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Functional and genetic evidence that nucleoside transport is highly conserved in *Leishmania* species: Implications for pyrimidine-based chemotherapy

Khalid J.H. Alzahrani [a, b, 1], Juma A.M. Ali [a, c, 1], Anthonius A. Eze [a, d], Wan Limm Looi [a], Daniel N.A. Tagoe [a, e], Darren J. Creek [f], Michael P. Barrett [a, e], Harry P. de Koning [a, *]

[a] Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom
[b] Department of Clinical Laboratory, College of Applied Medical Sciences, Taif University, Taif, Saudi Arabia
[c] Al Jabal Al Gharbi University, Gharyan, Libya
[d] Department of Medical Biochemistry, College of Medicine, University of Nigeria, Enugu Campus, Enugu, Nigeria
[e] Wellcome Trust Centre for Molecular Parasitology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom
[f] Department of Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

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A B S T R A C T

Leishmania pyrimidine salvage is replete with opportunities for therapeutic intervention with enzyme inhibitors or antimetabolites. Their uptake into cells depends upon specific transporters; therefore it is essential to establish whether various *Leishmania* species possess similar pyrimidine transporters capable of drug uptake. Here, we report a comprehensive characterization of pyrimidine transport in *L. major* and *L. mexicana*. In both species, two transporters for uridine/adenosine were detected, one of which also transported uracil and the antimetabolites 5-fluoruracil (5-FU) and 5F,2′deoxyuridine (5F,2′dUrd), and was designated uridine-uracil transporter 1 (UUT1); the other transporter mediated uptake of adenosine, uridine, 5F,2′dUrd and thymidine and was designated Nucleoside Transporter 1 (NT1). To verify the reported *L. donovani* model of two NT1-like genes encoding uridine/adenosine transporters, and an NT2 gene encoding an inosine transporter, we cloned the corresponding *L. major* and *L. mexicana* genes, expressing each in *T. brucei*. Consistent with the *L. donovani* reports, the NT1-like genes of either species mediated the adenosine-sensitive uptake of [^{3}H]-uridine but not of [^{3}H]-inosine. Conversely, the NT2-like genes mediated uptake of [^{3}H]-inosine but not [^{3}H]-uridine. Among pyrimidine antimetabolites tested, 5-FU and 5F,2′dUrd were the most effective antileishmanials; resistance to both analogs was induced in *L. major* and *L. mexicana*. In each case it was found that the resistant cells had lost the transport capacity for the inducing drug. Metabolomics analysis found that the mechanism of action of 5-FU and 5F-2′dUrd was similar in both *Leishmania* species, with major changes in deoxynucleotide metabolism. We conclude that the pyrimidine salvage system is highly conserved in *Leishmania* species - essential information for the development of pyrimidine-based chemotherapy.

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1. Introduction

Leishmaniasis is a neglected tropical disease caused by at least 21 species of obligate intracellular parasites of the genus *Leishmania*, and is transmitted by more than 30 species of female sand-flies from the genera *Phlebotomus* (Old World) or *Lutzomyia* (New World) (Rodrigues et al., 2014). The disease remains a major cause of morbidity and mortality worldwide that has been classified into three main categories on the basis of clinical symptoms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) (Alvar et al., 2012). Due, in part, to the fact that leishmaniasis with various clinical manifestations is caused by different species, treatment of the disease is still complicated, and often unsatisfactory (Croft and Olliaro, 2011; Sundar and Chakravarty, 2013). Nucleotide metabolism provides

* Corresponding author. Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, Sir Graeme Davies Building, University of Glasgow, 120 University Place, Glasgow G12 8TA, United Kingdom.
E-mail address: Harry.de-Koning@glasgow.ac.uk (H.P. de Koning).
[1] These authors contributed equally to this study.

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Nonstandard abbreviations

5-FURes 5-Fluorouracil resistant cells
5-F2′dURes 5-Fluoro-2′-deoxyuridine resistant cells
5-FU 5-Fluorouracil
5F-2′dUrd 5-Fluoro-2′-deoxyuridine
5F-Urd 5-Fluorouridine
5F-2′dCtd 5-Fluoro-2′-deoxycytidine

many promising therapeutic targets due to the fact that protozoan parasites are unable to synthesize the purine ring *de novo* and rely solely on salvage mechanisms for these important nutrients (De Koning et al., 2005). Nonetheless, purine analog-based chemotherapy has not emerged against kinetoplastid parasites due to redundancy of the interconversion pathways, making inhibition of single enzymes often ineffective (Lüscher et al., 2007a, 2013; Berg et al., 2010a). For the major protozoan pathogens most of the purine nucleoside and nucleobase transporters – which also sometimes exhibit a secondary transport activity for pyrimidines – have been cloned, and all of these transporters belonged to the Equilibrative Nucleoside Transporter (ENT) family (e. g. Vasudevan et al., 1998; Chiang et al., 1999; Burchmore et al., 2003; Sanchez et al., 2004; De Koning et al., 2005; Quashie et al., 2008). In contrast to purines, kinetoplastid parasites are known to possess both salvage and biosynthesis routes for pyrimidines (Wilson et al., 2012; Ali et al., 2013a, 2013b). It has recently been demonstrated that although both functions are important for infection, neither function is absolutely essential (Wilson et al., 2012; Ali et al., 2013b).

Although no single *Leishmania* purine or pyrimidine transporter can be considered essential (Ortiz et al., 2007; Wilson et al., 2012), they are vital links in the delivery of any nucleoside-based chemotherapy to these parasites. The first purine transporter genes to be identified from any parasite were LdNT1 (Vasudevan et al., 1998) and LdNT2, which were cloned from *L. donovani* (Carter et al., 2000). NT1 mediates the uptake of adenosine and the pyrimidine nucleosides uridine and thymidine, whereas NT2 recognizes the 6-oxopurine nucleosides inosine, guanosine, and xanthosine (Carter et al., 2001; Boitz et al., 2012). NT1 and NT2 also serve as the primary conduits for uptake of the antileishmanial adenosine analog tubercidin (7-deazaadenosine) and the antileishmanial inosine analog formycin B, respectively (Vasudevan et al., 1998; Carter et al., 2000), but the pharmacological exploitation of these transporters has not been investigated further. In addition, two purine nucleobase transporter genes have been described in *L. major*, encoding the broad specificity nucleobase transporter LmajNT3 active in promastigotes (Sanchez et al., 2004), and the acid-activated LmajNT4 that is presumed to be mainly functional in the intracellular amastigotes (Ortiz et al., 2009). It is assumed that LmajNT3 and LmajNT4 correspond to the previously characterized nucleobase transport activities in *L. major* promastigotes and *L. mexicana* amastigotes, respectively (Al-Salabi et al., 2003; Al-Salabi and De Koning, 2005), and that there are at a minimum some differences between nucleoside/nucleobase transport in the promastigote and amastigote stages (Ghosh and Mukherjee, 2000; De Koning et al., 2005). Finally, a uracil-specific transporter designated LmU1 was characterized in *L. major* promastigotes (Papageorgiou et al., 2005), but unlike the NT1-4 nucleoside and purine nucleobase transporters, which are members of the Equilibrative Nucleoside Transporter (ENT) family, the gene encoding this transporter is unknown and believed to be of a different gene family (De Koning, 2007).

There remain many caveats to the pharmacological exploitation of the purine and/or pyrimidine salvage pathways for antileishmanial chemotherapy, including whether there are significant differences in nucleoside transport activities between the various *Leishmania* species, what antimetabolites might be transported by *Leishmania* nucleoside transporters, or what metabolic activation steps might follow the uptake of pyrimidine antimetabolites. In this study we address some of these issues and find that (1) nucleoside transport is highly similar in multiple *Leishmania* species; (2) that the substrate binding of the LmajNT1 transporter depends on interactions with the 2-keto and N3 positions of the pyrimidine ring and the 3′ and 5′ hydroxyl groups of the ribose moiety; and that (3) the antimetabolite 5-fluoro-2′-deoxyuridine (5F-2′dUrd) is principally converted to 5F-dUMP, by thymidine kinase, causing the inhibition of thymidylate synthase and the consequent disruption of deoxynucleotide metabolism; 5-fluorouracil is first converted to 5F-2′dUrd and thence to 5F-dUMP.

2. Materials and methods

2.1. Kinetoplastid strains and cultures

Promastigotes of *L. mexicana* (MNY/BZ/62/M379 strain) and *L. major* (Friedlin strain) were grown in HOMEM medium (Gibco, Paisley, UK) (pH 7.4) supplemented with 10% fetal bovine serum (FBS) (Gibco) 1% Penicillin/Streptomycin antibiotic (Gibco) at 25 °C as described (Al-Salabi et al., 2003). The *T. b. brucei* strain B48 (Bridges et al., 2007) was used throughout as the expression system for *Leishmania* transporters, and maintained exactly as described previously in HMI-9 medium with 10% FBS (Gibco) under a 5% CO_2 atmosphere at 37 °C (Vodnala et al., 2013). This strain is derived from a Lister 427 clone from which the aminopurine transporter *TbAT1* has been deleted (Matovu et al., 2003) and was further adapted to high levels of pentamidine, causing it to additionally lose the High Affinity Pentamidine Transporter (HAPT1) activity, encoded by the gene *TbAQP2* (Munday et al., 2014).

2.2. Plasmid construction and transfection

Plasmid construction and transfection was performed according to Munday et al. (2013). The nucleoside transporter genes were isolated from *L. mexicana* and *L. major*. The sequences of these genes displayed a high degree of similarity to the known *L. donovani* nucleoside transporter genes (LdNT1.1, LdNT1.2 and LdNT2; Table S1). Since we could not differentiate between NT1.1 and NT1.2 genes in *L. major* and *L. mexicana* because both genes are highly similar to both LdNT1.1, LdNT1.2, we designated these genes as NT1A and NT1B (Table S1). The primers used in this study (Table S2) were designed to flank the gene of interest, one complementary to the sequence upstream of the 5′ end and the other complementary to the sequence downstream of the 3′ end. All of the nucleoside transporters genes were PCR-amplified from genomic DNA of each strain using the high-fidelity proof-reading polymerase Phusion (New England Biolabs) and cloned into the pGEMTeasy (Promega) vector prior to Sanger sequencing (Source BioScience, Glasgow, UK). For each gene, six independent clones were sequenced and verified as correct. After confirming the identity of each gene, the nucleoside transporter genes (LmajNT1A, LmajNT1B, LmajNT2, LmexNT1A, LmexNT1B, and LmexNT2) were ligated into the expression vector pHD1336 (Biebinger et al., 1997) and then were linearized with *Not*I digestion. All genes were verified by Sanger sequencing, prior to transfection into *T. b. brucei* clone B48. B48 parasites (1×10^7 cells) were washed into Human T Cell buffer for transfection using the desired cassette with an Amaxa Nucleofector using program X-001. Cells were transferred into pre-warmed HMI-

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