WHITE PAPER

Targeting leishmaniasis with an entamoeba molecule

Beating parasites with their own weapons

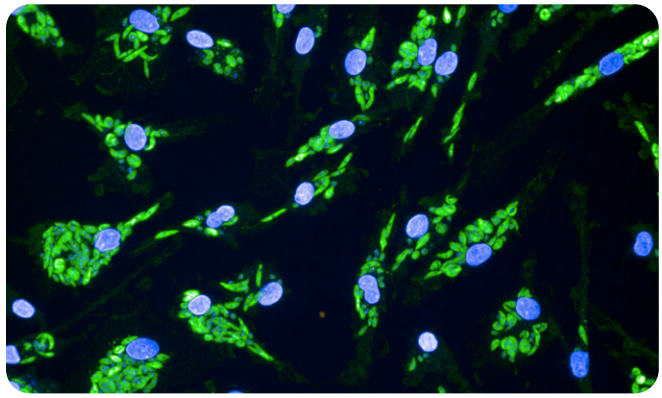
Introduction

Leishmaniasis is an increasingly prevalent parasitic disease found in tropical and subtropical regions throughout the globe. Leishmaniasis can be caused by 20 or more *Leishmania* species which are transmitted to humans via the bite of an infected female sand fly. These protozoa are obligate intracellular parasites that target host immune cells, especially macrophages. Once inside the immune cell, the parasite suppresses the cell's immune response in order to ensure its own survival.

Not all individuals infected with *Leishmania* become ill or even exhibit symptoms, thus the term leishmaniasis is used to refer only to those who become sick due to infection. There are three primary forms of leishmaniasis:[1]

- Cutaneous leishmaniasis, caused by *L. major*, is the most common form and causes cutaneous ulcers.
- Visceral leishmaniasis (also known as kala-azar), mainly caused by L. *donovani* or L. *infantum*, is the most severe form of the disease, having a fatality rate up to 100% within two years if left untreated.
- Mucocutaneous leishmaniasis, mainly caused by L. *braziliensis*, is the most disabling form, causing disfiguring mucosal ulcers that destroy mucous membranes.

According to WHO, roughly 700,000 to one million new cases of the disease occur annually, primarily in the >90 countries/territories considered endemic for leishmaniasis. More than one billion people are at risk of developing leishmaniasis in those areas.[2]



Current treatment regimens consist of chemotherapeutics such as pentavalent antimonials, miltefosine, amphotericin B, and others. Those approaches, however, face perennial challenges such as the risk of severe side effects, long treatment duration, emergence of drug-resistant *Leishmania* strains, high costs, and narrow therapeutic windows. Researchers continue to investigate new therapeutic approaches for leishmaniasis that could be used independently or in conjunction with chemotherapeutics.

Dr. Helena Fehling from the Department of Molecular Parasitology and Immunology at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany, is exploring an immune-therapeutic approach to leishmaniasis treatment. This white paper highlights Dr. Fehling's research and the promising results she and her colleagues are finding.

Immune-stimulatory molecule discovery

Dr. Fehling's current research was prompted by a discovery made in 2009 by group leader Prof. Dr. Hannelore Lotter. Dr. Lotter investigated the immunostimulatory properties of an

interesting molecule within the membrane of another human pathogenic protozoan parasite – *Entamoeba histolytica* (Eh). The molecule was a lipopeptidephosphoglycan (EhLPPG) composed of a highly-acidic polypeptide backbone modified with numerous linear glycan chains.

Further molecular studies revealed that EhLPPG contains a phosphatidylinositol (PI) anchor with two isoforms, EhPIa and EhPIb. These isoforms were found to have structural similarities with the strongly immunostimulatory molecule α-galactosylceramide (αGalCer). Unlike drugs that act directly on the disease-causing pathogen, immunostimulatory drugs help the patient's immune system combat the disease. This concept is well known for treating several forms of cancer, and now it is moving into focus for other difficult to treat diseases such as parasitic infections.

Based on its structure, the newly discovered molecule was screened for possible immune-stimulating properties. The initial screening assay exposed uninfected dendritic cells to EhLPPG isolated from E. *histolytica*. The results showed an increase in immune activity by the exposed dendritic cells.

These findings prompted researchers at the Institute to investigate the potential effectiveness of EhLPPG as a treatment for macrophage-targeting tropical parasitic diseases, including leishmaniasis.

Targeting *leishmania* infections with immune stimulation

The research team began their investigations of EhLPPG with *in vitro* and *in vivo* studies to examine its potential as a treatment for L. *major* infection. Initial studies used Eh-derived (native) LPPG, and later studies assessed numerous synthetic analogs of EhLPPG.

Native EhLPPG studies

The team's first *in vitro* test included treatment of L. major-infected murine bone marrow-derived macrophages with native EhLPPG and quantification of the resulting intracellular parasite load. The results showed significant reduction in the percentage of infected macrophages as well as reduced parasite load per macrophage. *In vivo* tests using a BALB/c mouse model of cutaneous leishmaniasis showed significant reductions in swelling at infection sites and significant reduction of parasite load.[3]

This encouraging evidence for the efficacy of EhLPPG as an antileishmanial treatment prompted the team to develop synthetic analogs of native EhLPPG and assess their potential as therapeutics. If effective, a synthetic source would be a great advantage for both research and future therapeutics because it would minimize the limitations involved in isolating and purifying native EhLPPG, such as time requirements, high costs, sophisticated purification steps, and challenging reproducibility.

Synthetic analogs studies

The research team evaluated numerous synthetic analogs of the EhLPPG anchor isoforms (EhPIa and EhPIb). In their initial studies, four synthetic analogs were created and evaluated. *In vitro* murine and human cellular assays and *in vivo* murine models were used to evaluate the analogs for cytotoxicity, capacity to stimulate cytokine production, and ability to reduce intracellular infection by L. major.[3]

The *in vitro* assay results indicated that the synthetic analogs would likely have low cytotoxicity *in vivo*. In addition, one EhPIa analog and one EhPIb analog showed substantial immunostimulatory activity. *In vivo* studies revealed that the same two analogs also resulted in significant inhibition of parasite infection and reduced disease severity, although the reduction was transient. mRNA studies revealed that EhLPPG and one EhPIa analog tended to induce expression of mRNA that encodes protective pro-inflammatory cytokines.

Based on these promising results, the researchers synthesized six new analogs of the EhPIb anchor and scrutinized them for antileishmanial activity. The analogs underwent *in vitro* murine and human cellular assays and *in vivo* testing using a murine model of cutaneous leishmaniasis.[4]

The cellular assays revealed almost no toxicity from the synthetic analogs. The *in vitro* assays showed that treatment with the analogs significantly decreased the parasite load in both murine and human macrophages. The *in vivo* studies revealed that topical application of one of the analogs significantly reduced cutaneous lesions in the murine model. This correlated with an increase in the production of selected Th1 cytokines in the murine model.

These data provided further support for the potential of synthetic analogs of the EhLPPG anchor to be an effective therapeutic for cutaneous leishmaniasis.

Moving into high-content screening

The research team then undertook to optimize their assay methods and conduct further evaluation of the synthetic analogs Eh-1 to Eh-6. They developed a high-content screening assay using murine primary macrophages, and validated the assay using the key leishmaniasis-causing species: L. *major* and L. *braziliensis* (dermotropic species), and L. *donovani* and L. *infantum* (viscerotropic species). The assay was also used to conduct additional investigations on the antileishmanial activity of the six EhPIb synthetic analogs.[5]

Parasite detection was based on a 90 kDA heat shock protein-specific staining that enabled the detection of multiple *Leishmania* species. Images were acquired using the Opera Phenix® high-content screening system. Image analysis was completed utilizing a sequence that was designed using the Harmony® high-content imaging and analysis software.

The image analysis generated output parameters for each well including:

- Total number of macrophages to determine the viability of host cells after drug exposure
- Total number of *Leishmania*-infected macrophages
- Percentage of infected macrophages to determine the overall infection rate
- The number of *Leishmania* parasites per infected macrophage
- Total number of *Leishmania* parasites to determine the overall parasite burden

The data revealed that macrophages infected with dermotropic species were more sensitive to treatment with the synthetic analogs than those infected with viscerotropic species, as evidenced by greater reductions in viability. Most compounds caused a significant reduction in infection rates and parasite burdens in at least one infecting species. Only compound Eh-6 showed activity against all *Leishmania* species.

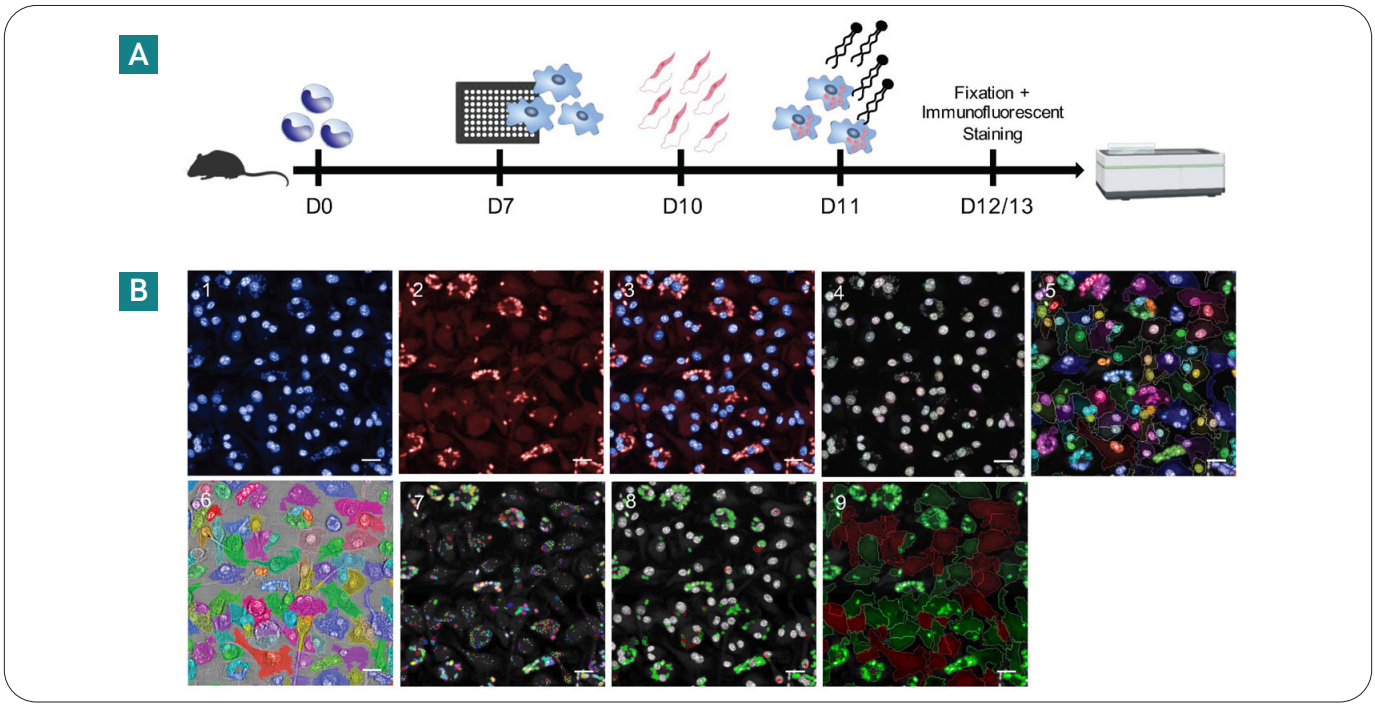


Figure 1: Schematic representation of the *Leishmania* ssp. Screening assay. (A) Experimental pipeline. D0: Isolation of mouse bone marrow-derived cells; D7: Harvest and seeding of mouse primary macrophages into CellCarrier-96 Ultra Microplates; D10: Infection with *Leishmania* (L.) parasites (4 h); D11: Adding immunostimulatory EhPIB-compounds to the cell cultures for 24 (D12) or 48h (D13) with subsequent fixation and immunofluorescent staining of the cells followed by image acquisition and analysis using the Opera Phenix® high-content screening (HCS) system. (B) Parameters for HCS analysis and output. (B1-3) Representative input images of bone-marrow derived macrophages (BMDMs) (DAPI);405nm) infected with L infantum (multiplicity of infection (MOI) 8:1) (Hsp90 staining; Alex 647;640 nm). (B4-6) Object segmentation of macrophages based on intensity and morphology properties of nuclei detection (B4;DAPI; >40 µm^2) and cytoplasm detection (B5; Alexa 647). (B7-8) Detection of immune-stained intracellular l. parasites. B7) Detection of all spots within the region of interest defined as cell cytoplasm (B5). (B8) Selection of correct spots (green=L.-parasites,>4 µm^2) and discarded spots (red) based on intensity and morphology properties. (B9) Output population L. -infected macrophages (green) and non-infected macrophages (red) based on merged images obtained from cell segmentation (B4-6) and parasites detection (B7-8). Scale bar, 20 µM. Figure 1 was created with a licensed version of Biorender.com

Despite the challenges in antileishmanial drug discovery, the researchers successfully developed and validated a multi-species high-content screening assay that can analyze multiple non-recombinant parasite strains. They then demonstrated the usefulness of the assay by screening macrophage-targeting EhPlb-based synthetic analogs and demonstrating their potential for the treatment of both cutaneous and visceral leishmaniasis.

Previously, the researchers had to rely on RT-PCR to detect the parasite load in each well. That method required significant time and resources to obtain only a small amount of data. The current approach, utilizing high-content screening, provides the ability to analyze more samples and obtain multi-parametric data from a single experiment. A single run provides data on cellular toxicity, total parasite load, percentage of infected cells, and other target parameters. "It's great to be able to get so much data from a single well," says Dr. Fehling. Furthermore, they can simultaneously run samples pertaining to multiple *Leishmania* species. This efficiency and depth of data extraction means they need fewer samples from fewer animals.

Dr. Fehling relates how much she and her colleagues are saving in both time and money, and she estimates they completed their latest round of studies at least one year faster than without HCS, significantly reducing their use of plates, animals, and other supplies. These and other savings are important in their day-to-day work, and will also help them secure – and make the most of – future research funding.

Looking to the future

Next up for Dr. Fehling and her colleagues are to use their high-content screening assay to conduct studies on the mechanisms and efficacy of EhLPPG-based treatment in human primary cells. They obtained buffy coats from healthy donors and ran them through the assay to compare those results to the murine data. The early results look promising, but many more samples must be screened for human application due to the greater variability in responses among humans than is seen in murine species.

The team is also conducting studies on the effects of co-treatments that pair chemotherapeutic drugs and immunotherapeutic treatments. The rationale behind such studies is three-fold: (1) to find a therapeutic approach that will be most effective against various *Leishmania* species, (2) find a combination that will reduce the side effects and cytotoxicity of high-dose traditional chemotherapeutics, and (3) help minimize the development of drug-resistant *Leishmania* strains.

Another important assay will be evaluating the effects of EhLPPG and its synthetic analogs on other immune cells to provide a broader picture of the effects of the molecules on the human immune response. Both of these steps – screening of human macrophages and of a broader range of immune cell types – will provide the more physiologically relevant data needed for the development of therapeutic applications.

A challenge noted by Dr. Fehling is the need to differentiate the responses of M1 and M2 macrophages to *Leishmania* species and also to immunostimulatory therapeutics. The research team is already using their high-content screening assay to generate data to address this challenge.

In addition to Leishmaniasis treatment, Dr. Fehling is optimistic that EhLPPG may have wide-ranging applications within the realm of immunotherapeutics. She anticipates research in the not-too-distant future on the potential for EhLPPG to be an effective immunostimulatory treatment for diseases caused by other protozoan parasites, such as *Trypanosoma* cruzi which causes Chagas disease. Researchers are also starting to investigate the effects of EhLPPG on pathogenic bacteria including *Mycobacterium tuberculosis, Salmonella spp.*, and *Rickettsia* spp.

Helena Fehling
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Helena Fehling studied biology at the University of Hamburg, where she already conducted initial studies on immune cell stimulation with EhLPPG as part of her diploma thesis in the Department of Molecular Parasitology at the Bernhardt Nocht Institute for Tropical Medicine (BNITM), Hamburg back in 2011 and completed her PhD in 2016 focusing on pathogenicity factors of Entamoeba histolytica.

Helena continued on to postdoctoral work at group for interdisciplinary neurobiology and immunology investigating the bi-directional communication between the nervous- and the immune system in the field of psychoneuroimmunology (INI-Research gGmbH, Hamburg). In 2018, she returned to Prof. Dr. Hanna Lotter`s Molecular Infection Immunology group at the BNITM, where she took over the project on immunostimulatory compounds for host-targeted therapies in intracellular infections.

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