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Shilpy Shakya*

Department of Zoology, Govt. Post Graduate College, Fatehabad, Agra (U.P.)

** Corresponding Author Email: shilpy.shilpy@gmail.com*

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Shilpy Shakya*

Department of Zoology, Govt. Post Graduate College, Fatehabad, Agra (U.P.)

Email: shilpy.shilpy@gmail.com

ABSTRACT

Wuchereria bancrofti, a parasitic filarial nematode is a major cause of global morbidity. These parasites are responsible for lymphatic filariasis (elephantiasis) and are transmitted by mosquitoes. Intracellular alpha-proteobacteria, *Wolbachia*, that were first observed almost 40 years ago reside within these filarial parasites. These obligate endosymbionts has been recognized as a target for anti-filarial nematode chemotherapy as evidenced by the loss of worm fertility and viability upon antibiotic treatment in an extensive series of human trials. While current treatments with doxycycline and rifampicin are not practical for widespread use due to the length of required treatments and contraindications, anti-Wolbachia targeting nevertheless appears a promising alternative for filariasis control in situations where current programmatic strategies fail or are unable to be delivered and it provides a superior efficacy for individual therapy. The mechanisms that underlie the symbiotic relationship between *Wolbachia* and its nematode hosts remain elusive. Comparative genomics, bioinformatics and experimental analyses have identified a number of potential interactions, which may be drug targets. There is need to find additional candidate targets, as well as new approaches for understanding the nature of the host-symbiont relationship.

Keywords: *Symbiosis, Wolbachia, Filaria, Drug target, Liposomes.*

INTRODUCTION

Filariasis affects over 150 million people in more than 80 countries, with over 1 billion at risk of infection [1]. The causative agents of human lymphatic filariasis (LF) are parasitic filarial nematodes, *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. The life cycle of these parasites requires anopheline and culicine mosquitoes for transmission. Adult worms can live for over a decade and are ovoviviparous, releasing millions of fully formed microfilariae (1st stage larvae) into the blood. Microfilariae are acquired by the insect vector during a blood meal and migrate from the midgut to the thoracic musculature where they develop into third stage larvae. These larvae then migrate to the proboscis from where they can infect another human via the insect bite wound resulting from a subsequent blood feed. The larvae enter the lymphatics and molt twice more as they develop into adults. LF is a disease associated with swellings of the limbs (lymphodema that can lead to elephantiasis) and scrotal sac (hydrocoele) as a result of damage and dysfunction of the lymphatics. In general, filarial infections cause little direct mortality but are both disfiguring and debilitating and cause much morbidity and economic loss in endemic countries.

Public health programs have been initiated for the control of LF infections by WHO in 1997 and later on a global coalition was forged between many organizations, each with a different mandate but all having a common goal. Currently, these programs use yearly mass application of drugs that mainly kill the microfilariae like diethylcarbamazine (DEC) or ivermectin, with an aim to block transmission and reduce the infections. Clinical trial studies with a combination of DEC or ivermectin with albendazole are producing promising results [2]. Treatment with DEC or ivermectin, results into reappearance of microfilaraemia after a period of withdrawal of the drug [3]. While the programs have been very successful in general, there are drawbacks like too low coverage of the population, reappearance of infection by migration of infected people into controlled areas, microfilaricidal efficacy while adults are causative agents of pathology in LF, and the potential threat of development of resistance against albendazole and ivermectin [4]. There is therefore an unequivocal call for the development of higher efficient, complementary chemotherapeutic approaches that lead to a long lasting reduction of the pathology.

*Author for correspondence

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Therapeutic Approaches using Conventional Drugs

Since last 60 years the diethylcarbamizine (DEC) is the most widely used drug for the treatment of LF. It has been shown to over stimulate parasite neuromuscular systems and increase motility, inhibit vital parasite metabolic enzymes, activate complement on the parasite's surface membrane, activate eosinophils and stimulate them to produce eosinophil-derived cationic proteins, enhance eosinophil-dependant antibody-mediated destruction of parasites and increase the adhesion of parasites to phagocytic and antibody-producing cells. Apart from this, it has also been suggested that nitric oxide mediates the microfilarial clearance. DEC therapy leads to elimination of circulating microfilariae but antigenaemia persists for up to 12 months, suggesting that DEC is only partly effective against adult filarial parasites [5]. The reason for the absence of microfilariae in spite of presence of live adult females indicated that either they were prevented from producing microfilariae or that DEC in some way facilitates the rapid removal of microfilariae from the circulation. In any case, the lack of microfilariae means interruption of transmission cycle.

Common cooking salt medicated with DEC in concentrations ranging from 0.1% to 0.6% [6] has been effectively used in mass treatment programs in India for bancroftian filariasis [7] and has shown to be effective even in brugian filariasis [8]. There are advantages and disadvantages of using DEC-fortified salt compared with other interventions for LF elimination programmes, but rather than being considered as a 'competing' intervention, DEC salt should be seen as an additional option. Based on these results, salt co-fortified with DEC and iodine should be considered as a concurrent intervention for lymphatic filariasis and iodine deficiency elimination programs.

Apart from DEC, ivermectin has also microfilaricidal activity and used very successfully for the treatment of oncocerciasis since long and a single annual dose, either alone or in combination with DEC, has shown to be very effective producing long-term suppression of microfilariaemia in bancroftian lymphatic filariasis in a number of countries [9]. Apart from DEC and ivermectin, albendazole that has been used for the treatment of intestinal helminths, have recently been trialed as an antifilarial drug.

***Wolbachia*: An Endosymbiotic Bacteria of Filarial Parasites**

Electron microscopy studies showed the presence of intracellular bacteria, *Wolbachia* in the body of various species of filarial nematodes infecting both humans and animals. The bacteria are concentrated in intracytoplasmic vacuoles within the hypodermal lateral chords of both male and females, in the reproductive apparatus of females (i.e. in the oogonia, oocytes, embryos and microfilariae) and also in the larvae present in the mosquito vector [10]. The rapid multiplication continues throughout L4 development, so that the major period of bacterial population growth occurred within 4 weeks of infection of the definitive host. In adult male worms up to 15 months of age, the bacterial populations are maintained, whilst in females, bacterial numbers increases as the worms mature and as the ovary and embryonic larval stages became infected. *Wolbachia* are speculated to play an important role in nutrition and metabolism or in subverting the host immune responses as being present in high concentrations in the hypodermal syncitial cells. Alternatively, they may play an important role in protecting the nematodes against the host immune responses. The detrimental effects of anti-rickettsial antibiotics on filarial nematodes which harbours *Wolbachia* had no effects on filarial nematodes which do not possess *Wolbachia* e.g. *A. viteae* [11]. These observations support the hypothesis that filarial nematodes harbouring *Wolbachia* require the presence of this bacterium, at least during some stages of their life cycle. *Wolbachia* could thus represent a useful target for the control of filariasis. In addition, *Wolbachia* seems to play an important role in the immunology and pathogenesis of filarial diseases.

Therapeutic approaches against *Wolbachia*: A Novel Drug Target

The mutualistic association between the *Wolbachia* and filarial parasite was demonstrated in animal models by depleting the endosymbiont from filariae using antirickettsial antibiotics. Tetracycline inhibited development of third stage larvae to adult worms and the development of microfilariaemia [12]. Several studies in a variety of animal models of filariasis have shown that antibiotic targeting of *Wolbachia* can have profound effects on the development, viability and fertility of filarial parasites. Dose response curves showed that when tetracycline was used for short period or at a dose

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insufficient to deplete *Wolbachia*, worm development and fertility remained unaffected [13]. The effects of tetracycline are sublethal with inhibition of L3 development, interference with L4-L5 molting and with the sex ratio distortion killing female worms earlier than male worms, inhibition of embryogenesis with cytotoxicity against developing embryos, inhibition of bacterial transovarial transmission and stunting of adult worm growth. Therefore, it appears that *Wolbachia* might have contributed for the long term survival of the nematode. The antifilarial efficacy of antibiotic therapeutic approach is due to result of its activity against *Wolbachia* because tetracycline was found to be ineffective against the rodent filarial parasite *A. viteae*, naturally devoid of *Wolbachia*. In addition to tetracycline, *Wolbachia* is also sensitive to few other tetracycline class of antibiotics (doxycycline, oxytetracycline), but all antirickettsial drugs cannot be assumed to be efficacious. The effectiveness of doxycycline against human lymphatic filarial parasite *B. malayi* has also been examined in a clinical study where the individuals were treated with doxycycline (100mg/kg/day) for 6 weeks and followed by a single standard dose of DEC and albendazole at 4 months. At four months after the onset of treatment *Wolbachia* was reduced by 98%, while 77% reduction was observed in prevalence of microfilaraemia [14].

In contrast to ivermectin and DEC, which mainly affect mature mf, doxycycline blocks mf generation during embryogenesis, thus leading to a reduction of microfilariae [15]. Besides, doxycycline targets metabolic pathways unique to *Wolbachia* and thereby causing little harm to mammalian host. Therefore, doxycycline may be considered as a first step to safely reduce high microfilarial loads and avoid severe adverse effects of microfilaricidal drugs. Further studies are being carried out to prove the efficacy on other filariids and evolving measures for shortening the treatment schedule of doxycycline.

Delivery System/s in Improving the Effectiveness of Therapeutic Agents

The lymphatic system comprises a network of lymphatic vessels and lymph nodes throughout the body. The lining of lymphatic vessels consists of very thin endothelium and intercellular junctions which allow absorption of interstitial fluid containing macromolecules (proteins) and particulate cellular matter. Unfortunately, this route of entry to the lymphatics is also utilized by tumor cells and pathogens, such as viruses, bacteria and certain nematodes as well. For example, many human cancers spread via this route and form secondary tumors in the lymphatic system. For treatment of diseases with lymphatic involvement, it is desirable to develop approaches to deliver diagnostic, therapeutic and immunomodulatory agents to lymph nodes [16]. The distribution of an active molecule in the body is mainly a function of its physicochemical properties. Due to which, the lymphatic system has generated interest in the use of colloidal systems for the targeting of agents to regional lymph nodes after local parenteral administration such as subcutaneous (s.c.), intramuscular (i.m) and intraperitoneal (i.p.) injection. The need for intracellular chemotherapy has been recognized for many years. An alternative approach to the classical delivery of antibacterial therapy consists of associating the drug to a submicroscopic carrier thereby hiding and protecting the molecule from degradation and delivering it to inaccessible pathogens in a controlled manner [17]. Indeed, the challenge is to design the means for carrying an antibiotic in a form that is able to be endocytosed by phagocytic cells and then released into these cells. Liposomes and nanoparticles are the main carriers developed for these logistic targeting strategies and are colloidal in nature, biodegradable and possess behavior similar to intracellular pathogens. When administered by the intravenous route, phospholipidic and polymeric particles localize preferentially in organs with high phagocytic activity and in circulating monocytes, ensuring their clearance [18].

The ability of circulating carriers to target these cells is highly dependent on tissue characteristics and on the carrier's properties. The liver rather than the spleen or bone marrow captures the submicronic particles. Immediately after injection, the foreign particles are subjected to opsonization by plasma proteins. In this way, 'classical or conventional carriers' are recognized by the mononuclear phagocytic system (mononuclear phagocytic system cells), ('passive targeting'). In contrast, surface of 'stealthy carriers' is intentionally modified to avoid the opsonization process and increase their systemic circulation time.

Despite the discovery of new antibiotics, the treatment of intracellular infections often fails completely to eradicate the pathogens. By loading antibiotics into the colloidal carriers, liposomes and nanoparticles, one can expect improved delivery to

infected cells. Among the colloidal systems proposed for lymphatic targeting, emulsions, nanoparticles (nanospheres and nanocapsules) and liposomes are probably well-known particulate carriers with comparatively long histories of research. Most of these carriers accumulate to the target site during continuous systemic circulation to deliver the drug substance thereon.

Nanoparticles

Nanoparticles are biodegradable and polymeric/polyalkylcyanoacrylate in nature and have therapeutic applications [19]. Owing to their polymeric nature, nanoparticles may be more stable in biological fluids and during storage. These polymers which are bioresorbable have been in use for several years and are still being used as surgical glues. Polyalkylcyanoacrylate nanoparticles are degraded *in vivo* and avoid side effects due to intracellular polymeric overloading, therefore extensively studied because of ease of manufacture and their physicochemical properties. They may be freeze-dried and rehydrated without modifying the size and drug content. Their structure allows better retention of the drug inside the polymeric network and then can be slowly degraded by esterase action. Monomers with long side chains are preferred since the acute toxicity of these polymers is greatly reduced.

There are two types of nanoparticles: nanospheres (solid framework) and nanocapsules (liquid central cavity surrounded by a wall). Only nanospheres have been well studied for antibiotic delivery. Since the conventional polymers are hydrophobic, highly polar compounds that makes them less suitable for efficient loading. The classical nanoparticles are prepared by emulsion polymerization of alkylcyanoacrylic monomers in the presence of the drug. This reaction is induced by a nucleophilic agent. In some cases, the antimicrobial agent may itself induce the anionic polymerization of the monomer. The possible linkage of the drug to the polymer by a covalent bond could lead to its partial or complete non availability. Therefore, the antimicrobial activity of the nanoparticle drug formulation must always be compared *in vitro* with the free drug as control.

The release rate of antibiotics from nanoparticles was found to highly correlate with the degradation rate of the polymer by esterases. Thus, the release from nanoparticles is low in esterase-free medium but is greatly increased in the presence of carboxyesterase [20]. Endocytosed by phagocytic cells, the colloidal carriers can be degraded in endosomes by lysosomal esterases. Usually, the entrapment efficiency is increased by using monomers with long side chains.

Liposomes

Liposome is a wonderful artificial model for the biological membrane. These phospholipid dispersions in water solutions are able to trap and release solutes, to which they are selectively permeable. The property of sequestration of solutes by liposomes was used to formulate the concept of the liposome drug-carrier. Interest in liposomes is directed upon their vesicular structure limited by one or more outer protecting shell/s consisting of lipids arranged in a bilayer configuration and upon their ability to interact with living cells in one of four ways: adsorption, endocytosis, lipid exchange and fusion. Depending on the method of preparation, liposomes can vary widely in size (0.02–10μm) and in the number of lamellae [21]. Usually, liposomes are classified into three categories on the basis of their size and lamellarity (number of bilayers); small unilamellar vesicles (SUVs) or oligolamellar (OLVs), large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). Entrapment efficiency is limited by the volume of the liposomes and drug solubility. The active compound can be located either in the aqueous spaces, if it is water-soluble, or in the lipid membrane, if it is lipid-soluble. Liposomes have been administered in a variety of ways but intravenous injection is the most practical route. Organ distribution can be altered by reducing the mononuclear phagocytic system uptake thereby maintaining high concentrations in the circulation and allowing penetration into other organs. The half-lives of liposomes in the blood stream range from a few minutes to many hours depending on the size and lipid composition of the vesicles [22].

The natural opsonization of liposomes, especially by blood lipoproteins, and their osmotic fragility can destabilize them leading to leakage of the entrapped drug. However, satisfactory stability is obtained with MLVs containing phospholipids with a long saturated chain and with a negative surface charge. Numerous studies of long-term stability have been undertaken and liposomes in the dry state were shown to be preserved in presence of sugars.

Liposomes are extensively used as drug delivery vehicles with their potential application in cancer chemotherapy, enzyme therapy, immunomodulation,

antimicrobial therapy, metal detoxification, diagnostics, vaccination and topical therapy [23]. Liposomes as drug delivery systems offer several advantages over conventional dosage forms especially for parenteral (i.e. local or systemic injection and infusion), topical and pulmonary route of administration. The benefits of drug loaded liposomes are summarized into eight categories:

i. *Improved solubility of lipophilic and amphiphilic drugs*- Examples include Porphyrins, Amphotericin B, Minoxidil, some peptides, and anthracyclines, furthermore, in some cases hydrophilic drugs, such as anticancer agent Doxorubicin or Acyclovir can be encapsulated in the liposome interior at concentrations several fold above their aqueous solubility. This is possible due to precipitation of the drug or gel formation inside the liposome with appropriate substances encapsulated;

ii. *Passive targeting to the cells of the immune system*- especially cells of the mononuclear phagocytic system. Examples are antimonials, Amphotericin B, porphyrins and also vaccines, immuno-modulators;

iii. *Sustained release system of systemically or locally administered liposomes*- Examples are doxorubicin, cytosine arabinose, cortisones, biological proteins or peptides such as vasopressin;

iv. *Site-avoidance mechanism*- liposomes do not dispose in certain organs, such as heart, kidneys, brain, and nervous system and this reduces cardio-, nephro-, and neuro-toxicity. Typical examples are reduced nephrotoxicity of Amphotericin B and reduced cardiotoxicity of Doxorubicin liposomes;

v. *Site specific targeting*- in certain cases liposomes with surface attached ligands can bind to target cells (‘key and lock’ mechanism), or can be delivered into the target tissue by local anatomical conditions such as leaky and badly formed blood vessels, their basal lamina, and capillaries. Examples include anticancer like cytosine arabinose, antiinfection like praziquantel, benznidazole, albendazole etc. and anti-inflammatory drugs like corticosteroids;

vi. *Improved transfer of hydrophilic charged molecules*- such as chelators, antibiotics, plasmids and genes into cells;

vii. *Intracellular targeting with greater concentration of the drug*- Example includes N-(phosphonacetyl)-L-aspartate (PALA) and antibiotics

viii. *Improved penetration into tissues*- especially in the case of dermally applied liposomal dosage forms. Examples include anaesthetics, corticosteroids, and insulin.

DISCUSSION

Three drugs namely diethylcarbamazine, ivermectin and albendazole are being currently used to control and treat the lymphatic filariasis. Most of these drugs interrupt transmission by eliminating microfilariae; ironically, they do not reliably kill the adult worms. There is an urgent need to develop drugs acting on adult worms, and in this regard several compounds are under-going *in vitro* and *in vivo* testing [24]. After the discovery of endosymbiont *Wolbachia*, efforts were made to study the relationship between the bacteria and the worm and the disease progression. Several studies have been conducted to target *Wolbachia* which may reduce the worm burden and the intensity of the inflammation. Thus, *Wolbachia* and its proteins are good targets for filarial control and new delivery mode for antifilarial chemotherapy should be explored to achieve better results such as promising macrofilaricidal activity at low dose, short treatment regimen and least side effects.

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