Comprehensive characterization of purine and pyrimidine transport activities in *Trichomonas vaginalis* and functional cloning of a trichomonad nucleoside transporter

Manal J. Natto | Yukiko Miyamoto | Jane C. Munday | Tahani A. AlSiari | Mohammed I. Al-Salabi | Neils B. Quashie | Anthonius A. Eze | Lars Eckmann | Harry P. De Koning

---

**Abstract**

Trichomoniasis is a common and widespread sexually-transmitted infection, caused by the protozoan parasite *Trichomonas vaginalis*. *T. vaginalis* lacks the biosynthetic pathways for purines and pyrimidines, making nucleoside metabolism a drug target. Here we report the first comprehensive investigation into purine and pyrimidine uptake by *T. vaginalis*. Multiple carriers were identified and characterized with regard to substrate selectivity and affinity. For nucleobases, a high-affinity adenine transporter, a possible guanine transporter and a low affinity uracil transporter were found. Nucleoside transporters included two high affinity adenosine/guanosine/uridine/cytidine transporters distinguished by different affinities to inosine, a lower affinity adenosine transporter, and a thymidine transporter. Nine Equilibrative Nucleoside Transporter (ENT) genes were identified in the *T. vaginalis* genome. All were expressed equally in metronidazole-resistant and -sensitive strains. Only *TvagENT2* was significantly upregulated in the presence of extracellular purines; expression was not affected by co-culture with human cervical epithelial cells. All *TvagENTs* were cloned and separately expressed in *Trypanosoma brucei*. We identified the main broad specificity nucleoside carrier, with high affinity for uridine and cytidine as well as purine nucleosides including inosine, as *TvagENT3*. The in-depth characterization of purine and pyrimidine transporters provides a critical foundation for the development of new anti-trichomonal nucleoside analogues.

**KEYWORDS**

adenosine transporter, anaerobic parasite, protozoan parasite, purine salvage, trichomonad
1 | INTRODUCTION

Trichomoniasis can be said to be a neglected and underestimated sexually transmitted infection (STI), even though several hundred million people world-wide are infected annually (World Health Organization, 2012). Although symptoms are usually mild or even absent (Edwards et al., 2016), the potential sequelae are severe. These include adverse pregnancy outcomes (Silver et al., 2014) and increased transmission of viral infections as a result of damage to the epithelial layers of the reproductive tracts, including HIV (Kissingler & Adamski, 2013), HSV-2 (Gottlieb et al., 2004; Kissingler, 2015), and HPV (Feng et al., 2018; Raffone et al., 2021), increasing the incidence of AIDS, genital herpes, and cervical neoplasia, respectively. Vertical transmission during birth has also been documented (Peters et al., 2021). Although the infection is particularly ignored in men, as a result of its often-asymptomatic nature, here too are severe long-term risks, including diminished fertility, urethritis, prostatitis and, again, higher risk of HIV infection (Van Gerwen et al., 2021).

The infection is routinely treated with the relatively cheap nitroimidazole drug metronidazole or, in a minority of cases, its more recent derivative tinidazole (Kissingler, 2015). However, reports of clinical resistance to these drugs have increased. Although the level of resistance is generally mild-to-moderate (Marques-Silva et al., 2021) the adverse effects of the drugs, including neurologic maladies, nausea, metallic taste, and hypersensitivity (Muzny et al., 2021) or pyrimidines (Heyworth et al., 1984; Wang & Cheng, 1984) de novo. This makes the parasites vulnerable to inhibitors of key enzymes of the nucleoside salvage pathways and to subversive substrates. Nucleoside analogues with strong antitrichomonal activity have been identified and include formycin A (Munagala & Wang, 2003), adenine arabinoside, 2′- F,2′- deoxyadenosine, and 2′, F,2′- deoxyarabinoadenosine (Shokar et al., 2012). Very recently, we reported on a range of 7- substituted,7- deazaadenosine analogues with mid-nanomolar activity against T. vaginalis in vitro and one compound, 7-(4-Cl-phenyl),7-deazaadenosine, was shown to be efficacious in a murine model of vaginal trichomonad infection (Natto et al., 2021).

Trichomonas vaginalis must salvage extracellular purines and pyrimidines, and expresses 5′-ecto-nucleotidases and NTPDases to hydrolyse nucleotides to their corresponding nucleosides (Menezes et al., 2016; Tasca et al., 2003), which are then internalized by transporters. Most nucleoside antimetabolites rely on those same transporters and thus their substrate selectivity is a key determinant as to which analogues will be efficiently taken up (Campagnaro & De Koning, 2020). In Trypanosoma brucei, for instance, sensitivity to tubercidin (7-deazaadenosine) and cordycepin (3′-deoxyadenosine) depends on the expression of the TbAT1 aminopurine transporter (Geiser et al., 2005), while sensitivity to 7-Br,3′-deoxytubercidin does not (Hulpia et al., 2019). Similarly, sensitivity to tubercidin and formycin B in Leishmania donovani depends on the NT1 and NT2 nucleoside transporters, respectively (Galazka et al., 2006; Vasudevan et al., 2001), and sensitivity to adenine arabinoside (AraA) in Toxoplasma gondii on the TgAT1 adenosine transporter (Chiang et al., 1999). However, nucleoside and nucleobase transport have been poorly studied in T. vaginalis. A single report from 1988 describes two nucleoside transport activities, one that transports all nucleosides and one selective for adenosine, guanosine and uridine (Harris et al., 1988); neither was inhibited by nucleobases, although adenine and guanine have both been shown to be incorporated into the T. vaginalis nucleotide pool (Munagala & Wang, 2003). Yet, the genome of T. vaginalis contains nine genes of the Equilibrative Nucleoside Transporter (ENT) family (TrichDB.org), to which, to date, all protozoan nucleoside and nucleobase transporters have been attributed (Campagnaro & De Koning, 2020; De Koning et al., 2005), although there are some indications that there may be some protozoan nucleobase transport activities that are not encoded by ENT genes (Campagnaro, Alzahrani, et al., 2018; De Koning, 2007). We therefore performed a comprehensive examination of nucleoside and nucleobase transport in T. vaginalis trophozoites with the objective to begin the process of assigning specific transport activities to individual genes, as well assessing their relative levels of expression and their regulation in the presence and absence of substrate and feeder cells. Expression of the individual TvaENT genes in a Trypanosoma brucei cell line allowed us to identify the main high affinity, broad specificity nucleoside transporter, which turned out to be encoded by TvaENT3.

2 | RESULTS

2.1 | Nucleoside transport in T. vaginalis trophozoites

For all permeants (i.e., the substrate for which permeation is being measured, as opposed to inhibitors that are only potential permeants), time course experiments were first undertaken to establish (1) whether uptake could be discerned at a certain radiolabel concentration, and (2) that uptake is linear and through zero over a given period. If these conditions are not met, the parameters determined might be of the rate-limiting step, which could be a metabolic enzyme rather than the transporter for this radiolabel. In all cases, care was taken to use a very low starting permeant concentration so as to obtain the most accurate $K_m$ and $K_i$ values and Hill slopes, and avoid (partial) saturation of very high-affinity transporters as much as possible. This has the added benefit of extending the linear range of uptake as the low rate of permeant entry at those concentrations will not easily saturate the downstream metabolic reactions. For some permeants, the lower concentration limit was determined by detectability of low rates of uptake. Higher radiolabel concentrations were used when specifically probing the existence of lower-affinity transporters and in these cases, again, linearity of uptake...
was first established. Dose–response experiments used incubation times near the middle of the established linear uptake period. Linearity was queried using the Prism runs test for deviation of linearity. Uptake was considered significant if the slope was significantly non-zero (F-test, Prism) and saturability by high levels of unlabeled permeant was tested with the function for significant difference between linear regression lines (F-test). All transporter data presented in figures are single experiments with data points representing the mean and SEM of triplicate determinations (unless otherwise indicated), and are representative of multiple similar repeats. For the transport experiments with *T. vaginalis* trophozoites, close attention was paid to the Hill slope of inhibition experiments, as a Hill slope above −1 (usually between −1 and −0.5) will indicate the uptake of permeants by more than one transporter with non-identical *Kₘ* for the inhibitor. In a complex cellular system with multiple transporters of overlapping substrate selectivity this is an important parameter and a Hill slope that is consistently >−1 permits the plotting of the inhibitor data to a bi-phasic inhibitor model.

### 2.1.1 Adenosine uptake

Submicromolar concentrations of [³H]–adenosine were rapidly taken up by *T. vaginalis* trophozoites. Figure 1a shows that transport of 0.25 μM [³H]-adenosine was linear over 60 s with a rate of 1.15 pmol(10⁷ cells)⁻¹s⁻¹. The uptake was >99% inhibited by 1 mM unlabeled adenosine, and the remaining was not significantly different from zero (p = .35, F-test). The uptake was therefore saturable and most likely carrier-mediated. The inset in Figure 1a is a technical control showing that stopping the uptake with 1 ml of 1 mM ice-cold adenosine did stop all uptake, as previously reported for other cell types.

A series of dose–response inhibition experiments with unlabeled adenosine (Figure 1b) allowed the determination of *Kₚ* as 6.2 ± 0.6 μM (n = 5) and the *Vₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚ¢ an average Hill slope of 1.80 ± 0.04, with values ranging from −1.01 to −0.76 (n = 5), leaving open the possibility of more than one transporter with very similar but non-identical *Kₚ* values. Indeed, the Hill slope for guanosine inhibition of [³H]-adenosine uptake was even more indicative of multiple carriers (−0.68 ± 0.14, n = 3). If taken as monophasic (plotted to a sigmoid equation with variable slope), the apparent guanosine *Kₚ* would be 12.2 ± 2.4 μM (Table 1; Figure 1c). The biphasic inhibition of adenosine uptake was clearest when using inosine as inhibitor (Figure 1c) and here an equation for two binding sites was in all cases the significantly better fit, yielding a high affinity *Kₚ* of 5.2 ± 1.2 μM and a low affinity interaction *Kₚ* of 347 ± 127 μM (n = 3). Thus, the combined inhibitor evidence strongly suggests that two similar high-affinity adenosine transporters are expressed in *T. vaginalis*, with similar affinity for adenosine and guanosine as well as uridine (*Kₚ* = 3.7 ± 1.0 μM; Hill slope = −0.97 ± 0.10; n = 3) and cytidine (*Kₚ* = 179 ± 4.4 μM; Hill slope = −0.73 ± 0.13; n = 3) (Figure 1d). These two adenosine transporters both displayed much lower affinity for thymidine (*Kₚ* = 274 ± 45 μM; n = 3) and the nucleobases hypoxanthine (*Kₚ* = 185 ± 37 μM; n = 2) and adenine (*Kₚ* > 1 mM).

### 2.1.2 Guanosine uptake

The above findings suggest the expression of two similar adenosine transporters with, judging by the Hill slopes, slightly different affinities for adenosine, cytidine, and guanosine. This should be mirrored when using [³H]-guanosine as radiolabel, unless there is a separate adenosine-insensitive guanosine transporter as well. Uptake of 1 μM [³H]-guanosine was linear for at least 60 s and almost completely saturable (98.6% reduced) by 250 μM guanosine (Figure 1e). Sigmoid inhibition plots showed that the Hill slopes for nucleoside inhibitors were indeed indicative of two transporters with somewhat different affinity: −0.67 ± 0.03 (adenosine), −0.78 ± 0.08 (guanosine), and −0.72 ± 0.06 (cytidine), respectively. Using the *EC₅₀* values from plotting to a monophasic sigmoid curve with variable slope apparent *Kₚ* values of 4.1 ± 0.9 μM and 18.6 ± 0.9 μM were obtained for adenosine and cytidine, respectively, and a *Kₚ* of 9.0 ± 0.6 μM was calculated for guanosine (n = 3 for all) (Figure 1f). It is acknowledged that most of these *Kₚ* values are highly likely to be composites of at least two separate transport activities. Also consistent with the [³H]-adenosine transport data was that uridine again displayed a Hill slope close to −1 (−0.91 ± 0.02) and high affinity (*Kₚ* = 7.8 ± 1.3 μM) while the affinity for thymidine was much lower (*Kₚ* = 206 ± 62 μM). Finally, the Hill slope for inosine (−0.75 ± 0.07) was again indicative of at least two separate transport activities, with *Kₚ* = 6.3 ± 2.6 and 146 ± 26 μM, respectively. It can be tentatively concluded on the evidence that guanosine is taken up by the same transporters as adenosine, at least at low permeant concentrations.

### 2.1.3 Uridine uptake

Uptake of 1 μM [³H]-uridine was linear for at least 240 s (*r² = 0.97; not significantly non-linear [runs test, *p* = .14]*) and fully saturable with 1 mM unlabeled uridine (not significantly different from zero [runs test, *p* = .15]) (Figure 2a). Subsequent inhibition plots were performed with an incubation time of 30 s. Unlabeled uridine dose dependently (0.1–2,500 μM) inhibited uptake of [³H]-uridine, with an average Hill slope of −1.04 ± 0.05 (n = 3), yielding an average *Kₚ* of 5.49 ± 1.80 μM and *Vₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚ¢ from the equation for two binding sites (n = 3) (Table 1). Inhibition by adenosine and cytidine yielded *Kₚ* values of 8.16 ± 0.59 μM and 15.6 ± 2.3 μM (n = 3), respectively, with Hill slopes = 0.90 (Figure 2b,c). Inosine inhibition was again clearly biphasic (Figure 2c), with a Hill slope of −0.51 ± 0.02 (n = 3) and plotting to a 2-site sigmoidal curve yielded *Kₚ* values of 3.80 ± 0.90 μM and 231 ± 23 μM.
2.1.4 Cytidine uptake

Uptake of 0.25 μM [3H]-cytidine was linear for at least 5 min ($r^2 = 0.99$), and saturable with 1 mM unlabeled cytidine although the inhibition was not complete (97.0% and significantly non-zero by F-test, $p = 0.029$), indicating the presence of at least one low(er)-affinity carrier for cytidine (Figure 2d). Indeed, inhibition profiles, using just 0.15 μM [3H]-cytidine, consistently showed a mixed high affinity and low affinity component,
|                 | Adenosine (HA) | Guanosine (HA) | Inosine (LA) | Uridine (HA) | Cytidine (HA) | Thymidine (HA) | Adenosine (LA) | Cytidine (LA) | Thymidine (LA) |
|----------------|----------------|----------------|--------------|--------------|---------------|----------------|----------------|---------------|----------------|
| $K_{m,app}$   | 6.2 ± 0.6      | 9.0 ± 0.6      | 196 ± 18     | 5.5 ± 1.8    | 7.2 ± 1.8     | 13.2 ± 0.8     | 59 ± 6         | 348 ± 18      | 470 ± 104      |
| $V_{max}$     | 9.5 ± 2.4      | 0.91 ± 0.25    | 2.6 ± 0.4    | 1.5 ± 0.5    | 0.83 ± 0.06   | 6.7 ± 1.8      | 31 ± 2         | 6.7 ± 1.8     | 9.4 ± 2.5      |
| $V_{max}/K_m$ | 1.5            | 0.10           | 0.013        | 0.27         | 0.12          | 0.05           | 0.52           | 0.019         | 0.020          |
| $K_{i,app}$   |                |                |              |              |               |                |                |               |                |
| Adenosine     | 4.1 ± 0.9      | 6.4 ± 1.7      | 8.2 ± 0.6    | 2.2 ± 0.3    | 39 ± 8        | 357 ± 32       | 73 ± 6.9       |               |               |
| Inosine (HA)  | 5.2 ± 1.2      | 14.1 ± 5.7     | 3.8 ± 0.9    | ND           | 78 ± 15$^c$   | ND             | ND             | ND            |               |
| Inosine (LA)  | 348 ± 127      | ND             | 231 ± 23     | ND           | N/A           | ND             | ND             | ND            |               |
| Guanosine     | 12.2 ± 2.4     | 7.4 ± 2.9      | ND           | 16.7 ± 2.6   | 36 ± 7        | >250           | ND             | ND            |               |
| Uridine       | 3.7 ± 1.0      | 79 ± 1.3       | 3.6 ± 1.1    | 2.0 ± 0.5    | 12 ± 1        | 376 ± 63       | ND             | ND            |               |
| Thymidine     | 274 ± 45       | 206 ± 62       | ND           | ND           | ND            | 557 ± 135      | ND             |               |               |
| Cytidine      | 179 ± 4.4      | 19 ± 0.9       | ND           | 15.6 ± 2.3   | -15$^b$       | 116 ± 5        | ND             |               | -250$^c$      |
| Adenine       | -1,000         | ND             | ND           | ND           | ND            | >1,000         | 1,000          | ND            | ND            |
| Hypoxanthine  | >500           | ND             | ND           | ND           | ND            | >1,000         | 1,000          | ND            | ND            |
| Uracil        | ND             | ND             | ND           | ND           | >1,000        | >10,000        | ND             | ND            |               |

Abbreviations: HA, high affinity; LA, low affinity; ND, not determined; N/A, not applicable.

$^a$Units for $V_{max}$ are pmol(10$^7$ cells)$^{-1}$s$^{-1}$.

$^b$Estimated rough average from three separate experiments yielding biphasic plots.

$^c$Based on monophasic plots. This transport activity has only one $K_{i,app}$ for inosine.
with an average Hill slope of $-0.74 \pm 0.06$ ($n=4$). The higher affinity component was dominant at the chosen radiolabel concentration and resolved to a $K_{\text{m,app}}$ of 7.19 ± 1.77 µM ($n=4$) (Figure 2e) but no consistent estimate for the lower affinity component could be obtained. Dose-response experiments yielded $K_{\text{i,app}}$ values of 1.98 ± 0.50 µM for uridine ($n=3$; Hill slope $-0.88 \pm 0.09$; Figure 2e) and 2.18 ± 0.29 for adenosine ($n=4$; Hill slope $-0.71 \pm 0.06$).

### 2.1.5 | Thymidine uptake

Uptake of 1 µM thymidine was linear for at least 180 s ($r^2 = 0.98$) with a rate of $0.139 \pm 0.008$ pmol(10$^7$ cells)$^{-1}$s$^{-1}$. This was strongly (90.6%, $p < .0001$, $F$-test) but incompletely inhibited by 1 mM unlabeled thymidine (slope significantly non-zero, $p = .020$; Figure 3a). At the lower concentration of 0.125 µM, the averaged rate was
determined as 0.0045 ± 0.0012 pmol(10^7 cells)^-1 s^-1 (n = 4; slope non-zero, p = .0002) and was likewise only partially saturated by 1 mM thymidine (Figure 3b), consistent with a mixed higher and lower affinity uptake of thymidine. At the lower per cent concentration of 125 nM [3H]-thymidine, the higher affinity component was highly dominant and dose-dependent thymidine uptake was near-monophasic (Hill slope = −0.88 ± 0.01, n = 3), yielding a K_{m,app} of 13.2 ± 0.8 µM and a V_{max} of 0.69 ± 0.37 pmol(10^7 cells)^-1 s^-1 (n = 3) (Figure 3c). This transporter appeared not to discriminate between thymidine and uridine (Figure 3c), which displayed a K_{i,app} of 12.2 ± 1.2 µM with a Hill slope of −0.76 ± 0.12 (n = 3), but had lower affinity for purine nucleosides, with moderate affinity for adenosine, guanosine and inosine (K_{i,app} of 39.5 ± 8.1 µM (n = 4), 36.5 ± 7.3 µM (n = 4) and 77.7 ± 15.1 µM (n = 3), respectively) (Table 1). The three purine nucleosides all displayed near-monophasic inhibition with Hill slopes consistently near −1. In contrast, inhibition by cytidine appeared to be biphasic (Figure 3d), although the two individual K_i values could not be determined separately from the available data, as large 95% confidence intervals (95% CI) were returned in each case. From a series of three experiments, they could be estimated as being approximately 15 and 250 µM. None of the nucleobases, adenosine, hypoxanthine, and uracil, inhibited transport of 125 nM [3H]-thymidine by more than 50% at 1 mM (n = 3).

At 1 µM [3H]-thymidine, transport was more clearly a mixture of the higher and lower affinity thymidine flux (Figure 3e), reflected in an average Hill slope of −0.69 ± 0.08 (n = 3). For the lower affinity component, a Michaelis-Menten curve could now be obtained and this yielded a K_{m,app} of 470 ± 104 µM (Inset of Figure 3e). A double reciprocal Lineweaver-Burk plot (Figure 3f) yielded highly similar estimates as well as estimates for the higher affinity K_{m,app}, which averaged −20 µM over 3 experiments. This low-affinity thymidine flux was sensitive to inhibition by adenosine (Figure 3e) with K_{i,app} 73.0 ± 6.9 µM, which indicated that the same transporter could potentially also function as a low affinity adenosine transporter.

2.1.6 | Low affinity adenosine transport

The presence of a low-affinity adenosine transport capability was suspected not just on the basis of inhibition on the low affinity thymidine flux but on the Hill slope for adenosine inhibition of multiple permeants being above −1, including the inhibition curves for 150 nM [3H]-adenosine (section 1). We probed the presence of this low-affinity carrier using a concentration of 20 µM [3H]-adenosine to fully saturate the high-affinity adenosine uptake. The K_{m,app} was determined as 59.4 ± 6.1 µM and V_{max} as 31 ± 2 pmol(10^7 cells)^-1 s^-1 (n = 3; Figure 4a), not statistically different from the K_i value for adenosine on the low-affinity thymidine transport activity (p = .29, Student’s unpaired t-test). Similarly, the K_{i,app} for thymidine, at 557 ± 135 µM (n = 4; Figure 4b) was not significantly different from the thymidine low affinity K_{m,app} (p = .66), an indication that this may be the same transporter. Apparent K_i values for uridine (Figure 4b) and cytidine were 376 ± 63 µM and 116 ± 5 µM, respectively (n = 3). No inhibition above 50% was observed at the highest tested concentrations of guanosine (250 µM, n = 2), adenosine (1 mM, n = 3), hypoxanthine (1 mM, n = 3), or uracil (10 mM, n = 2). We conclude that this is a high capacity transporter with modest affinity for adenosine and low affinity for the pyrimidine nucleosides.

2.1.7 | Inosine transport

Inosine uptake could be measured at 1 µM at a rate of 0.0081 pmol(10^7 cells)^-1 s^-1, which was significantly (p < .0001, F-test) but incompletely inhibited by 1 mM unlabeled inosine (88%, significantly non-zero uptake, p = .0018; Figure 5a). Dose–response experiments yielded a K_{m,app} of 196 ± 18 µM, with a Hill slope −0.95 ± 15, consistent with a single transporter and with the approximately 90% inhibition by 1 mM adenosine in the time course experiment: the V_{max} was 2.25 ± 0.37 pmol(10^7 cells)^-1 s^-1 (n = 3) (Figure 5b). Inhibition by adenosine was monophasic (Hill slope −0.93 ± 0.04) and produced an apparent K_i of 6.36 ± 1.69 µM (n = 3; Figure 5b). K_{i,app} values for guanosine and uridine were 7.41 ± 2.92 µM and 3.60 ± 1.14 µM, respectively (n = 3), both with Hill slopes of approximately −1. This inhibition pattern is very close to that of the high-affinity adenosine transport described above. However, the high-affinity adenosine transport split in two parts, with high and with low affinity for inosine (Figure 1c). The uptake described in this section clearly delineates the transport component with the lower inosine affinity. We did not observe any high-affinity inosine transport, the rate of which may have been insufficient to be clearly observed over the high capacity lower affinity uptake activity.

2.1.8 | Uracil transport

We next probed whether T. vaginalis is able to salvage nucleobases from its environment, starting with uracil. Uptake of 0.5 µM [3H]-uracil was linear for at least 60 s and partially inhibited by 1 mM unlabeled uracil (69.2%. p < .0001) (Figure 5c). The rate in the presence of 1 mM uracil was significantly different from zero (p < .031), apparently indicating low affinity uptake. Accordingly, dose-dependent inhibition of 0.5 µM [3H]-uracil uptake over 20 s yielded mono-phasic curves (Hill slope −1.00 ± 0.09, n = 3) and a K_{m,app} of 257 ± 58 µM, V_{max} 1.58 ± 0.08 pmol(10^7 cells)^-1 s^-1 (Figure 5d). Other pyrimidine nucleobases were similarly able to inhibit this transporter, with K_{i,app} for cytosine, thymine and 5F-uracil at 334 ± 73 µM, 57 ± 8 µM and 265 ± 57, respectively (all n = 3). In contrast, pyrimidine nucleosides (uridine, thymidine, up to 10 mM) and purine nucleosides (adenosine, inosine, up to 1 mM) were unable to inhibit uracil transport, even at high concentrations. We conclude that this is a low-affinity pyrimidine nucleobase transporter. However, in preliminary experiments saturable uptake of cytosine and thymine over 120 s was not significant at 0.25 µM and 0.5 µM, respectively.
Transport of \[^3H\]-guanine was measurable at 2 µM (as lower concentration yielded insufficient signal), producing a rate of 0.017 ± 0.002 pmol(10⁷ cells)⁻¹s⁻¹ (Figure 6a). The slope of the linear regression line was significantly non-zero (p = .0035) and not significantly non-linear (p = .50; \(r^2 = 0.96\)) but limits to guanine solubility did not allow stringent testing of saturability or a dose-dependent inhibition with unlabeled guanine.
Uptake of 0.1 μM [3H]-hypoxanthine was low and non-linear over 60 s (Figure 6b), which seems to indicate that hypoxanthine is not (or very slowly) metabolized by T. vaginalis, as the unmetabolized hypoxanthine equilibrates across the plasma membrane. At 1 μM [3H]-hypoxanthine no specific uptake was observed over 300 s, as the rate was not significantly different in the presence or absence of 1 mM unlabeled hypoxanthine (p = .73; data not shown) and we conclude that, at least in culture, T. vaginalis takes up at most very little hypoxanthine.

In contrast, we observed significant uptake of [3H]-adenine, both at 50 nM (0.00073 pmol(10^7 cells)^−1s^−1; non-zero p < .0001) (Figure 6c) and 1 μM (0.016 pmol(10^7 cells)^−1s^−1; non-zero p < .0001) (Figure 6d). At both label concentrations, saturation by 1 mM unlabeled adenosine was highly significant (p < .0001): uptake of 50 nM [3H]-adenine was 86.7% inhibited by 1 mM unlabeled adenosine (not significantly non-zero, p = .064), whereas uptake of 1 μM [3H]-adenine was inhibited by 73.8% (significantly non-zero, p = .018). The significant uptake at 50 nM pointing to a high-affinity transporter and the incomplete inhibition with 1 mM adenosine pointing to a low affinity transporter, a dose-dependent inhibition experiment was performed with 50 nM of [3H]-adenine, which favors uptake through the high-affinity system, yielding a K_{m,app} of 0.41 ± 0.097 μM and V_{max} of 0.091 ± 0.027 pmol(10^7 cells)^−1s^−1 (n = 4) (Figure 6e). The adenosine transporter displayed only low affinity for adenosine (K_{m,app} 155 ± 48 μM, n = 3) (Figure 6f).

Can be taken up by multiple transporters (Table 1). This is an important conclusion but these observations make construction of an unambiguous model for purine and pyrimidine salvage based on analysis of whole-organism cellular uptake studies challenging. Equally, it is difficult to construct a full model of nutrient salvage by an organism through a reductive approach of characterizing single transporters through heterologous expression alone—clearly, the two approaches are complementary in order to arrive at a full understanding of purine/pyrimidine salvage. Nonetheless, the cells express at least two high affinity, broad specificity transporters for purine and pyrimidine nucleoside, one of which has high affinity for inosine, one low affinity. Neither has significant affinity for nucleobases. Of the potential substrates, adenosine appears to be taken up more efficiently than the others, as expressed by V_{max}/K_{m,app} (Table 1) and uridine appears to be the best pyrimidine substrate. Thymidine was salvaged relatively poorly, and through separate higher and lower affinity transporters, which may make T. vaginalis vulnerable to antifolates. We also found low affinity uptake of cytidine but are unable to say, based on the current data, whether this is a separate transport activity or the same carrier that is responsible for the low affinity thymidine uptake. Separate nucleobase transporters were also found, including a high-affinity and a low-affinity adenosine carrier as well as likely uptake of guanine, although this remains largely uncharacterized. The only pyrimidine nucleobase uptake we observed was low affinity, low capacity uracil uptake.

2.1.10 | Summary of purine and pyrimidine transport by T. vaginalis trophozoites

Our data show that T. vaginalis trophozoites grown in suspension culture express multiple distinct transporters for salvage of nucleosides and nucleobases, with overlapping substrate specificities. It appears that most natural purines and pyrimidine nucleosides can be taken up by multiple transporters (Table 1). This is an important conclusion but these observations make construction of an unambiguous model for purine and pyrimidine salvage based on analysis of whole-organism cellular uptake studies challenging. Equally, it is difficult to construct a full model of nutrient salvage by an organism through a reductive approach of characterizing single transporters through heterologous expression alone—clearly, the two approaches are complementary in order to arrive at a full understanding of purine/pyrimidine salvage. Nonetheless, the cells express at least two high affinity, broad specificity transporters for purine and pyrimidine nucleoside, one of which has high affinity for inosine, one low affinity. Neither has significant affinity for nucleobases. Of the potential substrates, adenosine appears to be taken up more efficiently than the others, as expressed by V_{max}/K_{m,app} (Table 1) and uridine appears to be the best pyrimidine substrate. Thymidine was salvaged relatively poorly, and through separate higher and lower affinity transporters, which may make T. vaginalis vulnerable to antifolates. We also found low affinity uptake of cytidine but are unable to say, based on the current data, whether this is a separate transport activity or the same carrier that is responsible for the low affinity thymidine uptake. Separate nucleobase transporters were also found, including a high-affinity and a low-affinity adenosine carrier as well as likely uptake of guanine, although this remains largely uncharacterized. The only pyrimidine nucleobase uptake we observed was low affinity, low capacity uracil uptake.

2.2 | ENT genes of T. vaginalis

In order to gain further insights into purine and pyrimidine salvage in T. vaginalis, we next probed its genome for genes of the Equilibrative Nucleoside Transporter (ENT) family, which to date is the only gene family linked to nucleoside/nucleobase transport in protozoa (Campagnaro & De Koning, 2020). BLAST and keyword searches of
TrichDB identified nine putative ENT family members, which we designated TvagENT1-9, with 1,011–3,377 bp and 9–11 predicted transmembrane domains (TMDs) (Table 2). Their sequences and a multiple alignment of the nine genes are shown in the Supporting Information (Figure S1). The alignment shows that most of the variation in length is from high variability in the size of the N-terminal domain, most pronounced for TvagENT2, which lacks 135 N-terminal amino acids relative to TvagENT1, including all of TMD1 and part of TMD2, posing the question whether this transporter can be fully functional; it is the only TvagENT with less than 10 TMDs. From a phylogenetic tree (Figure 7) it can be seen that all TvagENTs are more similar to each other than to other protozoan or human ENT genes, and that the TvagENTs essentially split into two clades, with one consisting of TvagENT1, 2, 3, 6, and 8, and the other of TvagENT4, 5, 7, and 9 (Table 3).

2.3 | Expression levels of TvagENTs in trophozoites

The relative level of expression of the TvagENTs in trophozoites was determined using quantitative real time polymerase chain reaction (qRT-PCR), standardized to the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in a panel of three metronidazole-sensitive and three resistant T. vaginalis isolates. The expression pattern of the TvagENTs was very similar in all six isolates, with the highest expression consistently found for TvagENT5 (Figure 8). An exception was TvagENT9, which was expressed at a low level in isolates G3 and S1469 but robustly in all other strains (Figure 8). To explore whether substrate availability had an effect on TvagENT expression, we assessed expression in the presence of 100 µM of adenosine, hypoxanthine, or inosine. For TvagENT3-9, no significant effects on expression were observed but, interestingly,
expression of TvaGENT1 was reduced under these conditions and expression of TvaGENT2 increased, although the latter trend only reached significance for incubation with inosine (Figure 9a). We also tested whether the presence of (and feeding on) HeLa cell layers would change the level of ENT expression, but no significant changes were observed (Figure 9b). Together, these results indicate that the TvaGENT expression is largely constitutive regardless of high substrate availability or the presence of human feeder cells.
The TbAT1-KO strain is particularly useful for the expression vector (Biebinger et al., 1997; Munday et al., 2015) Index described by Rashmi and Swati (2013). The thus optimized Campagnaro & De Koning, 2020; Sanchez et al., 2002), is also capable of recombination (Matovu et al., 2003). Plasmid DNA was then used for expression in the aminopurine transporter TbAT1 had been deleted by homologous recombination (Matovu et al., 2003). Plasmid DNA was then used for expression in the

To start assigning gene identities to the observed nucleoside and nucleobase transport activities of T. vaginalis trophozoites, each individual gene was to be expressed in a T. b. brucei strain, TbAT1-KO, in which we had successfully characterized nucleoside transporters from several other protozoan species including Trypanosoma cruzi (Campagnaro, de Freitas Nascimento, et al., 2018), Trypanosoma congoense (Munday et al., 2013), Leishmania spp (Alzahrani et al., 2017), and Toxoplasma gondii (Campagnaro et al., manuscript in preparation).

As T. b. brucei is able to synthesize its own pyrimidine nucleotides (Ali, Tagoe, et al., 2013), bloodstream forms are known to have a very limited ability to take up any pyrimidines (Ali, Creek, et al., 2013; Ali, Tagoe, et al., 2013; Gudin et al., 2006) except uridine which is efficiently taken up by the U3 transporter (Ali, Tagoe, et al., 2013). However, the P1 transporter sub-family, consisting of at least eight distinct ENT-family genes (Al-Salabi et al., 2007; Campagnaro & De Koning, 2020; Sanchez et al., 2002), is also capable of some thymidine uptake (Ali, Creek, et al., 2013) with affinity of 44 µM (De Koning & Jarvis, 1999), although P1-type transporters are principally high affinity, broad specificity purine nucleoside carriers (Campagnaro & De Koning, 2020; De Koning et al., 1998). For these reasons, the TbAT1-KO strain is particularly useful for the expression and characterization of transporters of uridine and, particularly, cytidine, which was fortuitous as these are high-affinity substrates of the main broad specificity nucleoside transporter(s) of T. vaginalis (Table 1).

The nucleotide sequences of the nine TvagENTs were codon-optimized for T. brucei codon usage bias using the Codon Adaptative Index described by Rashmi and Swati (2013). The thus optimized sequences were custom synthesized and cloned into the pHDI336 expression vector (Biebinger et al., 1997; Munday et al., 2015) for expression in the T. b. brucei strain TbAT1-KO, from which the aminopurine transporter TbAT1 had been deleted by homologous recombination (Matovu et al., 2003). Plasmid DNA was then used to transfect bloodstream forms of T. b. brucei TbAT1-KO followed by selection on blasticidin, and cloned out by limiting dilution. The

| Name      | Accession code | Size (bp/aa) | TM domains |
|-----------|----------------|-------------|------------|
| TvagENT1  | TVAG_166380    | 1,377/458   | 11         |
| TvagENT2  | TVAG_192810    | 1,011/336   | 9          |
| TvagENT3  | TVAG_271560    | 1,275/424   | 10         |
| TvagENT4  | TVAG_441760    | 1,203/400   | 10         |
| TvagENT5  | TVAG_483030    | 1,200/399   | 11         |
| TvagENT6  | TVAG_053320    | 1,266/421   | 10         |
| TvagENT7  | TVAG_101510    | 1,206/401   | 11         |
| TvagENT8  | TVAG_271570    | 1,287/428   | 10         |
| TvagENT9  | TVAG_341290    | 1,227/408   | 10         |

Source: TrichDB.org.

2.4 Heterologous expression of TvagENTs in Trypanosoma brucei brucei TbAT1-KO

To test assigning gene identities to the observed nucleoside and nucleobase transport activities of T. vaginalis trophozoites, each individual gene was to be expressed in a T. b. brucei strain, TbAT1-KO, in which we had successfully characterized nucleoside transporters from several other protozoan species including Trypanosoma cruzi (Campagnaro, de Freitas Nascimento, et al., 2018), Trypanosoma congoense (Munday et al., 2013), Leishmania spp (Alzahrani et al., 2017), and Toxoplasma gondii (Campagnaro et al., manuscript in preparation).

As T. b. brucei is able to synthesize its own pyrimidine nucleotides (Ali, Tagoe, et al., 2013), bloodstream forms are known to have a very limited ability to take up any pyrimidines (Ali, Creek, et al., 2013; Ali, Tagoe, et al., 2013; Gudin et al., 2006) except uracil, which is efficiently taken up by the U3 transporter (Ali, Tagoe, et al., 2013). However, the P1 transporter sub-family, consisting of at least eight distinct ENT-family genes (Al-Salabi et al., 2007; Campagnaro & De Koning, 2020; Sanchez et al., 2002), is also capable of some thymidine uptake (Ali, Creek, et al., 2013) with affinity of 44 µM (De Koning & Jarvis, 1999), although P1-type transporters are principally high affinity, broad specificity purine nucleoside carriers (Campagnaro & De Koning, 2020; De Koning et al., 1998). For these reasons, the TbAT1-KO strain is particularly useful for the expression and characterization of transporters of uridine and, particularly, cytidine, which was fortuitous as these are high-affinity substrates of the main broad specificity nucleoside transporter(s) of T. vaginalis (Table 1).

The nucleotide sequences of the nine TvagENTs were codon-optimized for T. brucei codon usage bias using the Codon Adaptative Index described by Rashmi and Swati (2013). The thus optimized sequences were custom synthesized and cloned into the pHDI336 expression vector (Biebinger et al., 1997; Munday et al., 2015) for expression in the T. b. brucei strain TbAT1-KO, from which the aminopurine transporter TbAT1 had been deleted by homologous recombination (Matovu et al., 2003). Plasmid DNA was then used to transfect bloodstream forms of T. b. brucei TbAT1-KO followed by selection on blasticidin, and cloned out by limiting dilution. The

| Name      | Accession code | Size (bp/aa) | TM domains |
|-----------|----------------|-------------|------------|
| TvagENT1  | TVAG_166380    | 1,377/458   | 11         |
| TvagENT2  | TVAG_192810    | 1,011/336   | 9          |
| TvagENT3  | TVAG_271560    | 1,275/424   | 10         |
| TvagENT4  | TVAG_441760    | 1,203/400   | 10         |
| TvagENT5  | TVAG_483030    | 1,200/399   | 11         |
| TvagENT6  | TVAG_053320    | 1,266/421   | 10         |
| TvagENT7  | TVAG_101510    | 1,206/401   | 11         |
| TvagENT8  | TVAG_271570    | 1,287/428   | 10         |
| TvagENT9  | TVAG_341290    | 1,227/408   | 10         |

Source: TrichDB.org.
In this study, we have attempted to map out the nucleoside and nucleobase transport activities of *T. vaginalis*. The effort is complicated as trophozoites express multiple such transporters, with overlapping substrate selectivities and a wide range of substrate affinities. This makes it hard to characterize single carriers by kinetic analysis of whole-cell transport assays only. Yet, the whole-cell analysis helped to establish which purines and pyrimidines are salvaged by the parasite and with what affinities and rates. The efficiency of uptake, defined as \( V_{\text{max}}/K_m \), was highest for high-affinity adenosine transport, at 1.5, compared to 0.10 for high-affinity guanosine uptake, making adenosine the preferred purine substrate. Uptake of adenine was relatively weak with low \( V_{\text{max}} \) and an efficiency of 0.22. For pyrimidines the highest affinity and efficiency was observed for uridine followed by cytidine and then thymidine. Although uracil uptake is robust in kinetoplastid parasites (Alzahrani et al., 2017; De Koning & Jarvis, 1998; Papageorgiou et al., 2005) and *Toxoplasma gondii* (Natto and De Koning, unpublished), as well as other microbes including *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *E. coli* (Campagnaro & De Koning, 2020; De Koning & Diallinas, 2000), uptake of uracil (efficiency 0.0061) and other pyrimidine nucleobases was marginal.
The observations of efficient uptake of purine nucleosides rather than nucleobases are largely consistent with observations and models from before the T. vaginalis genome was published (Carlton et al., 2007). Hypoxanthine has been reported to not or barely be incorporated into the T. vaginalis nucleotide pool (Heyworth et al., 1982; Munagala & Wang, 2003) and indeed we found little or no uptake of this nucleobase. However, adenine and guanine could be salvaged and incorporated into nucleotides (Munagala & Wang, 2003). Miller and Lindstead (1983) reported that they were unable to detect any phosphoribosyltransferase activity with any of the purine nucleobases, only with uracil, but the same authors did find that the purine nucleobases, including hypoxanthine and xanthine, could be converted by cell-free extracts of T. vaginalis to nucleosides using purine nucleoside phosphorylase (PNP). This enzyme has been isolated from extracts (Miller & Miller, 1985) and cloned (Munagala & Wang, 2002), and the structure has been elucidated (Rinaldo-Matthis et al., 2007). For the pyrimidine nucleobase, a uracil phosphoribosyltransferase activity was reported in T. vaginalis extracts by Miller and Lindstead (1983) but not by Wang and Cheng (1984), who attributed the incorporation of uracil to a uridine phosphorylase. We were unable to identify a candidate gene for uracil phosphoribosyltransferase in the T. vaginalis genome but did identify a candidate uridine phosphorylase (XP_001323814.1; TrichDB).

We observed very little overlap between nucleoside transporters and nucleobase transporters in T. vaginalis, as is generally the case for protozoan purine transporters (Campagnaro & De Koning, 2020; De Koning et al., 2005), although there are some notable exceptions like the TbAT1 adenosine/adenine transporter of T. b. brucei (De Koning & Jarvis, 1999), the UUT1 uridine-uracil transporter of Leishmania major (Alzahrani et al., 2017) and the Plasmodium falciparum NT1 carrier that transports both hypoxanthine and adenine (Quashie et al., 2008). The strict separation of nucleoside and nucleobase transport activities in T. vaginalis appears to fit well with the organism’s overall preference for nucleosides over nucleobases. Indeed, it is possible that the nucleobase carriers have more of a sensory/regulatory role than one of providing significant amounts of nutrients, judging by their very low V\textsubscript{max} and/or efficiency, and the observation that the presence of hypoxanthine influenced the expression of some of the TvigENTs, especially TvigENT1.

The most active nucleoside transporter in T. vaginalis trophozoites appears to most efficiently transport adenosine, in keeping with adenosine in the form of ATP being most likely the most abundant purine available to it for salvage and T. vaginalis expressing a number of apyrases and ecto-phosphohydrolases to convert extracellular nucleotides to the corresponding nucleosides (de Aguiar Matos et al., 2001; de Jesus et al., 2006). The same transporter also displayed high affinity for uridine, guanosine and cytidine (Table 1). Our analysis strongly suggests there are at least two such transporters expressed in trophozoites, one with high affinity for inosine, here provisionally designated NT-a until the gene ID is definitively known, and one with low affinity, NT-b. In addition, we found a lower affinity adenosine >cytidine > uridine >thymidine transporter (NT-c), and a thymidine transporter with similar affinity for uridine and moderate affinity for adenosine and guanosine (NT-d). Together, our analysis suggests there are at least two such transporters expressed in trophozoites, as is generally the case by the NT-b activity as the $K_i$ values for adenosine, guanosine, and uridine are all very close to those obtained with $[^3]$H-adenosine. Similarly, high affinity uptake of uridine and cytidine appears to be mediated jointly by NT-a and NT-b. The low affinity $[^3]$H-thymidine uptake could well be mediated by NT-c, considering the adenosine $K_i$ value is highly similar to the $K_m$ for NT-c and the NT-c $K_i$ for thymidine is similar to the $K_m$ for low affinity thymidine uptake.

Although our comprehensive transporter characterizations give a well-supported view of nucleoside and nucleobase uptake in T. vaginalis, the data cannot resolve how many and which ENT-family (or other gene family) genes are involved in the various transport processes. As a complement to the functional transport studies in trophozoites, we therefore identified the TvigENT genes from the

| TvigENT1 | TvigENT2 | TvigENT3 | TvigENT4 | TvigENT5 | TvigENT6 | TvigENT7 | TvigENT8 | TvigENT9 |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 100      | 42.2     | 39.6     | 28.4     | 26.8     | 49.9     | 24.2     | 41.2     | 22.2     |
| 42.2     | 100      | 83.2     | 22.4     | 26.6     | 56.6     | 29.2     | 86.4     | 24.8     |
| 39.6     | 83.2     | 100      | 24.0     | 25.9     | 54.3     | 28.4     | 77.7     | 25.1     |
| 28.4     | 22.4     | 24.0     | 100      | 53.7     | 25.2     | 38.6     | 23.5     | 42.0     |
| 26.8     | 26.6     | 25.9     | 53.7     | 100      | 26.7     | 44.8     | 26.8     | 42.6     |
| 49.9     | 56.6     | 54.3     | 25.2     | 26.3     | 100      | 26.2     | 52.2     | 24.7     |
| 24.2     | 29.2     | 28.4     | 38.6     | 44.8     | 26.2     | 100      | 26.7     | 72.8     |
| 41.2     | 86.4     | 77.7     | 23.5     | 26.8     | 52.2     | 26.7     | 100      | 23.9     |
| 22.2     | 24.8     | 25.1     | 42.0     | 42.6     | 24.7     | 72.8     | 23.9     | 100      |

Note: https://blast.ncbi.nlm.nih.gov/Blast.cgi.
TrichDB database, had them synthesized in the *Trypanosoma brucei* codon preference and introduced them individually in the TbAT1-KO strain of *T. b. brucei*. The new strains were screened for the transport of pyrimidine nucleosides and uptake of cytidine was the most effective tool, identifying TvagENT3 as the carrier that mediated its uptake most strongly, at conditions designed to identify the high-affinity flux. The inhibition profile aligned quite closely to that of NT-a, with high affinity for inosine, adenosine, guanosine, and uridine but not thymidine or nucleobases. TvagENT3 is functionally related to the broad specificity *T. brucei* P1-type transporters (De Koning & Jarvis, 1999), although the relatively high affinity for cytidine may be unique amongst protozoan transporters characterized to date, and like P1, its broad specificity/high affinity should be valuable in targeting cytotoxic nucleoside analogues to the *T. vaginalis* interior (Geiser et al., 2005; Hulpia et al., 2019; Ranjarbain et al., 2017). Indeed, we have very recently reported the identification of a series of strongly antitrichomonal nucleoside analogues (Natto et al., 2021) and other nucleoside analogues with activity against this parasite have been reported (Munagala & Wang, 2003; Shokar et al., 2012).
After TvagENT3, TvagENT6 displayed the most significant cytidine uptake as well as the most robust uridine uptake, and we used uptake of \[^{3}H\]-uridine in the presence of 250 \(\mu\)M uracil for a partial characterization of this carrier. In this case, the characterization indicated alignment with the NT-\(c\) activity observed in trophozoites, as the affinities for adenosine and uridine, in particular, are very similar. Definitive assignment any gene ID to a specific transport activity in \(T.\) vaginalis trophozoites, however, will require further studies, such as gene deletions or the identification of sufficiently specific inhibitors.

In this work, we present a model of multiple high- and low-affinity, broad specificity nucleobase transporters and separate nucleobase transporters in \(T.\) vaginalis. Only a single study of purine or pyrimidine transport in this species has previously been reported (Harris et al., 1988). That study did not address nucleobase uptake but did describe the expression of at least two high affinity, broad specificity nucleoside transporters with overlapping substrate specificity. Indeed, their \(K_{m}\) values for adenosine (3.9 \(\mu\)M), guanosine (13.9 \(\mu\)M), and uridine (2.5 \(\mu\)M) are remarkably similar to those we report here for the NT-\(a\) activity (6.2, 12.2, and 3.7 \(\mu\)M, respectively using \[^{3}H\]-adenosine as substrate). In addition, we identified three additional nucleoside transport activities and three nucleobase transport activities in trophozoites and identified the genes encoding two of the main nucleoside transporters through heterologous expression. This study thus presents the most complete model yet of nucleoside metabolism in the parasite.

### EXPERIMENTAL PROCEDURES

#### 4.1 Parasite strains and cell cultures

The following \(T.\) vaginalis strains were used: Metronidazole-sensitive G3 (ATCC PRA-98), F1623 (Brown et al., 1999), and
S1469 R88 (kindly provided by Dr. Evan Secor, US Centers for Disease Control and Prevention); metronidazole-resistant B7268 (Bradic et al., 2017), LA1 (Goldman et al., 2009) and R88 (kindly provided by Dr. Secor). Trophozoites were grown in vitro, as described, at 37°C in modified Diamond’s media (MDM) with 10% heat-inactivated horse serum (HIHS; Gibco Life Technologies), with the pH adjusted to 6.3 – 6.4 (Natto et al., 2015). The MDM composition was 20 g/L trypticase peptone (BD Biosciences, Sparks, USA), 10 g/L yeast extract (Formedium Ltd., UK), 5 g/L maltose monohydrate (Sigma, U.K.), 1 g/L L-ascorbic acid (Sigma), 1 g/L KCl, 1 g/L KHCO₃, 1 g/L KH₂PO₄, 0.5 g/L K₂HPO₄, and 0.1 g/L FeSO₄·7H₂O (Natto et al., 2012). Culture flasks were completely filled and tightly capped to maintain anaerobic conditions. Cell density was determined using a haemocytometer (Camlab, Cambridge, U.K.) and phase-contrast microscopy at 40x magnification, or a Beckman Coulter Z2 particle counter.
Human cervix epithelial HeLa (ATCC CCL-2) cells were grown in DMEM medium containing 10% FBS and 5% CO2. For co-culture of T. vaginalis with HeLa cells, subconfluent HeLa monolayers were exposed to doses of 10^6 G3 trophozoites at a 1:1 ratio in 75% DMEM and 25% MDM under 5% CO2 atmosphere at 37°C for 6 hr. Total cellular RNA (attached trophozoites and HeLa cell) was harvested from the co-cultures for subsequent analysis. HeLa cells alone and trophozoites alone were used as controls.

4.2 | Transport assays

Transport assays with T. vaginalis trophozoites and T. brucei bloodstream forms were performed identically and essentially as described previously for T. brucei (Wallace et al., 2002) and Leishmania promastigotes (Alzahrani et al., 2017). Briefly, cells of either species were grown in larger culture flasks (75 cm² U-shaped Canted Neck Culture Flask with Vent Cap (Corning) for T. brucei, 25 ml glass Universals, completely filled with medium for T. vaginalis), harvested and washed twice by centrifugation (10 min, 1,000 × g, room temperature) into an assay buffer (AB (Campagnaro, Alzahrani, et al., 2018)) and resuspended at 10^8 cells/mL. Aliquots of 100 µl (i.e. 10^7 cells) were incubated for predetermined times with 100 µl of a solution containing [3H]-labeled nucleoside or nucleobase plus a competitive inhibitor, depending on the experiment, each at 2x the final concentration, on top of an oil layer (7:1 v/v mix of di-n-butylphthalate (DBH) and light mineral oil (Sigma)) in a microfuge tube. To stop the uptake of radiolabel, 1 ml of an ice-cold solution of unlabeled permeant, usually at 1 mM or 2.5 mM, was added, followed by immediate centrifugation of the cells through the oil layer (90 s, 13,000 × g). The tubes were flash-frozen in liquid nitrogen and the tips of the tubes, containing the cell pellet cut off into scintillation tubes. Cell were lysed with 2% SDS for at least 1 hr under agitation on a rocking platform, and after scintillation fluid (Scintlogic U, Lablogic) was added, the vials were shaken and left to stand overnight in the dark. Radioactivity
was determined using a Hidex 300SL scintillation counter and background radiation (from vials to which no radiolabel was added) was subtracted. Radiolabel associated with the cell pellet but not internalized was also subtracted, and defined as radioactivity in the pellet after incubation of the same number of cells in the presence of saturating levels of non-radiolabeled permeant. All assay points were determined in triplicate in each experiment. Results were plotted and analyzed with GraphPad Prism (versions 8 and 9), using the inbuilt statistics packages to determine linearity, difference between two lines, goodness of fit, and significances from zero slope etc. Time courses were commonly plotted by linear regression and inhibition experiments used incubation times well within the linear phase of uptake. Dose-response inhibition experiments for the determination of 50% inhibition concentrations (IC_{50}) were plotted to a 4-parameter sigmoid curve with variable slope and Km values through a Michaelis-Menten saturation plot (V_p = V_{max} × [S]/(K_m + [S]) in which [S] is the substrate concentration) or double reciprocal Lineweaver-Burk plot. Plots typically contained 6–8 points over the relevant range plus a zero-inhibitor control, all in triplicate. Km values were calculated from IC_{50} values through a Cheng-Prusoff equation (Cheng & Prusoff, 1973). In all cases, care was taken to use a very low permeant concentration so as to obtain the most accurate Km and Ki values and Hill slopes, and avoid saturation of very high-affinity transporters. This has the added benefit of extending the linear range of uptake as the low rate of permeant entry at those concentrations will not easily saturate the downstream metabolic reactions. The following radiolabels were used: [5-^3H]-cytidine from American Radiolabeled Chemicals (ARC), 20 Ci/mmol; [5^-3H]-uridine from ARC, 60 Ci/mmol; [methyl-^3H]-thymidine from ARC, 71.7 Ci/mmol; [^3H]-adenosine from Amersham, 27.0 Ci/mmol; [^3H]-inosine from Moravek, 7.9 Ci/mmol; [^3H]-guanosine from Moravek, 12.9 Ci/mmol; [^3H]-guanine from Moravek, 8.4 Ci/mmol; [^3H]-adenine from Moravek, 27 Ci/mmol; [^3H]-hypoxanthine from Amersham, 19 Ci/mmol; [^3H]-uracil from Perkin-Elmer 30.3 Ci/mmol; [^3H]-cytosine from Moravek, 25.6 Ci/mmol; [^3H]-thymine from Moravek, 56.3 Ci/mmol.

**4.3 | RNA extraction and real-time RT-PCR of T. vaginalis**

Total cellular RNA was extracted from lysates made with TRIzol (Invitrogen, 1 ml per 10^7 cells or confluent 10 cm^2 flask) and purified with DirectZol kit (Zymo) according to the manufacturer’s protocol. RNA was quantified by absorbance at 260 nm. RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and qRT-PCR analysis was done with PerfeCTa SYBR Green FastMix (Quantabio) in a Step-one real-time PCR machine (Applied Biosystems) using the primer pairs listed in Supplement table. Relative changes in target mRNA levels were calculated by the 2ΔΔCt method (Dann et al., 2015), with GAPDH mRNA as the reference standard and normalized to ENTP mRNA levels. Primers for GAPDH: forward 5'-GCCCGCAAGCTCTATCCAAAG-3'; reverse primer 5'-CG GCCACCGATTGACTTAAC-3'.
4.4 | Expression of TvagENTs in T. brucei

The full-length coding sequences of 9 TvagENT genes (Table 2) were retrieved from TrichDB.org by BLASTP and keyword searches. The nucleotide sequences were optimized for expression in T. brucei using a codon optimization algorithm at https://www.idtdna.com/CodonOpt (Rashmi & Swati, 2013). The thus optimized sequences were synthesized by BaseClear BV, except for TvagENT8, which was synthesized by Genewiz and all delivered in the vector pUC57-Amp. Each gene was amplified by PCR using primers that introduced 5'–HindIII and 3'–BamHI restriction sites (Table S2); the same enzymes were used to digest pHDI336 (Biebing et al., 1997) and the PCR products were ligated in using T4 DNA ligase (NEB). The constructs were used to transform E. coli XL1-blue competent cells (Agilent), which were then grown on ampicillin agar plates. Colonies were screened by PCR using the vector specific primer HDK528 (CTCTAGAGGATCCTATGCGTGACTGAGTGGCC) and the relevant reverse primer for the inserted gene (Table S2) and, if yielding the correct size of amplicon, further verified by Sanger sequencing (Source BioScience, Nottingham, UK). Plasmid DNA from selected colonies was linearized by digestion with NotI before transfection into T. brucei strain TbAT1-KO (Matovu et al., 2003) using an Amaxa Nucleofector, program X-001 (Burkard et al., 2007). Transfectants were grown and cloned out, by limiting dilution, in complete HMI-11/FBS media containing 5 μg/ml blasticidin S. Correct integration of the expression cassettes was analyzed by PCR.

4.5 | qRT-PCR of TvagENTs expressed in T. brucei

Cells were harvested from 11 ml flasks, each containing ~3 × 10⁶ cells/ml for one clonal line expressing a TvagENT in T. brucei TbAT1-KO, by centrifugation at 2,000 RPM for 10 min. The supernatant was discarded and RNA was isolated using the NucleoSpin® RNA isolation kit in accordance with the manufacturer’s instructions. RNA was eluted off the column with 15 μl RNAase-free water and the concentration determined using a Nanodrop spectrophotometer.

cDNA was produced using the Precision nanoScript2 Reverse Transcription kit (Primer Design). Primers were annealed to 9 μl of RNA sample using 0.5 μl oligo-dT primers and 0.5 μl Random nonamer primers for 5 min at 65°C and subsequently cooled on ice for 5 min. The extension master mix consisted of 5 μl nanoScript2 4x buffer, 1 μl 10 mM dNTP, 2.5 μl RNAse free water, and 1 μl nanoScript2 enzyme. This was added to 10 μl RNA in water, briefly vortexed, vortexed and incubated at room temperature for 5 min before extending at 42°C and a heat denaturation step at 75°C for 10 min.

qPCR primers (Table S1) were designed using Primer Express 3000 software, selecting for primers with >50% GC content and the forward and reverse being of similar length. Primers were diluted in RNase free water to 100 μM stock concentration and stored at −20°C. The forward and reverse primers were then combined at a concentration of 3 μM each. 15 μl of master mix, consisting of 10 μl PrecisionPLUS, 1 μl primer mix and 4 μl RNase free water, was added to wells in a 96-well plate. cDNA was diluted to 4 ng/μl using RNase free water and 5 μl of cDNA from each clone was added to the master mix. Plates were designed to have three repeats for the TvagENT and the GPI8 control gene, and four repeats for the empty vector control as well as four no cdNA controls for both the TvagENT and the GPI8 primers. The qPCR was run on a 7500 Real Time PCR system for 40 cycles in accordance with the PrecisionFAST Mastermix protocol and analyzed using SDS software.

4.6 | Data analysis

All values for IC₅₀, Kₘ and Vₘₐₓ are presented as means ± SEM of at least three independent determinations in triplicate but individual plots shown as Figures are always single experiments with data points being the means ± SEM of the triplicates. Hill slopes were calculated by plotting dose-response inhibition data to an equation for a sigmoid plot with variable slope (4 parameter) in GraphPad Prism. Rates of transport in timecourses were calculated by linear regression in Prism and the in-build statistical analysis were used to determine deviation from linearity, significance of deviation from zero slope (flat line), and significance of difference in slope between two lines within the same experiment.

For mRNA measurements, experiments were repeated at least three times, fold changes were expressed as mean and SEM of log10-transformed expression values. Significance was evaluated by ANOVA with Dunnett’s post-hoc test using Prism (Graphpad software).

ACKNOWLEDGMENTS

The authors thank Professor Jeremy Mottram (University of York, UK) and Dr. Evan Secor for gifts of T. vaginalis strains. This research was supported by a personal fellowship to MJN from the Saudi Arabian Ministry of Education, a studentship to TAA from the Saudi Arabian Ministry of Education, MIA by a studentship from the Libyan government, NBQ by a Getfund studentship from the Ghanaian government, a project grant from the Medical Research Council (84733), and National Institutes of Health grants DK120515 and AI158612.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

Harry P. De Koning contributed to concept and overall strategy. Harry P. De Koning and Lars Eckmann contributed to supervision. Manal J. Natto, Yukiko Miyamoto, Jane C. Munday, Tahani A. AlSiari, Mohammed I. Al-Salabi, Neils B. Quashie, and Anthonius A. Eze contributed to experiments. Harry P. De Koning and Lars Eckmann contributed to manuscript writing.

DATA AVAILABILITY STATEMENT

Sequences used are those of the publicly available database trichDB. All other relevant data are presented in the main text and Supporting Information sections of the paper.
Munday, J.C., Rojas López, K.E., Eze, A.A., Delespaux, V., Van Den Abbeele, J., Rowan, T. et al. (2013) Functional expression of TcoAT1 reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake. International Journal for Parasitology Drugs and Drug Resistance, 3, 69–76. https://doi.org/10.1016/j.ijpddr.2013.01.004

Munday, J.C., Tagoe, D.N.A., Eze, A.A., Krezdorn, J.A., Rojas López, K.E. et al. (2015) Functional analysis of drug resistance-associated mutations in the Trypanosoma brucei adenosine transporter 1 (TbAT1) and the proposal of a structural model for the protein. Molecular Microbiology, 96, 887–900.

Muzny, C.A., Van Gerwen, O.T. & Kissinger, P. (2020) Updates in Trichomonas treatment including persistent infection and 5-nitroimidazole hypersensitivity. Current Opinion in Infectious Disease, 33, 73–77. https://doi.org/10.1097/QCO.0000000000000618

Natto, M.J., Eze, A.A. & De Koning, H.P. (2015) Protocols for the routine screening of drug sensitivity in the human parasite Trichomonas vaginalis. Methods in Molecular Biology, 1263, 103–110.

Natto, M.J., Hupla, F., Kalkman, E.R., Baillie, S., Alhejeli, A., AlSiari, T. et al. (2021) Deazapurine nucleoside analogues for the treatment of Trichomonas vaginalis. ACS Infectious Diseases, 7, 1752–1764. https://doi.org/10.1021/acsinfecdis.1c00075

Papageorgiou, I.G., Yakob, L., Al-Salabi, M.I., Dialinas, C., Soteriadou, K. & De Koning, H.P. (2005) Identification of the first pyrimidine nucleo-base transporter in Leishmania: similarities with the Trypanosoma brucei U1 transporter and antileishmanial activity of uracil analogues. Parasitology, 130, 275–283. https://doi.org/10.1017/S0031182004006262

Peters, R.P., Feucht, U.D., De Vos, L., Ngwepe, P., McIntyre, J.A., Klauser, J.D. et al. (2021) Mother-to-child transmission of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis in HIV-infected pregnant women in South Africa. International Journal of STD & AIDS, 32, 799–805. https://doi.org/10.1177/0956462421990218

Quashie, N.B., Dorin-Semblat, D., Bray, P.G., Biagini, G.A., Doerig, C., Ranford-Cartwright, L.C. et al. (2008) A comprehensive model of purine uptake by the malaria parasite Plasmodium falciparum: Identification of four purine transport activities in intraerythrocytic parasites. Biochemical Journal, 411, 287–295. https://doi.org/10.1042/BJ20071460

Raffone, A., Travaglini, A., Angelino, A., Esposito, R., Orlandi, G., Toscano, P. et al. (2021) Gardnerella vaginalis and Trichomonas vaginalis infections as risk factors for persistence and progression of low-grade precancerous cervical lesions in HIV-1 positive women. Pathology Research and Practice, 219, 153349. https://doi.org/10.1016/j.prp.2021.153349

Ranjbarian, F., Vodanla, M., Alzahrani, K.J.H., Ebloma, G.U., De Koning, H.P. & Hofer, A. (2017) 9-[(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)] is a potent antityrpanosomal adenosine analogue that circumvents transport-related drug resistance. Antimicrobial Agents and Chemistry, 61, e02719–e2816.

Rashmi, M. & Swati, D. (2013) Comparative genomics of trypanosoma-tid pathogens using codon usage bias. Bioinformation, 9, 912–918. https://doi.org/10.6026/97320630009912

Rinaldo-Matthis, A., Wing, C., Ghanem, M., Deng, H., Wu, P., Gupta, A. et al. (2007) Inhibition and structure of Trichomonas vaginalis purine nucleoside phosphorylase with picomolar transition state analogues. Biochemistry, 46, 659–668.

Sanchez, M.A., Tryon, R., Green, J., Boor, I. & Landfair, S.M. (2002) Six related nucleoside/nucleobase transporters from Trypanosoma brucei exhibit distinct biochemical functions. Journal of Biological Chemistry, 277, 21499–21504. https://doi.org/10.1074/jbc.M202319200
Shokar, A., Au, A., An, S.H., Tong, E., Garza, G., Zayas, J. et al. (2012) S-Adenosylhomocysteine hydrolase of the protozoan parasite Trichomonas vaginalis: potent inhibitory activity of 9-(2-deoxy-2-fluoro-D- arabinofuranosyl)-adenine. Bioorganic and Medicinal Chemistry Letters, 22, 4203–4205. https://doi.org/10.1016/j.bmcl.2012.03.087

Silver, B.J., Guy, R.J., Kaldor, J.M., Jamil, M.S. & Rumbold, A.R. (2014) Trichomonas vaginalis as a cause of perinatal morbidity: A systematic review and meta-analysis. Sexually Transmitted Diseases, 41, 369–376. https://doi.org/10.1097/OLQ.0000000000000134

Tasca, T., Bonan, C.D., Carli, G.A., Battastini, A.M. & Sarkis, J.J. (2003) Characterization of an ecto-5'-nucleotidase (EC 3.1.3.5) activity in intact cells of Trichomonas vaginalis. Experimental Parasitology, 105, 167–173. https://doi.org/10.1016/S0020-7519(01)00191-6

Van Gerwen, O.T., Camino, A.F., Sharma, J., Kissinger, P.J. & Muzny, C.A. (2021) Epidemiology, natural history, diagnosis, and treatment of Trichomonas vaginalis in men. Clinical Infectious Diseases, 73, 1119–1124.

Vasudevan, G., Carter, N.S., Drew, M.E., Beverley, S.M., Sanchez, M.A., Seyfang, A. et al. (1998) Cloning of Leishmania nucleoside transporter genes by rescue of a transport-deficient mutant. Proceedings of the National Academy of Sciences, 95, 9873–9878. https://doi.org/10.1073/pnas.95.17.9873

Wallace, L.J., Candlish, D. & De Koning, H.P. (2002) Different substrate recognition motifs of human and trypanosome nucleobase transporters: selective uptake of purine antimetabolites. Journal of Biological Chemistry, 277, 26149–26156. https://doi.org/10.1074/jbc.M202835200

Wang, C.C. & Cheng, H.-W. (1984) Salvage of pyrimidine nucleosides by Trichomonas vaginalis. Molecular and Biochemical Parasitology, 10, 171–184. https://doi.org/10.1016/0166-6851(84)90005-7

World Health Organization. (2012). Global incidence and prevalence of selected curable sexually transmitted infections – 2008. ISBN 978 92 4 150383 9. Available from: https://www.who.int/reproductivehealth/publications/rtis/stisestimates/en/

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Natto, M.J., Miyamoto, Y., Munday, J.C., AlSiari, T.A., Al-Salabi, M.I., Quashie, N.B., et al (2021) Comprehensive characterization of purine and pyrimidine transport activities in Trichomonas vaginalis and functional cloning of a trichomonad nucleoside transporter. Molecular Microbiology, 116, 1489–1511. https://doi.org/10.1111/mmi.14840