A major challenge in developing drugs against eukaryotic pathogens such as *Leishmania* is target specificity - selectively killing the pathogen while avoiding toxicity against the human host. Currently, millions suffer from leishmaniasis, and new infections increase annually, making it a major global health problem (1,2). Available chemotherapeutic agents for leishmaniasis are less than ideal because they are toxic to humans (3). This problem is compounded by the emergence of drug-resistant strains of *Leishmania*, and species-variation in their drug-sensitivity (3), prompting continued efforts to find novel, broad-spectrum anti-leishmanial agents (4). Aminoacyl-tRNA synthetases (aaRSs) are excellent drug target candidates because they are essential enzymes that often display marked evolutionary differences between human and parasite orthologs (5-7). To date, efforts focused directly on the amino acid binding-pockets of parasite aaRSs (8-16) have been hindered by both the high degree of sequence conservation between active sites and the potential to rapidly evolve resistance (17-21). In comparison, targeting aaRSs via their interactions with tRNAs have underexplored and unanticipated potential for drug intervention (22), and several of these enzymes have recently emerged as promising targets for antifungal and antimalarial agents (23-26). Because of the great evolutionary divergence between *Leishmania* and humans, tRNA/aaRS interactions present a promising avenue for the development of novel specific anti-*Leishmania* agents, the focus of this proposal.

Our *long-term goal* is the development of a general discovery pipeline applicable to any eukaryotic parasite, which takes genome data as its input and yields nontoxic and resistance-proof combination chemotherapy as its output. Our *objective here* is the discovery and validation of multiple, nontoxic and complementary chemotherapeutic leads against Visceral Leishmaniasis (VL) sourced from a novel marine natural products library. Our two *central hypotheses*, supported by published and preliminary data, are that macromolecular interactions are better targets than small molecule interactions for species-specific chemotherapy, and that co-discovery of leads against multiple targets in the same biological network facilitates combination therapy. Our *rationale* lies in the great need for new nontoxic drugs to combat drug-resistant parasites and in the extensibility of our approach to any genomically sequenced pathogen. We will test our central hypothesis and achieve our objectives through the following two phases of *specific aims*:

Phase one (R21, years 1-2)

Aim 1 - *In vitro* characterization and validation of *Leishmania*-specific aaRS inhibition using reconstituted tRNA aminoacylation and amino acid recognition assays with purified lead compounds.

Aim 2 - Testing of inhibition of cultured parasite growth by lead compounds.

Aim 3 - Integrate genome annotations to improve *in silico* target prediction in *Leishmania*

Transition to Phase two will be based on assessment of Phase one outcomes with respect to three Milestones:

1. Identify two leads with $K_i \leq 2$ μM and at least 100-fold higher K_i for human versus parasite aaRS.
2. Validate one lead with efficacy as an inhibitor of *Leishmania* growth in cell culture.
3. Identification of one aaRS to target for development of broad spectrum leads.

Phase two (R33, years 3-5)

Aim 4 - Purification and/or semi-synthesis of pilot scale quantities of lead compounds and their derivatives.

Aim 5 - Characterization of *in vivo* mode of action of lead compounds and characterization of potential mechanisms of resistance acquisition.

Aim 6 - Evaluation of the efficacy of leads in treatment of VL using an established pre-clinical animal model.

Aim 7 - Improve *in silico* target prioritization algorithm using *in vitro* and *in vivo* data on lead efficacy, mechanism and resistance and expand models to other *Leishmania* and Kinetoplastids.

The *expected outcomes* of this work are multiple novel natural products chemical leads against *Leishmania*, and a potentially universal pipeline for bioinformatics-to-leads ready for deployment against other eukaryotic pathogens or parasites. The *positive impact* of this research lies in its novel approach against intractable infectious disease, and in its application of evolutionary systems biology of tRNA-protein interactions towards combination chemotherapy refractory to the evolution of drug resistance.

Research Strategy

Significance

Infections caused by parasites like *Leishmania* are a leading cause of human morbidity and mortality worldwide, especially in developing countries (1,2). *Leishmania* are transmitted by a sand fly vector and cause a wide range of diseases, including visceral leishmaniasis (VL). It is estimated that VL causes over 50,000 deaths annually (2). Over 12 million people currently suffer from leishmaniasis, and more than 2 million are infected annually, making it a major global health problem and a neglected tropical disease (2). Leishmaniasis is increasingly seen in U.S. soldiers serving in *Leishmania*-endemic countries (27,28), such as Iraq and Afghanistan. Antimonials, amphotericin B, and miltefosine, the standard drugs used to treat leishmaniasis, are toxic (3). This leads to poor patient compliance and low-dose administration (5), ultimately contributing to the rise of strain-dependent drug resistance (29,30). There is a strong need for new nontoxic drugs with broad-spectrum activity against different species of *Leishmania*.

Eukaryotic pathogens like *Leishmania* are particularly challenging subjects of drug discovery efforts because their molecular machineries are structurally and mechanistically similar to our own, contributing to the risk of human drug toxicity. The proposed research is significant because it will identify multiple drug leads against *Leishmania* while avoiding toxicity in human hosts. Our proposal is intended to initiate a highly cross-disciplinary collaboration to systematically chemically interrogate the translation machinery of the protist parasite *Leishmania.* The translation machinery has historically proven to be a productive target for the development of therapeutically effective antimicrobials, particularly for bacterial infections, due to significant differences between essential components of the host and pathogen. We will probe multiple targets early in the translation pathway with a unique and chemically diverse panel of marine natural products (MNPs) developed by one of our team (Roger Linington, Simon Fraser University, Canada). This will allow a systems biology approach to discover multiple tRNA-synthetase inhibitors with anti-leishmanial activity from a natural products library. Such drugs could be used in multidrug combination therapies with current anti-leishmanial agents, thereby reducing the probability of adaptive resistance.

The proposed work will also predict targets for future efforts to control other pathogenic kinetoplastids such as trypanosomes. Cellular viability in all organisms depends on highly specific recognition of tRNAs by their cognate synthetases, but previous attempts to exploit this interaction as a therapeutic target for anti-infectives have often failed due to limited species specificity and subsequent toxicity problems (31). Accurate translation and cellular viability require the faithful aminoacylation of particular tRNAs by their cognate aaRSs. This is achieved by the recognition of specific combinations of structural and chemical features (identity elements) within particular tRNAs that establish a so-called “identity set” (32). While identity sets sometimes show a reasonable degree of conservation across species, one of our team (David Ardell, University of California, Merced) has developed novel computational predictions that suggest many *Leishmania* rely on unique tRNA identity elements, which we seek to exploit here to identify new therapeutic targets (Fig. 1). The Ardell laboratory can successfully estimate, from genomic tRNA sequence data alone, the tRNA identity elements (32) that structurally underlie the entire system of tRNA-enzyme interactions in any organism (33,34). We can estimate the specific diverged differences in tRNA-protein interactions that coevolve between different clades

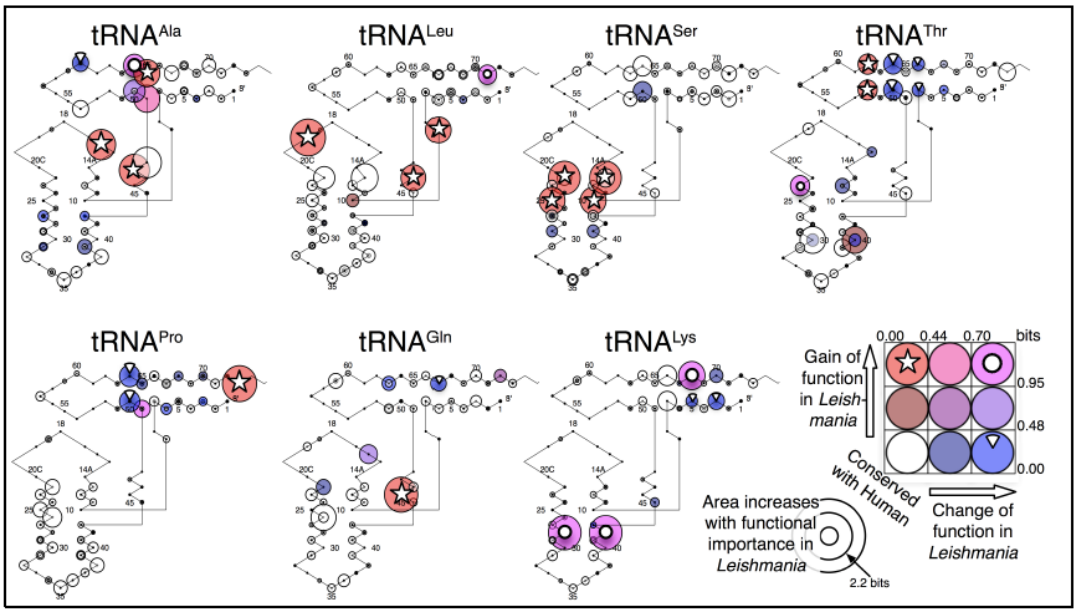

Figure 1. Predictions of functional elements in tRNAs from four species of *Leishmania* contrasted against human tRNAs. Several classes of tRNAs in *Leishmania* show evidence of gains and recruitments of function. 609 human and 293 *Leishmania* predicted tRNA genes were structurally aligned with COVEA..tRNA identity elements were estimated using function logos. *Leishmania*-specific gains and conversions of function were estimated by Information Difference and KLD statistics.

or species (34). Our tRNA-based predictions have found excellent correlations in tRNA-interacting proteins (34,35) that have been experimentally verified by independent laboratories (36-38), correlate with molecular dynamic models of tRNA-protein interactions (39), and were recently shown to resist genome-wide mutation-driven changes in base-composition (40). Our hypothesis of cryptic variation in the structural basis of class-specific tRNA functions is based on statistical results in bacteria (35,40), which are likely to be universal (41), and is the basis for the systems and network approach to drug development proposed here. The advances of systems biology and chemogenomics have fueled interest in combination chemotherapy driven in pursuit of therapeutic benefits from synergistic drug interactions (42-46) and combating the evolution of resistance (47).

Innovation

The innovation in our approach lies in our "bioinformatics-to-leads" strategy to discover resistance-proof chemotherapies targeting multiple synthetases from genome data alone. Combination chemotherapy is a naturally effective strategy, for example, in the pathogenic defenses of arthropods (48). Advances in the systems biology of combination chemotherapies have aided prediction of synergistic drug interactions (42-46) and evolution of resistance (47) and yielded exciting antifungal (49) and antihelminthic (50) therapies. Although monotherapeutic inhibitors of aaRSs are highly effective (51), combination therapies involving multiple aaRSs have not yet been explored. Because aminoacylation pathways are integrated in parallel into protein synthesis (52), their multiple inhibition is not expected to be synergistic, but rather "Bateson-Gaddum non-interaction masked" (53). Unlike some synergistic combination therapies that can facilitate the evolution of drug-resistance (54,55), aaRS inhibitor combination therapies are predicted to be comparatively resistance-proof (47).

R21 Objectives:

Aim 1 – *In vitro* characterization and validation of *Leishmania*-specific aaRS inhibition using reconstituted tRNA aminoacylation and amino acid recognition assays with purified lead compounds (Ibba/Linington).

Preliminary data - To provide supporting data for the discovery pipeline proposed here, the Ibba lab reconstituted an *in vitro* aminoacylation system using the *Leishmania major* alanyl-tRNA synthetase (AlaRS; aaRSs are abbreviated according to the three letter code for their cognate amino acid substrate) based on bioinformatic predictions prioritized by the Ardell laboratory. To facilitate rapid cloning and overproduction, the AlaRS-encoding gene was commercially synthesized (GenScript) based on *L. major* genomic data (56). This allowed for codon-optimized production in *E. coli* of His_6 epitope-tagged AlaRS. A gene encoding an *L. major* $tRNA^{Ala}$ isoacceptor substrate was synthesized from oligonucleotides and used for T7 RNA polymerase-directed transcription of mg quantities of $tRNA^{Ala}$. *In vitro* reconstitution of AlaRS activity was confirmed by observation of the ATP-dependent accumulation of ^{14}[C]-Ala-$tRNA^{Ala}$, and this activity was used to screen our MNP library for inhibitors of *L. major* aaRSs. The MNP library was pre-screened against *L. donovani* and the initial hits were serially diluted and rescreened in the axenic assay. These samples comprise 120 active extracts arrayed as 8 x 2-fold dilutions, for a total of 3 x 384 well screening plates. Reactions measured accumulation of ^{14}[C]-Ala-$tRNA^{Ala}$ after 20 min incubation at 37 °C to allow for time-dependent effects from slow-tight-binding inhibitors (23), and were amenable to medium throughput format assays as required here (Fig, 2).

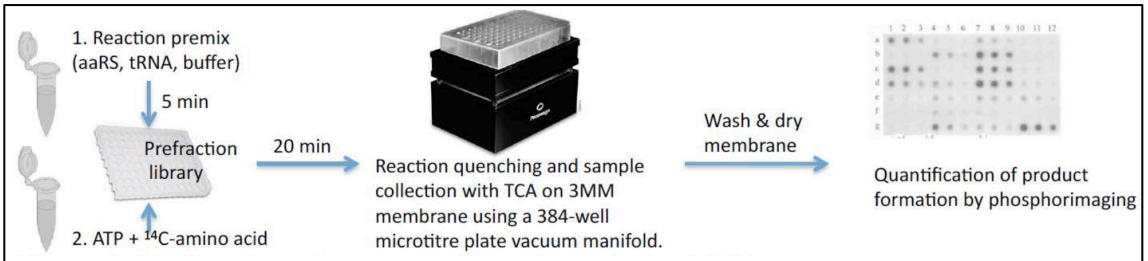

Figure 2. Medium throughput screen for aminoacylation inhibitors.

Screening identified 4 compound pools that inhibited aminoacylation at estimated levels of 10 μM or less. These 4 compound pools were assayed for inhibition of an aminoacylation time course assay, which confirmed inhibitory activity for 3 of the 4. To determine if the MNP leads were inhibiting amino acid activation or tRNA aminoacylation, ATP/^{32}[P]PPi exchange was assayed. Preliminary work confirmed that all 3 candidates inhibited amino acid activation. The candidates were then screened for activity against the human AlaRS enzyme. At 1 μM concentrations, alanine activation by *L. major* AlaRS is inhibited but there is no effect on the activity of the human enzyme (Fig. 3). These findings validate our use of *in silico* system-level modeling coupled to medium throughput *in vitro* screens to identify *Leishmania* aaRSs that are promising targets for the development of antiparasitic therapeutics derived from MNPs.