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Therapeutic targets for the treatment of microsporidiosis in humans

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Abstract

Introduction: Microsporidia have been increasingly reported to infect humans. The most common presentation of microsporidia infection is chronic diarrhea which can be extremely debilitating and carries a significant mortality risk in immune compromised patients. Albendazole, which inhibits tubulin, and fumagillin, which inhibits methionine aminopeptidase type 2 (MetAP2), are currently the two main therapeutic agents used for microsporidiosis treatment. In addition, to their role as emerging pathogens in humans, the Microsporidia are important pathogens in commercially important insects, aquaculture, and veterinary medicine. New therapeutic targets and therapies have become a recent focus of attention for medicine, veterinary and agricultural use.

Areas covered: Herein, we discuss the detection and symptoms of microsporidiosis in humans and the therapeutic targets that have been utilized for the design of new drugs for the treatment of this infection, including Triosephosphate isomerase (TIM), Tubulin, Methionine aminopeptidase 2 (MetAP2), Topoisomerase IV, Chitin synthases, and Polyamines.

Expert opinion: *Enterocytozoon bieneusi* is the most common microsporidia in human infection. Fumagillin has a broader anti-microsporidian activity than albendazole and is active against both *Ent. bieneusi* and Encephalozoonidae. Microsporidia lack methionine aminopeptidase type 1 (MetAP1) and are, therefore, dependent on MetAP2, while mammalian cells have both enzymes. Thus, MetAP2 is an essential enzyme in the Microsporidia and new inhibitors of this pathway have significant promise as therapeutic agents.

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Declaration of interest

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1. Introduction

Microsporidia are unicellular, obligate intracellular parasites. They have a wide infection range from invertebrate to vertebrate hosts. Phylogenetic studies suggest that microsporidia are related to the Fungi^{1–3} probably as a sister group along with the Cryptomycota. Microsporidia were first identified in the 19th century when *Nosema bombycis* was identified as the cause of pebrine disease in *Bombyx mori* which almost destroyed the European silkworm industry⁴. Microsporidia are still responsible for economic losses due to their adverse effects on farming and other industries^{5, 6}. There are over 200 genera and 1400 species of microsporidia. While they are extremely diverse, they all contain a unique invasion apparatus the polar tube and are surrounded by a spore wall⁷. The spore wall contains two layers, the exospore layer, and a chitin-containing endospore layer^{8–10}. Chitin is probably essential in maintaining spore rigidity and function. The polar tube is a highly specialized invasion organelle, which is coiled around the sporoplasm inside of spore before germination and invasion. Upon appropriate environmental stimulation, the polar tube extrudes out of spore rapidly and polar tube proteins interact with host cell surface proteins creating an invasion synapse, the sporoplasm and nucleus travel down the hollow polar tube and enter the host cells in this invasion synapse^{11–14}. The sporoplasm then undergoes its life cycle which consists of a proliferative phase (merogony), spore production phase (sporogony), and formation of mature spores (infective phase), either in a parasitophorous vacuole or in the host cytoplasm depending on the species of microsporidia^{15, 16}.

Microsporidiosis has been recognized as a significant problem for immune deficient patients including those with AIDS, organ transplantation, bone marrow transplantation, those with neoplastic disease on chemotherapy, those receiving immunomodulatory therapy for collagen vascular diseases, and is being increasingly recognized in the elderly and pediatric population^{17, 18}. Most of microsporidia infections are thought to result from oral infection due to contaminated water and food containing microsporidian spores and the site of initial infection is the gastrointestinal tract¹⁹. However, other transmission routes including direct contact through broken skin, the eye, and genital mucosa have been reported¹⁹. Microsporidia infections in humans can cause gastrointestinal, brain, kidney, liver, eye, muscle, sinus, respiratory, or disseminated infections. Infection in immune competent mammals are often chronic and asymptomatic, while immune compromised hosts often develop lethal infections²⁰. Diarrhea and chronic wasting associated with microsporidia infection in AIDS patients has been widely reported, with microsporidia being detected in some studies in 70% AIDS patients with chronic diarrhea without other known causes^{21–23}.

Since the first description of human infection with microsporidia causing encephalitis in 1959, there have been an increasing number of species of microsporidia recognized in human infections which are summarized in Table 1. Microsporidia seen in human infection

include: *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Anncaliia algerae*, *Anncaliia connori*, *Anncaliia vesicularum*, *Microsporidium sp.*, *Nosema ocularum*, *Pleistophora ronniaeae*, *Trachipleistophora sp.*, *Tubulinosema acridophagus* and *Vittaforma corneae*^{2, 24–26}. *Enterocytozoon* and *Encephalitozoon* are the most common genera causing human infections. *Ent. bieneusi* was the first microsporidia reported causing diarrhea in patients with AIDS⁶. *Ent. bieneusi* has not been cultivated continuously *in vitro* and there are no reliable rodent models of this infection, limiting its use for studies of immune responses and for drug screening²⁷. *Enc. intestinalis* was first identified in AIDS patients with diarrhea and is considered the second-most prevalent species reported to infect humans^{28, 29}. *Enc. cuniculi* was the first mammalian microsporidian species that was isolated and successfully grown in long-term culture³⁰. *Enc. hellem* was first identified from conjunctival specimens of AIDS patients and can cause systemic infections in humans^{31–33}. All three *Encephalitozoon* species infecting humans have been successfully grown in tissue culture and there are rodent models of all three of these organisms.

Other species of microsporidia have been reported less frequently in human infections. *V. corneae* was first identified in a non-HIV-infected individual and has been reported to cause keratitis^{34, 35}. *A. algerae* was found in mosquitoes and other insects and has caused myositis in immune deficient humans^{27, 36, 37}. The natural hosts of *Trachipleistophora sp.* (*T. hominis* and *T. anthropophthera*) are insects, and these pathogens have caused brain infections, myositis and keratitis in immune deficient patients³⁸. All of these microsporidia have been successfully grown in tissue culture and there laboratory animal models of infection with these organisms.

2. Clinical presentations of microsporidiosis in humans

2.1 Gastrointestinal infection

Most microsporidian infections are transmitted by oral ingestion of spores in spore-contaminated water or food leading to initial infection of the gastrointestinal tract. Symptomatic infection of the epithelium of the gastrointestinal tract can cause chronic diarrhea and wasting in patients, which is the most common presentation of microsporidiosis in both immune competent and immune deficient patients. Most of these infections are caused by either *Ent. bieneusi* or one of the Encephalitozoonidae (e.g. *Enc. intestinalis*)^{15, 39–41}. *Ent. bieneusi* has been identified as causing diarrhea in travelers, children, the elderly, and patients with AIDS, organ transplantation or other immune deficiency^{42–44}. Other gastrointestinal manifestations of microsporidiosis include hepatitis, peritonitis, and biliary disease with sclerosing cholangitis^{33, 45, 46}.

2.2 Central Nervous System Infection

Enc. cuniculi in mammals is known to cause granulomatous encephalitis. Spores consistent with *Encephalitozoon spp.* were found in the cerebrospinal fluid from a child with serological evidence of *Enc. cuniculi* infection⁴⁷. In immune compromised patients, *Enc. cuniculi* has presented as a mass lesion with associated seizures and focal neurological deficits. *Trachipleistophora anthropophthers* has also been reported to cause cerebral disease

^{48, 49}. Two patients with AIDS who died from disseminated *T. anthropophthera* infections had central nervous system involvement with multiple ringlike lesions on computed tomography scanning ⁴⁹.

2.3 Ocular Infection

Microsporidian keratoconjunctivitis is often due to infection by the Encephalitozoonidae with the majority of infections being due to *Enc. hellem* ^{50–52}. Most of these infections occur in the patients of immune dysfunction like HIV patients, however, there are case reports of superficial epithelial keratitis caused by *Encephalitozoon spp.* in immune competent patients. Most of these infections of immune competent patients were in contact lens wearers ¹⁵. Seasonal keratoconjunctivitis due to *Vittaforma corenea* has been reported in South East Asia, and associated with water or soil exposure.

2.4 Musculoskeletal Infection

Myositis has been reported due to infection with *A. algerae*, *Anncaliia vesicularum*, *Tubulinosema spp.*, *Endoreticulatus spp.*, *Pleistophora sp.*, *Trachipleistophora hominis*, and *Pleistophora ronneafiei*. *Trachipleistophora hominis* and *Trachipleistophora anthropophthera* musculoskeletal infection has been reported in patients with AIDS ⁴⁸. A *Tubulinosema* species was identified in two cases of myositis and disseminated microsporidiosis in immunosuppressed human patients ^{53, 54}. *P. ronneafiei*, a genus which was originally identified from fish muscle, was found in a muscle biopsy from an HIV-negative patient and identified as the cause of myositis in AIDS patients ^{55, 56}. *A. algerae* has been identified as the cause of myositis in immune deficient patients with organ transplants and in a patient with vocal cord lesions ^{36, 37, 57}. *Anncaliia connori* caused disseminated infection including myositis in a child with SCID and *Anncaliia vesicularum* myositis in a patient with AIDS ^{57, 58}.

2.5 Genitourinary Tract Infection

In mammals, infection of the genitourinary system with spores being seen in the urine is commonly seen with *Encephalitozoonidae*, *T. anthropophthera* and *V. cornea* ^{49, 59–61}. Granulomatous interstitial nephritis composed of plasma cells and lymphocytes is the most frequent pathologic finding in humans with genitourinary microsporidiosis ^{44, 62, 63}. Congenital transmission of human infection with microsporidia has not been reported, although this has been seen in other mammals.

2.6 Other infections

A. algerae has been reported as a cause of cellulitis in a child with leukemia ⁶⁴. *Encephalitozoon sp.* have been reported to cause nodular skin lesions ⁶⁵. Sinus and respiratory infections have been reported due to *Encephalitozoonidae*. In the cases of keratoconjunctivitis, due to *Enc. hellem*, spores were found in samples of patients' sputum indicating a co-existent respiratory infection ⁶⁶.

3. Diagnosis of Microsporidiosis

There are now at least ten Microsporidian genera that have been identified in human infections^{40, 67, 68}. Light microscopy is useful in the identification of microsporidian spores in various tissue samples and fluids. Stains such as chromotrope 2R, calcofluor white (fluorescent brightener 28), and Uvitex 2B^{69–71}, have been useful in the identification of spores in stool specimens and other body fluids. However, these techniques cannot provide species-specific diagnosis. Electron microscopy or molecular techniques can be used to identify the exact species of microsporidia causing an infection⁷². In cases of gastrointestinal infection, it is useful to also urine specimens for spores, as shedding of spores in the urine is common in infections due to Encephalitozoonidae, but not in infection by *Ent. bienersi*.

3.1 Light and Immunofluorescent Microscopy

Spores are the main targets for clinical diagnosis. Due to the small size of microsporidian spores (1~5 µm) adequate magnification using a 60X to 100X objective is required for their detection. Spores in fresh tissue samples are usually birefringent and retractile and can be seen using a phase-contrast microscopy¹⁵. The detection of spores in stool is difficult due to the presence of bacteria and yeast^{73–75}. In order to increase the specificity and sensitivity of detecting microsporidia, selective staining methods (calcofluor white, chromotrope 2R, Uvitex 2B, Warthin-Starry silver stain, Giemsa, acid-fast, and Brown-Hopps Gram) should be employed^{39, 40, 76, 77}. Antibodies for IFA techniques have been reported and, if available, can increase the sensitivity of this examination and provide species identification¹⁵.

3.2 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is the gold standard technique for the identification of microsporidian species due to its ability to identify the polar tube and other phylum- and species-specific ultrastructural characters^{40, 78}. However, it is expensive, time-consuming and requires specialized equipment that is not available in most laboratories⁷⁹.

3.3 Nucleic Acid-based Molecular Techniques

Nucleic acid-based diagnosis techniques have proven useful in the diagnosis of microsporidiosis⁸⁰. Molecular methods, such as PCR, can provide species and genotype diagnostic information^{80–83}. The genomes of most of the microsporidia that infect humans are available on MicrosporidiaDB (<http://microsporidiadb.org/micro/>) and primers for the diagnosis and speciation of these organisms based on rRNA genes⁸⁴ have been evaluated in many clinical and field settings^{80–83}. Nucleic acids can be isolated from clinical specimens such as urine or stool, in vitro cultures, and paraffin-embedded material by commercial DNA extraction kits. Real-time PCR assays have been developed and may provide higher sensitivity and specificity than traditional PCR⁸⁵ with detection limits of 102 to 103 spores/ml in stool^{86, 87}. Fluorescent in situ hybridization (FISH)-based techniques using probes against microsporidia small subunit (SSU) rRNA have also been used for the detection of *Ent. bienersi* and *Enc. hellem*^{88–90}; however, FISH is less sensitive than PCR due to the lack of signal amplification⁸⁰.

3.4 Serology

Serological techniques that have been reported for the detection of immune responses, both IgG and IgM, to microsporidiosis include: immunoblotting (IB), enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence test (IFAT), counter-immunoelectrophoresis (CIE), and carbon immunoassay (CIA) ^{91–97}. IFAT and ELISA are the most widely used tests and they correlate well with each other ⁹⁸. A limitation of serology is that it is difficult to distinguish when the infection has occurred as antibodies persist in the host for a long time following infection ^{99, 100}. To this end, the utility of serology is not as good as molecular diagnostic methods ⁸⁰. However, serology can be used for epidemiology to examine infection prevalence and will identify that an animal has been infected ^{101, 102}. Serological screening is used in laboratory research to eliminate potentially infected animals, particularly rabbits with *Enc. cuniculi* infection, to avoid infection related confounding effects on experimental results ¹⁰³.

4. Therapeutic targets for the treatment of microsporidiosis

4.1 Triosephosphate isomerase (TIM)

The genome of most microsporidia are highly reduced and their genome size is reported to be among the smallest of eukaryotic organisms ¹⁰⁴. In addition, microsporidia do not have typical mitochondria, but possess mitosomes, a mitochondrial remnant which cannot perform oxidative phosphorylation and microsporidia lack the tricarboxylic acid cycle ^{105–107}. This requires microsporidia to rely on other metabolic pathways such as the glycolytic pathway, pentose phosphate pathway, and trehalose metabolism for energy ¹⁰⁸. Thus, the enzymes involved in these pathways, which are critical for the microsporidia, are potential therapeutic drug targets.

Triosephosphate isomerase (TIM), which plays an important role in glycolysis of *Enc. intestinalis*, was analyzed as a drug target for the therapy of microsporidiosis ¹⁰⁹. TIM catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP), and is essential for efficient energy production. Studies of TIM in *Trypanosoma cruzi*, *T. brucei*, *Entamoeba histolytica*, and *Giardia duodenalis* have demonstrated that modification of cysteine (Cys) residues by sulfhydryl reagents could change its function and structure, leading to inactivation of this enzyme ^{110–113}. As a proof of concept, three sulfhydryl reagents (MMTS, MTSES, and DTNB) were used to modify the conserved Cys in *Enc. intestinalis* TIM (EiTIM). These three sulfhydryl reagents do not inactivate human triosephosphate isomerase (HsTIM) despite ^{109, 114}. Treatment with these compounds caused a significant reduction of EiTIM activity in a concentration-dependent manner. When EiTIM was inhibited the process of interconversion between DHAP and GAP was reduced, leading to an accumulation of either DHAP or GAP which are both toxic to the microsporidia spores (Figure 1A). A microsporidia lack the glyoxalase system, which can detoxify these compounds, the toxicity of TIM inhibition may be enhanced in microsporidia. Furthermore, three compounds that are widely used in the pharmaceutical industry (omeprazole, rabeprazole, and sulbutiamine) have also been demonstrated to modify EiTIM Cys residues and to significantly inactivate EiTIM [98], providing lead compounds for the development of this therapeutic pathway.

Overall, these data suggest Triosephosphate isomerase is a potential target for new therapeutic agents for microsporidiosis.

4.2 Tubulin

Microtubules are a characteristic feature of eukaryotic cells and critical in the formation of the mitotic spindle, cytoskeleton, flagella, and cilia. Microtubules are formed by polymerization of tubulin which is a dimeric protein composed of α -tubulin and β -tubulin (Figure 1B) ¹¹⁵. Benzimidazoles are a heterocyclic aromatic organic compound which consists of the fusion of benzene and imidazole. These drugs are inhibitors of microtubule polymerization by binding to tubulin and disturbing the self-association of tubulin subunit ^{116, 117}. The benzimidazoles include: albendazole, cambendazole, benomyl, carbendazim, fenbendazole, mebendazole, and triclabendazole. These compounds have diverse biological activity and applications including antifungal, anticancer, antihelminthic, antiviral, anti-histaminic, anti-inflammatory, anticoagulant properties and antihypertensive effects ^{118, 119}. They have been used since the 1960s as antihelminthic agents in veterinary and human medicine, and as antifungal agents in agriculture ^{115, 120–124}.

Benzimidazoles are also the most widely used drugs in the treatment of microsporidiosis. The only clear microtubules detected in microsporidia are intranuclear spindle microtubules formed during nuclear division ^{41, 125, 126}. Analysis of the microsporidia β -tubulin sequence identified similar amino acid residues that are present in various microsporidia (Encephalitozoonidae and *A. algerae*) which are associated with benzimidazole sensitivity and are consistent with the clinical response of these particular microsporidian infections to albendazole. For example, Val 268 has been identified in nearly all benzimidazole sensitive organisms, is present in these microsporidia, and is believed to play the role in benzimidazole activity ^{115, 120}. However, several other microsporidia, e.g. *Ent. bienewsi* and *V. corneae*, have a β -tubulin sequence with residues associated with benzimidazole resistance, and these microsporidia have been found not to respond to albendazole.

Albendazole is highly active against all of the *Encephalitozoonidae* including *Enc. hellem*, *Enc. cuniculi*, and *Enc. intestinalis* in vitro ¹²⁷. Patients with *Enc. intestinalis* diarrhea improved with albendazole treatment which is consistent with the analysis of β -tubulin of this organism ¹²⁸. Proliferative stages of microsporidia in albendazole treated individuals appeared larger than usual and unable to undergo cell division, probably due to the interaction of albendazole with tubulin resulting in the failure of these treated organisms to form spindle fibers and undergo mitosis (Figure 1B) ¹²⁹. Furthermore, not only albendazole but the metabolized albendazole sulfoxide and other benzimidazole derivatives inhibited the growth of *Enc. intestinalis* in vitro ¹²⁴.

A patient with disseminated microsporidiosis with advanced renal failure due to *Enc. cuniculi* infection was treated with albendazole, which resulted in improvement in serum creatinine levels, complete disappearance of spores from sputum, and a negative urine culture ¹³⁰. Albendazole also appears to have an attenuating effect on *A. algerae* infection in Rabbit Kidney (RK13) cells and long-term treatment could inhibit up to 98% of spore production ¹³¹.

In contrast, several studies using albendazole as a treatment for *Ent. bieneusi*, have found that it is less sensitive to this drug compared with the Encephalozoonidae^{6, 132}. As has been seen in other organisms, resistance has been demonstrated in diverse species including microsporidia¹³³. Benzimidazole derivatives have resistance in helminths is an active area of study and such compounds or new tubulin binding drugs could be useful in the treatment of microsporidiosis.

4.3 Methionine aminopeptidase 2 (MetAP2)

Methionine aminopeptidase (MetAPs) activity is essential for eukaryotic cell survival as the removal of the terminal methionine of a protein is often critical for its function and posttranslational modification. Two major classes of Methionine aminopeptidases, designated type 1 and type 2 (MetAP1 and MetAP2), were originally identified as cytosolic proteins in eukaryotes^{134–136}. In the genome of the cyanobacterium, *Synechocystis sp.*, a novel MetAP3 gene was also identified¹³⁷. MetAP2, as a member of the dimetallohydrolase family, is a cytosolic metalloenzyme that catalyzes the hydrolytic removal of N-terminal methionine residues from nascent proteins^{138–140}. Important functions of this enzyme, which is found in all organisms, are its role in tissue repair and protein degradation, as well as the role it plays in angiogenesis^{139, 141}. The MetAP2 genes were identified from the human pathogenic microsporidia *Enc. intestinalis*, *Enc. hellem*, *Enc. cuniculi*, *Ent. bieneusi*, and *A. algerae* using the strategy of homology polymerase chain reaction^{142–144}. And based on genome sequence data (Microsporidiadb.org) the microsporidia appear to only contain MetAP2¹⁴⁵. Since both MetAP1 and MetAP2 genes exist in mammalian genomes and the functions of these two MetAP genes overlap, this makes microsporidia MetAP2 an essential gene in microsporidia and a logical target for designing therapeutic agents for microsporidiosis¹⁴⁴.

MetAP2 is the target of two groups of anti-angiogenic natural products, ovalicin and fumagillin, and their analogs^{146, 147}. Fumagillin together with albendazole are two most widely used therapeutic agents for microsporidiosis, however, albendazole is not active against *Ent. bieneusi*^{41, 115, 132}. Fumagillin, ovalicin, and their analogs like TNP-470, have been demonstrated to have anti-microsporidial activity in vitro and in vivo in animal models and clinical cases of microsporidiosis, including activity in patients with *Ent. bieneusi* infection^{148–151}. Fumagillin also works in the treatment of *Nosema apis* and *Pleistopora anguillarum* in bees and fish^{152–154}.

Study of MetAPs in yeasts revealed that *Saccharomyces cerevisiae* deficient in MetAP1 could be killed by treatment with fumagillin or ovalicin, however, yeast deficient in MetAP2 were not killed by those drugs^{146, 155}. This confirmed that MetAP2, but not MetAP1 is the target of fumagillin. The activity of fumagillin is due to its ability to bind to MetAP2 via an epoxide ring that is the site of covalent binding to a histidine residue in the active site of MetAP2^{146, 155}. Structure analysis of MetAP2 demonstrated that His₂₃₁ in human MetAP2 is the residue that fumagillin forms a covalent bond with, and this residue is also highly conserved in microsporidian MetAP2 sequences^{142, 156}. Fumagillin treatment of microsporidia causes lipid and protein granules in host cell cytoplasm to increase suggesting effects on lipid incorporation into membranes¹⁵⁷. Ultrastructure demonstrated that the shape

of proliferative forms became distorted, membranous organelles were misshapen with numerous convoluted vacuoles, depletion or scattering of ribosomes, and breaking of parasite's plasma membranes ⁴¹. Even though human MetAP1 is not inhibited by fumagillin, there is inhibition of human MetAP2, and fumagillin and its analogs caused thrombocytopenia in patients treated with fumagillin ¹⁵⁰. Although this side effect did not limit treatment, improved and more selective microsporidian MetAP2 inhibitors would be useful, as well as compounds that lacked epoxide rings and were competitive inhibitors of MetAP2. There is a significant amount of interest by pharmaceutical companies in MetAP2 inhibitors due to their ability to affect angiogenesis, which has resulted in a pipeline of new compounds that could be evaluated for their activity against microsporidia.

4.4 Synthetic Polyamines

Polyamines are low molecular weight organic compounds having more than two amino groups. Natural polyamines are found in all forms of life. These small molecules are commonly known as putrescine (diamine), spermidine (triamine), and spermine (tetramine) and they can bind to DNA, chromatin and RNA affecting nuclear and cell division ¹⁵⁸. Ribosomes of the microsporidia contain polyamines (mainly spermine and spermidine), and these polyamines bind to rRNA helping to maintain the compact structure of the ribosome ¹⁵⁹. Polyamine concentrations in cells are carefully controlled by synthesis, degradation, excretion, and uptake from the environment ¹⁶⁰. In mammals and many protozoa, synthesis occurs from the conversion of ornithine to putrescine through ornithine decarboxylase (ODC). Spermidine and spermine are formed from putrescine by adding one or two aminopropyl groups using ODC and S-adenosylmethionine decarboxylase (AdoMetdc) as rate limiting enzymes ^{161, 162}. The key enzymes in interconverting of spermine and spermidine to spermidine and putrescine respectively, are spermidine/spermine *N*¹-acetyltransferase (SSAT) and polyamine oxidase (PAO) ¹⁶⁰. The trio of ODC, AdoMetdc and SSAT serves to keep intracellular polyamines at relatively stable levels ¹⁶¹. Research using purified ribosomes has demonstrated that they exchange polyamines with polyamines in the media suggesting that polyamine binding is a dynamic process in the ribosome ¹⁶³. Taking up exogenous polyamine analogs has been shown to down-regulate ODC and AdoMetdc, and up-regulate SSAT, leading to a down-regulation of polyamine synthesis while still allowing acetylation and excretion of intracellular polyamines. This leads to a decrease in the naturally occurring polyamines, and as polyamine analogues do not function in cell metabolism, unlike the naturally occurring polyamines, cell division stops and cells die ^{158, 161, 164}.

A study of polyamine metabolism in pre-emergent spores of *Enc. cuniculi* revealed that microsporidia are reliant on uptake and interconversion of polyamines, rather than synthesis, for maintaining polyamine levels, which makes it possible to use polyamine analogues as a drug for the treatment of microsporidia ¹⁶⁵. Several polyamine analogues have demonstrated activity against microsporidia *in vitro* and in a murine microsporidiosis model. Bis-ethylated 3–3–3 (bis-ethyl norspermine) or 3–4–3 (bis-ethyl homospermine) derivatives were effective in inhibiting microsporidia growth, but also had host cell toxicity ¹⁶⁶. Another class of polyamine analogues evaluated for the activity against microsporidia was 3–7–3 derivatives. Bis-phenylbenzyl-substituted 3–7–3 (i.e. 1.15-bis *N*-[*o*-(phenyl)benzylamino]–4,12-

diazapentadecane [BW-1]) was found to inhibit *Enc. cuniculi* PAO activity¹⁶⁰. Second generation alkyl-substituted polyamine analogues containing a 3–7–3 polyamine back bone were synthesized and several of these compounds had activity against microsporidia both *in vitro*, without producing overt cytotoxicity in their host cells, and *in vivo* in a murine infection model¹⁶⁷. The novel synthetic polyamines SL-11158 and SL-11144 were also active against *Enc. cuniculi* both *in vitro* and *in vivo*¹⁶⁸. In addition, another three polyamine analogues were tested in an *Ent. bienewsi* infection and were compared with the activity of fumagillin. Two of the analogues PG-11302 and PG-11157 had efficacy against *Ent. bienewsi* infection and were more potent drugs against *Ent. bienewsi* infection than fumagillin¹⁶⁹.

Due to the reliance of microsporidia being on uptake rather than synthesis of polyamines and the demonstrated anti-microsporidian activity of several polyamines analogues (e.g. BW-1, PG-11302, PG-11157, SL-11144, and SL-11158 [146, 152–155]), searching for new polyamine analogues could be an important future direction for the development of new therapeutic agents for this infection.

4.5 Other therapeutic targets

4.5.1 Chitin synthase—Chitin is part of the microsporidia spore wall. It is important for maintaining the spore cell structure and plays a crucial role in infection and extracellular survival^{8, 9, 170}. Chitin is synthesized by plasma membrane-associated chitin synthases (CS), which belong to the family of glycosyltransferases, specifically the hexosyltransferases^{171, 172}. Chitin synthases utilize UDP-N-acetylglucosamine as a substrate to synthesize linear chitin molecules (polymers of β -1,4-N-acetylglucosamine). All characterized fungi, including microsporidia, have chitin synthases and most have multiple genes encoding chitin synthases, with different type of chitin synthases being thought to be used in different stages of the fungal life cycles or for particular cell types^{172–175}. Due to the importance of chitin to the structure of microsporidia spore wall (Figure 1C) and its absence from mammalian cells, chitin synthase has been considered a prime target for the development of therapeutic agents for microsporidiosis. The best-known chitin synthesis inhibitors are nikkomycins and polyoxins which are the analogs of chitin synthesis substrate, UDP-N-acetylglucosamine^{172, 176}. They act as competitive inhibitors for chitin synthesis, leading to inhibition of septation, chaining, and osmotic swelling of the fungal cell¹⁷⁷. Nikkomycin was shown to inhibit replication of *Enc. cuniculi* and *Enc. hellem* *in vitro* and could induce cell damage in both mature and immature spores (Figure 1C)^{178, 179}. Polyoxin exhibits an even higher inhibition than Nikkomycin in controlling *Enc. hellem* *in vitro*¹⁷⁸. Lufenuron, another chitin synthesis inhibitor was proved to be effective in inhibiting *Enc. intestinalis* and *V. corneae* growth *in vitro* without any detectable toxicity to host cells¹⁸⁰.

4.5.2 Topoisomerase IV—While microsporidia are eukaryotic organisms related to fungi^{1–3, 181}, a partial gene encoding for the topoisomerase IV subunit (part C), previously only identified in prokaryotes, was seen in the *V. corneae* genome¹⁸². This suggested that, for some microsporidia, topoisomerase IV could be a potential target for therapy. Fluoroquinolones are antimicrobial compounds which target topoisomerase IV, thereby

causing decatenation of intertwined daughter chromosomes to allow for segregation of daughter cells and relaxing negative DNA supercoils^{183, 184}. Fifteen fluoroquinolones were tested both *in vivo* and *in vitro* of microsporidia infection. Lomefloxacin, gatifloxacin, nalidixic acid, and moxifloxacin inhibited both *V. corneae* and *Enc. intestinalis in vitro* and had minimal host cell toxicity. Ofloxacin and norfloxacin inhibited *Enc. intestinalis* replication by more than 70% compared to the control group. Using an *in vivo* athymic mouse infection model, it was found that ofloxacin, lomefloxacin, gatifloxacin, and norfloxacin increased time of survival of athymic mice infected by *V. corneae*; however, nalidixic acid and moxifloxacin failed to prolong survival¹⁸². These data illustrate that these drugs could be useful therapeutic agents, although more study is needed to define their therapeutic efficacy for microsporidiosis.

4.5.3 Calcium depended spore germination—Microsporidia process a unique invasion organelle termed polar tube, which coils under the spore wall in microsporidia. One of the mechanisms of microsporidia infection is by the germination of microsporidia spores and transferring the nucleic contained sporoplasm into host cell by the extruded polar tube. The process of spore germination has been reported to be calcium dependent¹⁸⁵. It has been hypothesized that displacement of calcium from the polaroplast membrane would either activate a contractile mechanism or combine with the polaroplast matrix causing polaroplast swelling^{186, 187}. The calcium antagonists (verapamil and lanthanum) and the calmodulin inhibitors (trifluoperazine and chlorpromazine) were demonstrated to prevent spore germination in *Spraguea lophii*¹⁸⁵. Nifedipine, which is a calcium channel blocker, has been reported to inhibit the germination of *Enc. hellem* and *Enc. intestinalis* spores *in vitro*^{188, 189}. However, no *in vivo* data exists regarding the use of calcium channel blockers in microsporidiosis¹⁹⁰.

5. Conclusion

Microsporidia have become important emerging human infections, particular with the increased use of immunomodulatory agents for disease therapy. These pathogens cause infection in both immune competent and immune compromised individuals. Clinically, microsporidiosis can cause a range of symptoms including chronic diarrhea, abdominal pain, fever, renal failure, and fatigue, which carry a significant mortality risk to patients. Albendazole and fumagillin are the most widely used drugs for the therapy of microsporidiosis which use β -tubulin and MetAP2 as targets, respectively. Albendazole is effective for *Encephalitozoon spp.*, however, it is not effective against *Ent. bienersi*. Fumagillin has a much broader range of effectiveness against microsporidia, including both *Encephalitozoon spp.* and *Ent. bienersi*. New therapeutic targets for microsporidia include triosephosphate isomerase, chitin synthase, polyamines, and topoisomerase IV. Drugs based on these targets are promising, but there is a need for additional studies before any are ready for clinical trials.

6. Expert opinion

Microsporidia are notoriously reduced and derived intracellular parasites. They have very reduced small genomes and relic mitochondria. Microsporidia lack the tricarboxylic acid

cycle and oxidative metabolism and many lack alternative oxidase. They, therefore, rely more on other energy metabolic pathways such as glycolysis, the pentose phosphate pathway, and trehalose metabolism. This reduction in energy metabolism pathways makes triosephosphate isomerase an intriguing target for drug design due to its important role in glycolysis in microsporidia. Similarly, the structural and catalytic mechanism differences between other key enzymes involved in microsporidian and human glycolysis pathways as well as other energy metabolic pathways could be compared, and those differences should offer opportunities for the design and synthesis of new anti-microsporidia drugs.

Two polyamine analogues PG-11302 and PG-11157 have been proven to be effective agents for the treatment of microsporidiosis in vitro and in murine infection models. There has been limited development of polyamine analogues and this pathway for the treatment of microsporidiosis and this area deserves further study.

Albendazole is one of the widely used drugs for microsporidiosis therapeutics, but it is less effective for *Ent. bienewsi* compared with the Encephalozoonidae, which limits its effectiveness in clinical practice. *Ent. bienewsi* is the most common microsporidia in human infection and the first microsporidia being reported as the causing of diarrhea in patients with AIDS, so drugs that developed for *Ent. bienewsi* therapy is even more important. Studies of new benzimidazole analogs that work for organisms resistant to traditional benzimidazoles may yield new therapeutic agents for microsporidia.

Microsporidia lack methionine aminopeptidase type 1 (MetAP1) and are, therefore, dependent on MetAP2, while mammalian cells have both enzymes. Meanwhile, MetAP2 plays a key role in angiogenesis which is required for tumor metastasis in cancer patients. Thus, MetAP2 is an essential enzyme in the Microsporidia and new inhibitors of this pathway have significant promise as therapeutic agents for microsporidiosis. The natural product fumagillin is a selective and potent irreversible inhibitor of MetAP2, and fumagillin and its analogs, TNP-470 have shown promise *in vitro* and in murine models for the therapy of microsporidiosis, and have been used in clinical studies for the treatment of cancer¹⁹¹. Fumagillin has a broader anti-microsporidian activity than albendazole and is active against both *Ent. bienewsi* and Encephalozoonidae; however, it is not commercially available for human infection in most countries. New compounds that bind MetAP2 have been developed by the pharmaceutical industry as potential anticancer therapies. For example, beloranib (CKD-732) which is a fumagillin derivative, has a much higher anti-angiogenic activity than fumagillin and has been used in a clinical trial¹⁹², and PPI-2458, another fumagillin derivative, has shown efficacy in rodent cancer models and has significant MetAP2 inhibitory activity^{193, 194}. Fumagillin analogues have been demonstrated to inhibit *Enc. cuniculi* *in vitro* and *in vivo*¹⁵. New MetAP2 inhibitors hold significant promise for the treatment of microsporidiosis.

A limitation of research is that *Enterocytozoon bienewsi* has not been cultivated continuously *in vitro* and are no reliable rodent models of this infection. Understanding what is needed to cultivate this pathogen and the development of a robust small animal would clearly facilitate the development of new therapies for this pathogenic microsporidian.

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Article Highlights

1. Microsporidia are opportunistic pathogens that are widely distributed in nature. They are related to the Cryptomycota and are probably a sister group to the Fungi. They have a broad host range including both invertebrates and vertebrates.
2. Microsporidiosis in immune deficient patients can be extremely debilitating and carries a significant mortality risk.
3. Multiple strategies have been used for the diagnosis of microsporidia including light microscopy, transmission electron microscopy, and nucleic acid-based molecular techniques.
4. Triosephosphate isomerase (TIM) is a new target identified recently for anti-microsporidia drug design. Modification of TIM cysteine (Cys) residues by three different sulfhydryl reagents could inactivate microsporidian TIM, but had little or no effect on human TIM, suggesting that this pathway could be useful for the design of new therapeutic agents
5. Microsporidia lack methionine aminopeptidase type 1 (MetAP1) and are, therefore, dependent on MetAP2, while mammalian cells have both enzymes. Thus, MetAP2 is an essential enzyme in the Microsporidia and new inhibitors of this pathway have significant promise as therapeutic agents.
6. Chitin is important for maintaining the structure of microsporidia spore wall, and it is absent in mammalian cells. To this end, like in classical Fungi, chitin synthase is an interesting target for the development of therapeutic agents for microsporidiosis. Chitin synthase inhibitors such as nikkomycins, polyoxins, and lufenuron, have been demonstrated *in vitro* to inhibit spore replication and alter spore morphology.

Figure 1. Therapeutic targets of microsporidia and the mode of action of their drugs.

(A) Sulfhydryl reagents can inhibit Triosephosphate Isomerase (TIM) in microsporidia reducing the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) leading to an accumulation of either DHAP or GAP which are toxic to microsporidia resulting in the death of this organism. (B) Albendazole inhibits the polymerization of tubulin, resulting in the failure formation of microtubules, which can lead to an inability to form spindle fibers during the mitosis, and cell death. (C) Chitinase inhibitors, such as nikkomycins and polyoxins, act as competitive inhibitors for chitin synthesis inhibiting the synthesis of chitin from UDP-N-acetylglucosamine resulting in cell damage in both mature and immature spores of microsporidia. Images were created using Motifolio Anatomy Drawing Toolkit available from <http://www.motifolio.com/anatomy.html>.

TABLE 1

Species of microsporidia infecting humans

Species	Synonym(s)	Common sites of infection in humans	Other mammalian hosts	Reported Non-mammalian hosts	Disease Manifestations Reported
<i>Anncaliia algerae</i>	<i>Brachiola algerae</i>	Eye		Mosquitoes	myositis, keratoconjunctivitis, cellulitis
	<i>Nosema algerae</i>	Muscle			
<i>Anncaliia connori</i>	<i>Brachiola connori</i>	Systemic			disseminated disease
	<i>Nosema connori</i>				
<i>Anncaliia vesicularum</i>	<i>Brachiola vesicularum</i>	Muscle			myositis
<i>Encephalitozoon cuniculi</i>	<i>Nosema cuniculi</i>	Systemic	Wide host range	Birds	hepatitis, encephalitis, peritonitis, urethritis, prostatitis, nephritis, sinusitis, keratoconjunctivitis, cystitis, diarrhea, disseminated infection
<i>Encephalitozoon hellem</i>		Eyes	Bats	Birds	superficial keratoconjunctivitis, sinusitis, pneumonitis, nephritis, prostatitis, urethritis, cystitis, diarrhea, disseminated infection
<i>Encephalitozoon intestinalis</i>	<i>Septata intestinalis</i>	Small intestine	Wide host range	Geese	diarrhea, intestinal perforation, cholangitis, nephritis, superficial keratoconjunctivitis, disseminated infection
<i>Enterocytozoon bienersi</i>		Small intestine	Wide host range	Birds	diarrhea, malabsorption with wasting syndrome, cholangitis, rhinitis, bronchitis
		Biliary tract			
<i>Microsporidium africanum</i>		Eyes			stromal keratitis
<i>Microsporidium ceylonensis</i>		Eyes			stromal keratitis
<i>Microsporium CU (Endoreticulatus-like)</i>		Muscle			myositis
<i>Nosema ocularum</i>		Eyes			stromal keratitis
<i>Pleistophora ronneaei</i>		Muscle		<u>Fish</u> *	myositis
<i>Trachipleistophora anthropoptera</i>		Eyes		<u>Insects</u> *	encephalitis, keratitis, disseminated infection
		Systemic			
<i>Trachipleistophora hominis</i>		Eyes		<u>Mosquitoes</u> *	myositis, keratoconjunctivitis, sinusitis, encephalitis
		Muscle			
<i>Tubulinosema acridophagus</i>		Muscle		<u>Fruit fly</u> *	myositis, disseminated infection
		Systemic			
<i>Vittaforma corneae</i>	<i>Nosema corneum</i>	Eyes			Keratoconjunctivitis, urinary tract infection
		Urinary tract			

* Putative host(s) based on phylogeny or host relationships of other species within the genus.