A Leishmania major Nucleobase Transporter Responsible for Allopurinol Uptake Is a Functional Homolog of the Trypanosoma brucei H2 Transporter.

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ABSTRACT
Nucleobase transporters play an important role in the physiology of protozoan parasites, because these organisms are purine auxotrophs and rely entirely on salvage of these vital compounds. Purine transporters have also been shown to mediate the uptake of important antiparasitic drugs. In the current study, we investigated the uptake of [3H]adenine, [3H]hypoxanthine, and [3H]allopurinol, an antileishmanial hypoxanthine analog, by Leishmania major. These compounds were all taken up by a single high-affinity transporter, LmNBT1, with $K_m$ values of 4.6 ± 0.9, 0.71 ± 0.07, and 54 ± 3 $\mu$M, respectively. Guanine and xanthine fully inhibited [3H]adenine transport, with $K_i$ values of 2.8 ± 0.7 and 23 ± 8 $\mu$M. Using purine analogs, an inhibitor profile for LmNBT1 was obtained, which allowed the construction of a quantitative model for the interactions between the transporter binding site and the permeant. The model predicts that hypoxanthine was bound through hydrogen bonds to N(1)H, N5, N7, and N(9)H of the purine ring, with a total Gibbs free energy of ~39.5 kJ/mol. The interactions with adenine were similar, except for a weak hydrogen bond to N1 (unprotonated in adenine). The predicted mode of substrate binding for LmNBT1 was almost identical to that for the Trypanosoma brucei H2 (TbH2) transporter. It is proposed that the architecture of their respective binding sites is very similar and that LmNBT1 can be named a functional homolog of TbH2.

Human leishmaniasis is still mostly treated with pentavalent antimony drugs such as pentostam (sodium stibogluconate) or glucantime (N-tavalent antimony drugs such as pentostam (sodium stibogluconate)) or glucantime (N-tavalent antimony drugs such as pentostam (sodium stibogluconate)) or glucantime (N-tavalent antimony drugs such as pentostam (sodium stibogluconate)), despite the severe side effects and emerging resistance (Croft, 2001; Sundar, 2001). Among the few alternatives is the hypoxanthine analog allopurinol, which, either alone or in combination with other drugs, has proved effective against cutaneous (Martinez and Marr, 1992; Baum and Berens, 1994; Becker et al., 1999), ocular (Abridshami et al., 2002), or visceral leishmaniasis (Llorente et al., 2000; Das et al., 2001; Momeni et al., 2002). Several of these reports describe combinations of allopurinol with low doses of other antileishmanials that are more effective than the usual dosage of the other drug alone, and the reduced dosage of pentamidine or antimony reduces or eliminates harmful side effects.

The metabolism of allopurinol in Leishmania species to the active metabolite, 4-aminopyrazolo(3,4-d)pyrimidine ribonucleoside triphosphate, has been described previously (Nelson et al., 1979; Marr and Berens, 1983). To date, however, no study has addressed allopurinol uptake by this parasite, even though the issue is important in understanding the selectivity of the drug as well as the potential for the development of resistance. In the related kinetoplast, Trypanosoma brucei, allopurinol is taken up through high affinity purine nucleobase transporters (De Koning and Jarvis, 1997a,b). We have therefore conducted a comprehensive study of purine nucleobase uptake in Leishmania major promastigotes. We have identified a single transporter, designated LmNBT1, with high affinity for all physiological purine bases and moderately high affinity for allopurinol. Studies with [3H]allopurinol confirmed that this transporter is its sole route of entry into promastigotes.

A model for the interactions of LmNBT1 with its substrates was constructed using the techniques developed to study Trypanosoma brucei and human purine transporters (De Koning and Jarvis, 1999; Wallace et al., 2002) to allow wider predictions about the potential of drug uptake by LmNBT1. The resulting model predicts that this transporter could mediate the uptake of an extensive range of nucleobase and guanosine analogs. In addition, it was shown that the architecture of the L. major and T. brucei nucleobase transporters are similar enough to bind purine

**ABBREVIATIONS:** LmNBT1, L. major nucleobase transporter 1; hFNT1, human facilitative nucleobase transporter.
bases in almost identical fashion, although the nucleobase transporter expressed in human erythrocytes and other cell types binds the same substrates in an entirely different way. This implies that many nucleobase analogs could be selectively internalized by both protozoan pathogens but remain excluded from many host cells. Although functional relationships such as those highlighted in the present article do not necessarily reflect evolutionary relationships, they have more pharmacological relevance than gene sequence alignments.

Materials and Methods

Leishmania major Culture. Promastigotes of Leishmania major promastigotes (Friedlin strain) were cultured in HOMEM medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen) at 25°C, and harvested for experiment at mid-log phase (typical density, 10^7 cells/ml) by centrifugation (2500 g, 10 min).

Transport Assays. Assays for transport of [3H]purines by L. major promastigotes was performed exactly as described for Trypanosoma brucei (De Koning, 2001; Wallace et al., 2002), using a rapid oil-stop protocol. Briefly, promastigotes were harvested and washed twice with the assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl_2, 0.07 mM MgCl_2, 5.8 mM NaH_2PO_4, 0.3 mM MgCl_2, 23 mM NaHCO_3, 14 mM glucose, pH 7.3) and resuspended at 10^8 cells/ml. Cells were then incubated with the radioligand in the presence or absence of competitive inhibitor and spun through oil (30 s, 13,000 rpm) after a predetermined time as indicated under Results. Radioactivity in the cell pellet was determined, after solubilization in 2% SDS, by liquid scintillation counting. [2,8-3H]Adenine (32.2 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA), [8-3H]hypoxanthine (32.0 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ), and [G-3H]allopurinol (1.9 Ci/mmol) from Moravek Biochemicals (Brea, CA). Unlabeled allopurinol, nucleosides and nucleobases were from Sigma.

Data Analysis. All experiments were performed in triplicate or more. Kinetic data, given as mean and S.E., were determined in at least three independent experiments and calculated by nonlinear regression using the Prism (GraphPad Software, San Diego, CA) software package from a minimum of 8 points over the relevant range. All uptake data are presented as total mediated uptake, defined as total uptake minus diffusion, taken to be uptake in the presence of saturating concentrations of unlabeled permeant.

Inhibition constants (K_i) were calculated from:

\[ K_i = IC_{50}/[1 + (L/K_m)] \]  
(1)

in which L is the permeant concentration (Cheng and Prusoff, 1973). Gibbs free energy \( \Delta G^\circ \) was calculated from

\[ \Delta G^\circ = -RT\ln(K_i) \]  
(2)

in which R is the gas constant and T the absolute temperature, as described previously (De Koning and Jarvis, 1999; Wallace et al., 2002).

Results

[3H]Adenine Transport by Leishmania major Promastigotes. Transport of 1 µM [3H]Adenine by L. major promastigotes was linear for at least 25 s, with a rate of 1.1 ± 0.1 pmol/10^7 cells/s, whereas in the presence of 1 mM unlabeled adenine, no uptake of [3H]adenine was detectable over 120 s (Fig. 1A), indicating that transport was saturable. [3H]Adenine uptake, measured over 10 s, followed Michaelis-Menten kinetics (Fig. 1B, inset) and displayed an apparent \( K_m \) of 4.6 ± 0.9 µM (n = 3). Transport of [3H]adenine was inhibited by a range of purine nucleosides and nucleobases (Table 1), but not by the pyrimidines uracil, cytosine, thymine, and thymidine at concentrations up to 1 mM. Nor was [3H]adenine transport significantly inhibited by 25 µM diazepam or dipyrindamole. The adenine transporter generally displayed far higher affinity for nucleobases than for their corresponding nucleosides, as illustrated in Fig. 1B for adenine and adenosine, which displayed a \( K_i \) value of >5 mM. Allopurinol was a moderately effective inhibitor of [3H]adenine transport (Fig. 1B) with a \( K_i \) of 56 ± 2 µM (n = 3). All inhibition profiles displayed Hill coefficients near −1 and maximum inhibition was invariably equal to the level of inhibition of the control (1 mM unlabeled adenine). These observations are consistent with a single transport activity for [3H]adenine.

A Single Transporter Is Responsible for Uptake of Adenine, Hypoxanthine, and Allopurinol. The inhibition

![Fig. 1. Transport of [3H]adenine uptake by L. major promastigotes. A, time course of 1 µM [3H]adenine uptake by L. major promastigotes in the presence (☐) or absence (□) of 1 mM unlabeled adenine. The inset shows the linear phase of this curve (25 s). The line was calculated by linear regression (r^2 = 0.97). B, inhibition of 0.1 µM [3H]adenine uptake by various concentrations of adenine (☐), allopurinol (○), and adenosine (●), with IC_{50} values of 5.9, 57, and 3.9 mM, respectively. Data were expressed as percentage of control, defined as uptake in the absence of inhibitor. The inset shows the conversion of the adenine inhibition plot to a Michaelis-Menten curve (in pmol/10^7 cells/s), showing total adenine transport as opposed to [3H]adenine transport only.](image-url)
of adenine transport by other purine bases does not establish whether these bases are in fact transported across the plasma membrane by this adenine transporter or whether additional transporters for purine nucleobases are expressed in L. major promastigotes. To further investigate these issues, additional transport studies were performed using [3H]hypoxanthine and [3H]allopurinol. [3H]Hypoxanthine uptake (0.1 μM) was linear for at least 15 s, with a rate of 0.22 ± 0.02 pmol/10⁷ cells/s (Fig. 2A). The apparent Kₘ value was 0.71 ± 0.07 μM and [3H]hypoxanthine transport was inhibited by adenine with a Kᵢ value of 3.0 ± 0.5 μM (Fig. 2B). The Kₘ is therefore almost identical to the Kᵢ value for hypoxanthine inhibition of adenine transport, and the Kᵢ value for adenine inhibition of [3H]hypoxanthine transport is equal to the Kₘ for adenine uptake (Table 1). In addition, purine nucleosides inhibited transport of 0.1 μM [3H]hypoxanthine in a manner similar to [3H]adenosine; 1 mM adenosine, 1 mM inosine, and 250 μM guanosine inhibited hypoxanthine uptake by 32 ± 13, 94 ± 1, and 87 ± 1%, respectively (n = 3). These results indicate that adenine and hypoxanthine most likely compete for uptake at a single transport unit. Likewise, 1 μM [3H]allopurinol, which was taken up with a rate of 0.026 ± 0.002 pmol/10⁷ cells/s (Fig. 3A), was inhibited by hypoxanthine (Fig. 3B) with a Kᵢ value very close to the Kₘ for [3H]hypoxanthine uptake (Table 1). It thus seems that L. major expresses a single high-affinity transporter for purine nucleobases. Given the substrate profile of this transporter, it was designated L. major nucleobase transporter 1 (LmNBT1). This transporter was not sensitive to the transporter inhibitors dilazep and dipyridamole, which inhibited [3H]hypoxanthine transport by just 20 ± 4 and 26 ± 4% at 50 μM (n = 3).

Structure-Activity Relationships of LmNBT1. The fact that LmNBT1 displays broad selectivity for purine nucleobases and mediates the uptake of allopurinol suggests that it may also transport other potential purine antimetabolites. The antineoplastic drug 6-thioguanine, for instance, displayed a Kᵢ value of 6.2 ± 0.8 μM for LmNBT1 and is probably efficiently taken up through this transporter. We have recently demonstrated that models for the interactions between a transporter binding pocket and its permeant can be constructed through the study of competitive inhibition by structural analogs and that such models have predictive value with respect to substrate recognition (De Koning and Jarvis, 1999; Wallace et al., 2002). A model for permeant recognition by LmNBT1 is displayed in Fig. 4A. The proposed hydrogen bond between LmNBT1 and N3 follows most directly from the observation that N3 is essential for high-affinity binding: 3-deazaguanine displays >10-fold lower affinity than guanine (Table 1). Conversion of the Kᵢ values for Gibbs free energy, using eq. 2, yields an energy difference δ(ΔG°) of 7.0 kJ/mol (Table 2), the apparent energy of the H-bond lost in 3-deazaguanine. Similar comparisons of guanine with 7-deazaguanine and 9-deazaguanine reveal H-bonds of 11.2 and 10.6 kJ/mol with N7 and N(9)H, respectively. The δ(ΔG°) of 3.3 kJ/mol between purine and 1-deazapurine is small but highly significant (P < 0.02) and shows that a weak interaction is formed between N1 of the purine ring and a H-bond donor in the LmNBT1 binding site. In contrast, no significant difference in affinity was observed between guanine and adenine, or between guanine and 6-thioguanine, showing that substitutions at position 6 do not contribute to binding. The δ(ΔG°) of 5.6 kJ/mol between purine and hypoxanthine must therefore be the result of binding through N(1)H. Because the weak bond with the unprotonated N1 of purine (3.3 kJ/mol) is lost in hypoxanthine, the Gibbs free energy of the H-bond with N(1)H must be 8.9 kJ/mol.

Corroborating evidence for this model is obtained from the Kᵢ values of other compounds listed in Tables 1 and 2. The structure of allopurinol differs from hypoxanthine in that N7 has shifted to position 8, and the δ(ΔG°) of 10.8 kJ/mol is virtually identical to the estimated bond energy for N7 (Fig. 4A). The low affinity for nucleosides [δ(ΔG°) > 10 kJ/mol] is consistent with the proposed H-bond to N(9)H. The δ(ΔG°) of xanthine versus hypoxanthine (8.6 kJ/mol) is identical to the estimated bond energy for N3, which is protonated in xanthine. Figure 4A also explains the total lack of recognition of

**Table 1**

Kinetic constants of purine nucleobase uptake in L. major promastigotes

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<tbody>
<tr>
<td>μM</td>
<td>n</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>Kᵢ Values</td>
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<tr>
<td>Adenine</td>
<td>4.6 ± 0.9</td>
<td>0.71 ± 0.07</td>
<td>54.3 ± 2.9</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>3.2 ± 0.3</td>
<td>2.8 ± 0.6</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>1.3 ± 0.3</td>
<td>3.0 ± 0.5</td>
<td>0.30 ± 0.09</td>
</tr>
<tr>
<td>Guanine</td>
<td>56 ± 1.5</td>
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<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>2.8 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>5150 ± 550</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inosine</td>
<td>125 ± 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanosine</td>
<td>68 ± 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purine</td>
<td>6.7 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Deazaguanine</td>
<td>26 ± 4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Deazaguanine</td>
<td>48 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Thioguanine</td>
<td>6.2 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-Deazaguanine</td>
<td>426 ± 140</td>
<td></td>
<td></td>
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<tr>
<td>9-Deazaguanine</td>
<td>204 ± 4</td>
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</table>

*Expressed as pmol/10⁷ cells/s.*
pyrimidine nucleobases. Finally, the sum of the individual bond energies is within 10% of the observed $\Delta G^\circ$ for adenine and hypoxanthine as calculated from their $K_m$ values. For hypoxanthine, $2(\Delta G^\circ) = -39.0$ kJ/mol, whereas the $\Delta G^\circ_{obs} = -35.1$ kJ/mol and for adenine these values are $-33.4$ and $-30.5$ kJ/mol, respectively.

The proposed model for substrate binding by LmNBT1 differs greatly from the one recently reported for the human facilitative nucleobase transporter hFNT1 (Fig. 4B), although they are both high-affinity purine nucleobase transporters (Wallace et al., 2002). However, the architecture of the LmNBT1 binding site seems to be very similar to that of T. brucei H2 (Fig. 4C; Wallace et al., 2002). Both transporters bind their highest affinity substrate, hypoxanthine, through the same interactions with the purine ring, and even the individual bond energies are very similar. The only substantial difference between the two transporters seems to be in the binding of adenine, where a weak H-bond with the amine group is now formed with N1 instead. It is certainly possible that this reflects merely a minor shift of position of one amino acid residue in the binding site. Alternatively a H-bond acceptor, such as aspartate, may have been replaced by a H-bond donor such as serine. The substrate recognition similarities of the two transporters extend to having far higher affinity for guanosine than for any other nucleoside, despite the fact that guanine is a lower affinity substrate than hypoxanthine. The only feasible explanation for this is that the optimal configuration for nucleoside binding is stabilized by an internal H-bond between the 5’hydroxyl and 2-NH$_2$ groups. The fact that this confers higher affinity than for the other purine nucleosides shows just how similar the binding site environment of LmNBT1 and TbH2 is. Table 2 lists the $\Delta G^\circ$ and $\delta(\Delta G^\circ)$ of H2 alongside those of LmNBT1.

Discussion

Nucleobase analogs are being widely used against infectious agents and malignancies (De Koning and Diallinas, 2000). The hypoxanthine analog allopurinol has shown considerable promise for the treatment of leishmaniasis, and
several nucleoside analogs have shown antiprotozoal activity, but no rational approach to a purine-based chemotherapy for protozoan infections has been developed. Such an approach needs to take account of 1) efficient uptake of the analog by the parasite and 2) conversion by the parasite’s metabolism to a form harmful to the organism. Selective toxicity therefore needs to be the result of either selective uptake or differences in metabolic enzymes. The current article deals with the issues related to specific and efficient uptake of purine analogs by the protozoan parasite Leishmania major.

We have investigated the transport of [3H]adenine, [3H]hypoxanthine, and [3H]allopurinol and found that all three bases were taken up by the same transporter, LmNBT1. This conclusion is based on 1) consistently monophasic inhibition profiles with Hill slopes of −1, leading to 100% inhibition of permeant uptake by the unlabeled inhibitor, 2) near identity of $K_m$ values for hypoxanthine uptake and the $K_i$ value for hypoxanthine inhibition of adenine uptake (and the equivalent observations for [3H]adenine and [3H]allopurinol), and 3) internally consistent $K_i$ and $DG^0$ values with a range of structural analogs, allowing the construction of a quantitative model for substrate recognition. The only previous report of nucleobase transport in Leishmania, by Hansen et al. (1982), also found that hypoxanthine and adenine were probably transported by a single transporter in Leishmania braziliensis panamensis. In contrast, nucleoside transporters have been relatively extensively studied in Leishmania donovani (Vasudevan et al., 1998, 2001; Carter et al., 2000; Ghosh and Mukherjee, 2000) and found to display high affinity for their substrates, with $K_m$ values typically between 0.3 and 5.0 μM. In view of the much higher $K_i$ values for nucleosides reported here for LmNBT1, it is unlikely that this transporter plays a significant physiological role in nucleoside salvage.

Allopurinol uptake has not previously been studied in Leishmania species. The discovery that, in contrast to T. brucei (De Koning and Jarvis, 1997b), allopurinol is taken up by only a single transporter, raises concerns about the ease with which resistance may develop, particularly because Leishmania species also express high-affinity purine nucleoside transporters (Vasudevan et al., 1998; Carter et al., 2000) and LmNBT1 is unlikely to be an essential gene. It would therefore be prudent to use allopurinol mainly as part of combination chemotherapy.

Allopurinol displayed only a moderate affinity for Lm-NBT1 (Table 2) suggestive of a more limited role for this transporter in allopurinol uptake. This conclusion is based on 1) consistently monophasic inhibition profiles with Hill slopes of −1, leading to 100% inhibition of permeant uptake by the unlabeled inhibitor, 2) near identity of $K_m$ values for hypoxanthine uptake and the $K_i$ value for hypoxanthine inhibition of adenine uptake (and the equivalent observations for [3H]adenine and [3H]allopurinol), and 3) internally consistent $K_i$ and $DG^0$ values with a range of structural analogs, allowing the construction of a quantitative model for substrate recognition. The only previous report of nucleobase transport in Leishmania, by Hansen et al. (1982), also found that hypoxanthine and adenine were probably transported by a single transporter in Leishmania braziliensis panamensis. In contrast, nucleoside transporters have been relatively extensively studied in Leishmania donovani (Vasudevan et al., 1998, 2001; Carter et al., 2000; Ghosh and Mukherjee, 2000) and found to display high affinity for their substrates, with $K_m$ values typically between 0.3 and 5.0 μM. In view of the much higher $K_i$ values for nucleosides reported here for LmNBT1, it is unlikely that this transporter plays a significant physiological role in nucleoside salvage.

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Allopurinol displayed only a moderate affinity for Lm-

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**Table 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>LmNBT1 $DG^0$</th>
<th>$\delta (DG^0)$</th>
<th>H2 $DG^0$</th>
<th>$\delta (DG^0)$</th>
<th>Control</th>
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<tr>
<td>Hypoxanthine</td>
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<td>−39.5</td>
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<td>−36.8</td>
<td>8.1</td>
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<td>−31.4</td>
<td>10.6</td>
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</tr>
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<td>−28.9</td>
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<td>Hypoxanthine</td>
</tr>
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</tr>
<tr>
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<td>0.4</td>
<td>Purine</td>
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<tr>
<td>3-Deazaguanine</td>
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<td>−28.5</td>
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<td>7-Deazaguanine</td>
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<tr>
<td>9-Deazaguanine</td>
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<td>10.6</td>
<td>−29.1</td>
<td>7.7</td>
<td>Guanine</td>
</tr>
</tbody>
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**Fig. 4.** Model for the interactions between the LmNBT1 (A), hFNT1 (B), and TbH2 (C) transporters and their permeants, adenine and hypoxanthine. Estimated Gibbs free energy for proposed bonds are indicated as −kJ/mol. The models for hFNT1 and TbH2 were adapted from Wallace et al. (2002).
NBT1 and its maximal rate of uptake is at least 10-fold lower than for adenine or hypoxanthine, but it is clinically active against leishmaniasis at high doses. It could be speculated that other purine analogs, if salvaged more efficiently, could have a higher efficacy. We have therefore studied the substrate selectivity of LmNBT1 in detail, constructing a quantitative model that allows predictions of the affinity of the transporter for potentially therapeutic analogs. Although such models yield estimates of \( K_a \) rather than \( V_{\text{max}} \), it does allow rational design or selection of purine analogs that are likely to be accumulated efficiently inside the parasite. Therapeutic action will then depend on such enzymes as the phosphoribosyltransferases, among others, that convert the analogs into nucleotides. The purine metabolic pathways of *Leishmania* have been studied in detail (Hassan and Coombs, 1988; reviewed by Berens et al., 1995), and most of the key enzymes have been characterized, cloned (Allen et al., 1995; Thiemann et al., 1998; Jardim et al., 1999; Sinha et al., 1999; Cui et al., 2001) and, in some cases, crystallized (Phillips et al., 1999; Shi et al., 1999), potentially allowing the design of specific inhibitors or subversive substrates. Compliance with the LmNBT1 model would ensure selective and efficient salvage of such designer drugs. And because the substrate recognition models for the nucleobase transporters and efficient salvage of such designer drugs. All because the design of specific inhibitors or subversive substrates.

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