene function in the sporocyst developmental stage of schistosomes. Boyle et al., (2003) knocked down SGT1P1, a facilitated diffusion glucose transporter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by RNAi. The expression of both genes in the sporocyst stage
was reduced when miracidia, a free living-larval stage of schistosome were soaked in its homologous dsRNA and allowed to transform to the sporocyst stage. Glucose transport activity was reduced when SGTP1 was knocked down, representing the function of the gene in schistosome. Fascinatingly, however, when dsRNAs were introduced at the sporocyst stage, the reduction was not observed, suggesting RNAi is not effective when introduced into this developmental stage. The effect of RNAi lasted for 28 days, which is similar to C. elegans (Tabara et al., 1998). RNAi can, therefore, be introduced at the miracidia stage and the effects will last well into the sporocyst stage. RNAi was used to reduce transcripts of scavenger receptors that are known to be substantial for the binding of low-density lipoprotein to the surface of schistosomula and adult S. mansoni. Because the parasites cannot produce sterols and fatty acids, uptake of host-lipoprotein by scavenger receptors is perilous in the synthesis of biological membranes (Kang and Hong, 2008). Miracidia were soaked in dsRNA targeting the scavenger receptors for 6 days and this resulted in a 60-70% reduction of the target gene transcript level. Decreasing of the scavenger receptors inhibited regular parasite development, showing a more rounded morphology in sporocysts and a shorter length in larval size (Dinguirard and Yoshino 2006). RNAi was also used to target the gynecophoral canal protein SjGCP, in Schistosoma japonicum, a major human pathogen in the Far East (Cheng et al., 2005). Schistosomes cannot develop without precise signaling during sexual pairing (Gupta and Basch et al., 1987), so disturbance of pairing would be good target in order to control schistosomes. It is known that S. mansoni GCP, an ortholog of SjGCP, is related to pairing and produced only by males (Bostic and Strand, 1996; Hoffmann, 2004). Cheng et al. (2005) utilized RNAi to demonstrate that the SjGCP can be silenced in a dosage dependent style in S. japonicum. The target gene transcripts were reduced by 75% with 100nM of dsRNA, but not affected at 12.5nM. This study recommended a potential therapeutic application of RNAi in S. japonicum. With reduced SjGCP, the mating in S. japonicum may be disturbed, which will cause inhibition of parasite development in the host.

**Fasciola spp.**

Fasciola spp. is a liver fluke that causes pernicious disease in humans and animals. Their present control is unmanageable due to anthelmintic resistance. Gene silencing (RNA interference, RNAi) has been shown to have potential to contribute to functional validation of new therapeutic targets. McVeigh et al. (2014), described that liver flukes reveal the persistence of gene silencing in vitro assay showing the dynamics of RNAi in juvenile Fasciola spp. They described the development of standardized RNAi protocols for a commercially-available liver fluke strain (the US Pacific North West Wild Strain), validated via robust transcriptional silencing of seven virulence genes, with in-depth experimental optimization of three: cathepsin L (FheCatL) and B (FheCatB) cysteine proteases, and an s-class glutathione transferase (FhesGST). In their studies, the robust transcriptional silencing of targets in both F. hepatica and Fasciola gigantica juveniles is attainable following exposure to long (200–320nt) dsRNAs or 27nt short interfering siRNAs. They described a set of simple, soaking-based methods that permit rapid, robust and persistent knockdown of target transcripts, and address target-specific aspects of the time course of this RNAi response, which is mainly evident in the time lag between transcript and protein knockdown. The Knockdown was noticeable following as little as 4h exposure to trigger (target-dependent) and in all cases silencing continued for ≥ 25 days following long dsRNA exposure (Cheng et al., 2005). Combinatorial silencing of three targets by mixing multiple long dsRNAs was similarly efficient. Despite profound transcriptional suppression, they found a significant time-lag before the incidence of protein suppression; FhesGST and FheCatL protein suppression were only detectable after 9 and 21 days, respectively. They established that in spite of marked variations in knockdown dynamics, a transient exposure to long dsRNA or siRNA activates
robust RNAi penetration and persistence in liver fluke NEJs supporting the development of multiple-throughput phenotypic screens for control target authentication Cheng et al. (2005). According to McVeigh et al. (2014), RNAi persistence in fluke encourages in vivo studies on gene function using worms exposed to RNAi-triggers prior to infection.

CURRENT CHALLENGES AND PROSPECTS OF RNAi IN HELMINTHS

Though genes are successfully silenced by RNAi in C. elegans, gene knockdown by RNAi has been either impossible or inconsistent in other helminths (Geldhof et al., 2007). For instance, Geldhof et al. (2006) tested efficacy of RNAi silencing in the strong lid parasitic nematode, H. contortus, using three different dsRNA delivery methods; feeding, soaking, or electroporation. In this study, no RNAi was observed with dsRNA feeding among four genes tested and only two genes out of eleven, beta-tubulin and sec-23, showed specific gene silencing by the soaking method. Similarly, two genes out of four had knockdown of target transcripts by electroporation of respective dsRNAs. Moreover, extents of gene silencing by RNAi also varied in the cattle parasitic helminths, O. ostertagi and the results were often challenging to reproduce (Visser et al., 2006). Disagreement of results of RNAi in parasitic helminthes may be due to different delivery methods employed for introducing dsRNAs into helminthes (Geldhof et al., 2006). There seems to be no consensus as to which is the most efficient delivery method to induce RNAi gene silencing in parasitic helminths. Numerous reports have showed electroporation was an efficient alternative to the soaking method in delivering dsRNA in T. colubriformis and schistosomes (Issa et al., 2005; Correnti et al., 2005; Krautz et al., 2007). In contrast, others suggested that electroporation was not effective in O. ostertagi and in fact lethal to certain stages of H. contortus (Geldhof et al., 2006; Visser et al., 2006). Feeding dsRNA is largely inefficient in reducing transcript levels of target genes except for tropomyosin in T. colubriformis (Issa et al., 2005). Soaking is another popular method for gene silencing, but this method was less efficient for the L1 / L2 stage of H. contortus (Geldhof et al., 2006) and for S. mansoni (Krautz et al., 2007). Apparently, all major methods normally used for RNAi in C. elegans have their limitations in the delivery of dsRNA into parasitic helminths. The disagreement in RNAi efficiencies among parasitic helminths may result from the absence of sid (systemic RNA interference-deficient)-1, sid-2 or rsd (RNAi spreading defective)-4 in some of parasitic helminths. SID-1, SID-2, and RSD-4 proteins are involved in cellular uptake and spread of dsRNA in C. elegans (Viney and Thompson, 2008). SID-1 is a trans-membrane protein that is required for RNAi uptake into cells and spread between cells. Therefore, the lack of these proteins in certain helminths can be stopped externally, preventing dsRNA from entering cells, making worms refractory to RNAi. In sid-1 mutant C. elegans, systemic spread of siRNA molecules was defective with any dsRNA delivery methods (feeding, soaking and microinjection) (Winston et al., 2002). SID-2 is also a trans-membrane protein, of which expression is incomplete in the apical membrane of the intestinal lumen. Therefore, SID-2 is believed to be essential for dsRNA uptake from the lumen to cells but not for spreading siRNA between cells (Kang and Hong, 2008). In sid-2 deficient C. elegans resistant to RNAi by soaking and feeding methods, dsRNA delivered to the pseudo-coelom (body cavity) by microinjection could initiate RNAi and siRNA that could independently spread into cells (Winston et al., 2007). As a result, sid-2 deficient C. elegans became vulnerable to RNAi, showing reduction of target transcripts. Likewise, rsd-4 mutant C. elegans resistant to RNAi by feeding was reverted to be susceptible to RNAi by supplying external dsRNA into the pseudocoelom using microinjection (Tijsterman et al., 2004). Therefore, it seems there is functional overlapping between sid-2 and rsd-4 proteins for systemic spread of siRNA (Kang and Hong, 2008). Many parasitic animal nematodes are deficient of sid-1, sid-2, or rsd-4 orthologs, or their homologs do not
share the same functions with their counterparts in *C. elegans* (Viney and Thompson, 2008; Winston et al., 2002; Tijesterman et al., 2004). The lack of sequence or functional conservation of *sid-1*, *sid-2*, or *rsd-4* may account for the discrepant RNAi results between *C. elegans* and animal parasitic nematodes. To oppose this explanation, despite the fact that there is no identifiable *sid-1* ortholog in *B. malayi*, a functional RNAi mechanism was detected by a soaking method (Aboobaker and Blaxter, 2003). This suggested, that there may be another molecules or pathways substituting *sid-1* for systemic spread of siRNAs in *B. malayi*. Therefore, it remains to be seen how systemic RNAi was achieved without *sid-1* orthologs in *B. malayi* (Kang and Hong, 2008). In order to have a phenotypic knock-down of specific gene for functional studies and therapeutic applications, definite modifications or enhancements for the RNAi delivery methods may be obligatory in animal parasitic helminths. To this end, two approaches have been recently suggested (Viney and Thompson, 2008).

Firstly, one may be able to trigger RNAi by microinjection of dsRNA into the pseudocoelom of parasitic helminths if failure of RNAi by soaking or feeding is due to the absence of *sid-2* orthologs in the gut lumen. This modification is based on the observation in *Caenorhabditis briggsae*, in which unlike *C. elegans* there is no *sid-2* ortholog functionally conserved to allow entry of dsRNA from the gut lumen to the body cavity. Therefore, RNAi cannot be initiated in *C. briggsae* by dsRNA using soaking and feeding methods. However, RNAi was possible when dsRNA was supplied to the pseudocoelom via microinjection, suggesting that microinjection can bypass barriers of dsRNA uptake in the lumen, making RNAi possible in *C. briggsae* (Winston et al., 2002; Baird and Chamberlin, 2006).

Secondly, RNAi may be feasible in *sid-1* or 2-deficient parasitic helminths by heterologous expression of *sid-1* and/or *sid-2* of *C. elegans* as shown by Winston et al., 2002.

In *C. briggsae*, for example, there is no functionally conserved *sid-2* and RNAi by soaking or feeding is thus impossible. However, transformation of *C. briggsae* with *C. elegans* *sid-2* allowed the uptake of dsRNA from the lumen in soaking experiments, generating systemic RNAi in *C. briggsae* (Winston et al., 2002). As transgenesis of helminths becomes available, development of an RNAi system through heterologous expression of *C. elegans* genes for soaking or feeding may have promising applications for gene functional studies and target identification for drug discovery (Kang and Hong, 2008). RNAi screen is a less challenging method for drug target studies in helminths compared to forward genetic research (Shuey et al., 2002). If, however, RNAi machinery requires complicated dsRNA delivery process in animal parasitic nematodes, *C. elegans* can be served as a model system to study gene functions. Since RNAi machinery is well characterized in *C. elegans*, this experimental system can provide a potent tool for unveiling gene functions in other nematodes via comparative genomics (Jones et al., 2005). The feasibility of this approach has been lately demonstrated in *B. malayi* (Kumar et al., 2007). Using sequence analyses and comparisons including gene functional studies with RNAi knockdowns in *C. elegans*, 589 *B. malayi* genes were recognized as being critical to the survival of the filarial worm, representing potential targets for anti-filarial drug discovery. Interestingly, among those 589 genes, 10 out of top 40 candidates were previously known to be promising targets for the drug discovery because of their roles in molting, central metabolism, and structural components (Kang and Hong, 2008). This method may be realistic only for animal parasitic nematodes whose genomes are fully sequenced such as *B. malayi* (Ghedin et al., 2007). Thus, genome sequencing of significant helminths should be a prerequisite for this model animal based drug target studies.
RNAi AND THE CONTROL OF TROPICAL DISEASES BY PARASITIC PROTOZOA

Trypanosomatids

RNAi in *T. brucei* has been studied comprehensively since the machinery was discovered in the organism (Ngo et al., 1998). *T. brucei* has been used to elucidate the functions of proteins comprising the RNAi machinery such as Ago1 and dicer (Shi et al., 2004, 2006, 2007), as well as basic RNAi mechanisms. Researchers constructed a stem-loop that generated hairpin-loop dsRNAs targeting the -tubulin gene under the control of the tetracycline-inducible promoter. This system inhibited β-tubulin expression in *T. brucei* (Shi et al., 2000). (Wang et al., 2000) used a vector (pZJM) in which a PCR amplified gene fragment was ligated between opposing promoters to inhibit specific gene expression in *T. brucei*. Consequently, sense and antisense RNAs were concurrently synthesized and hybridized, producing dsRNAs molecules. The synthesis of dsRNA targeting β-tubulin transcripts was induced by tetracycline. It was confirmed that *T. congolense*, which is the causative agent of nagana disease in cattle, also has RNAi machinery by utilizing a tetracycline-regulated vector expression system (Inoue et al., 2002). The constructed vector was transfected into *T. congolense* cells, inducing the same RNAi mechanisms as in *T. brucei*. The -tubulin synthesis was reduced when the vector was transfected into a *T. congolense* cell line in the existence of tetracycline, demonstrating similar results to the earlier experiment targeting β-tubulin gene in *T. brucei*. Induction of the RNAi pathway changed the morphology of transfected *T. brucei* cells after beta-tubulin transcripts were silenced (Ngo et al., 1998). This recommended that RNAi machinery exists in *T. congolense*. Conversely, *T. cruzi*, *Leishmania donovani* and *L. major* lack RNAi machinery although they belong to the same family as *T. brucei* that contains the required mechanisms of the RNAi pathway (Zhang and Matlashewski, 2000; Robinson and Beverley, 2003; Durocha et al., 2004). Upon searching genome databases, it was found that these species are deficient in orthologs of the Ago1 protein. It is not surprising that RNAi has not been observed without Ago1, since this protein is required for suppression of foreign and endogenous transgenes. Another missing component is the PAZ domain, generally present in Ago. The presence of a Piwi domain is indicative of a functional RNAi mechanism because it is a part of the Ago protein that links with siRNA to cleave target mRNA (Saito et al., 2006). However, species that do not have an RNAi mechanism lack proteins with a PAZ domain (Ullu et al., 2004), which is a subdomain of Ago and the dicer protein which binds to siRNA, contributing to firm incorporation of siRNA and miRNA into the RISC complex (Song et al., 2003). The deficiency of these domains may explain why certain species do not have an RNAi pathway. A vector system has been studied in *T. brucei* and can be applied to different parasitic protozoan species to achieve temporal and spatial induction of RNAi. Some researchers described the role of antisense RNA in knocking down target mRNA in *Plasmodium*. Although this is not a conventional RNAi pathway, it should be further exploited to use the antisense-knockdown system to silence specific genes (Noonpakedee et al., 2003).

RNAi IN INSECT VECTORS

RNAi has been used to silence target genes or investigate gene functions in many insect species. RNAi induced by dsRNA was revealed in *Drosophila* both in vivo and in vitro (Kennerdell et al., 1998; Tuschl et al., 1999). By eliminating *engrailed* (en) transcripts with RNAi, Marie et al. (2000) studied additional functions of the *en* gene that was previously known to control topography of axonal projections in *D. melanogaster*. Using larvae inserted with *en* dsRNA, a change in axonal branching and synaptic outputs was found, and thus demonstrating *en* controls synaptic choice as well as axonal projections. After fruitful trials of RNAi in *Drosophila*, the RNAi technique was applied to other insect species (Kang and Hong, 2008).
**Tsetse fly**

Tsetse fly is the main vector for trypanosomes. The trypanosomes life cycle is an intermediate between the tsetse fly and its mammalian host (Ebene et al., 2016; Mamoudou et al., 2016) RNAi was used to examine the role of a specific gene in tsetse flies (Nayduch and Aksoy, 2007). Tsetse flies, Glossina spp., transmit African trypanosomes which includes Africa Animal Trypanosomiasis and Human African Trypanosomiasis but the prevalence is low because the flies are refractory to trypanosome infection (Oyibo et al., 2009). Scientists have compared the transcription levels of an antimicrobial peptide gene attacin, between refractory G. palpalis palpalis and susceptible G. morsitans morsitans. The levels of attacin expression were higher in the refractory species, signifying that attacin may be involved in the refractoriness of G. palpalis palpalis. Subsequently, microinjection of dsRNAs targeting attacin was carried out in order to explain the role of the gene. When the attacin was silenced by dsRNAs, the infection rate meaningfully increased from 20% to 40%. This study showed that attacin is a refractory gene against African trypanosomes and also demonstrated that RNAi can be used as a powerful tool to examine gene functions in tsetse flies (Kang and Hong, 2008).

**Mosquito Species**

Mosquito species are commonly known to be vectors for malaria. The occurrence of malaria in Africa, together with the lack of functioning vaccine, and the occurrence of parasite resistance to conventional drugs makes it necessary to hunt for antimalarial drug or vaccine candidate for the treatment of malaria (Ngbolua et al., 2011). RNAi can be employed to experiment a novel method for the control of this vector-borne diseases. Malaria control using germ-line transformation and RNAi has been suggested (Osta et al., 2004). Malaria is prevalent in Sub-Saharan Africa and Southeast Asia where resources are limited for disease treatments and vector control. RNAi exists in several mosquito species, many of which are important disease vectors (Kang and Hong, 2008). When a pre-membrane coding section of the dengue virus type 2 genome was expressed in C6 / 36 cells derived from Aedes albopictus in sense and antisense orientation, the titer of specific type 2 virus was declined, signifying that dsRNA can induce resistance to virus infection in mosquito cells (Gaines et al., 1996). The same resistance pathway against dengue virus type 2 was shown when the pre-membrane coding region of the virus was expressed in Aedes aegypti adult mosquitoes (Olson et al., 1996). These experiments clearly established the existence of RNAi in Aedes species and suggested the possibility of the use of RNAi to control vector-borne diseases. An. gambiae, a vibrant vector species for malaria, also has the RNAi pathway (Blandin et al., 2002). Injection of dsRNAs targeting the endogenous Defensin gene, an antimicrobial peptide gene, silenced the Defensin transcripts, which augmented the activity of gram-positive bacteria in An. gambiae. This showed the possibility of RNAi in elucidating gene functions in anopheline mosquitoes. Analysis of gene functions using RNAi was employed to identify antiplasmodial genes in An. gambiae. Microarray analysis was performed in mosquitoes infected by P. falciparum or P.berghei, and 11 candidate immune genes (Tep1, AgMDL1, FBN8, FBN9, FBN39, SPCLIP1, APOD, IRSP1, IRSP5, LRRD7, and gambicin) were identified, and the functions of these genes were assessed by RNAi gene silencing to show their antiplasmodium activities. The dsRNAs of the 11 target genes were injected into mosquitoes and gene silencing was established by real-time RT-PCR. Silencing of each immune gene increased Plasmodium levels. However, insects do not share the same immune pathway. Thus, typical mammalian off-target effects may not cause concerns in insect vectors. Other common examples of off-target effects of RNAi may be over-dose lethality and toxic effects (Geldhof et al., 2007). Soaking and electroporation are the main RNAi delivery systems in protozoans and helminths, which may cause overall toxic effects due to long exposure time to dsRNA or injury by electroshock (Kang and Hong, 2008). Unlike...
parasitic pathogens, insect RNAi delivery relies on mostly microinjection. Thus, off-target effects caused by injection of non-physiological amounts of dsRNAs may be preventable by careful titration of dsRNAs to be used for RNAi experiments in insects (Kang and Hong, 2008).

Conclusion
The technique of RNAi showed great potential in genetic manipulation and the development of therapeutic and control applications in many tropical disease pathogens and vectors. Its mechanism makes it efficient to analyze most gene functions in a way that reduces the gene expression without having to alter the genotypes. Hence, RNAi gives us a better insight in understanding gene functions in parasites, find better drug targets and vaccine candidates, or reduce the vector competence to transmit most tropical diseases. Optimization of methods for delivering a specific RNAi system into a particular organism gives the technique its success in application. Soaking and electroporation have been effectively used for the introduction of dsRNAs into parasites, and a microinjection protocol has been established to deliver dsRNAs into vector insects. These provide general guidelines for performing RNAi gene silencing. However, there is need for modification of conditions for different gene knockdowns, in order to suit each system or organism since varying degrees of gene silencing have been observed among different target genes and delivery methods. The conventional RNAi produces transient gene suppression effects; however, long-lasting effects may be necessary for permanent gene silencing. Germline transformation and a vector-based RNAi system would be an answer to this transient knockdown issue. Factors that cause off-target effects need to be identified and avoided so as to ensure effective gene knockdown.

COMPETING INTERESTS
The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS
All authors contributed in writing and editing the manuscript.

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