Different Substrate Recognition Motifs of Human and Trypanosome Nucleobase Transporters

SELECTIVE UPTAKE OF PURINE ANTIMETABOLITES

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The therapeutic index of antimetabolites such as purine analogues is in large part determined by the extent to which it is selectively accumulated by the target cell. In the current study we have compared the transport of purine nucleobase analogues by the H2 transporter of bloodstream form Trypanosoma brucei brucei and the equilibrative nucleobase transporter of human erythrocytes. The H2 transporter forms hydrogen bonds with hypoxanthine at positions N3, N7, N(1)H, and N(9)H of the purine ring, with apparent \( \Delta G^o \) of 7.7–12.6 kJ/mol. The transporter also appears to H-bond with the amine group of adenine. The human transporter forms hydrogen bonds that form to (6)NH2 and N1 of adenine. An H-bond is also formed with N3 and the 6-keto and amine groups of guanine but not with the protonated N1, thus explaining the low affinity for hypoxanthine. N7 and N9 do not directly interact with the human transporter in the form of H-bonds, and it is proposed that \( \pi-\pi \) stacking interactions contribute significantly to permeant binding. The potential for selective uptake of antimetabolites by the parasite transporter was demonstrated.

Purine and pyrimidine antimetabolites are widely used to combat a variety of infectious diseases and other pathologies. However, many therapies suffer from a lack of selectivity, leading to severe side effects. The selectivity and efficacy of purine antimetabolites is achieved at two levels: the cell-surface transporters that mediate access to the cell, and the enzymes of the purine metabolic pathways that convert the pro-drug to the cytotoxic metabolite, usually a nucleotide analogue. This report elucidates the mechanisms of selectivity at the transporter level.

We have chosen Trypanosoma brucei, the etiological agent of sleeping sickness, as a model organism, because it lives freely in the host bloodstream rather than within a host cell. Therefore, the accumulation of most nucleobase and nucleoside drugs depends exclusively on the transporters expressed by the parasite itself. In addition, African trypanosomes are entirely dependent on purine salvage because they lack the capacity for de novo synthesis (1), and they consequently express several proton symporters that actively accumulate nucleosides (2, 3) and nucleobases (4–6). For trypanosomes, nucleobases may make more efficient drugs than their corresponding nucleosides, because purine ribonucleobases are rapidly hydrolyzed in bloodstream form T. brucei, limiting their incorporation into the nucleotide pool (7). In contrast, nucleobases are efficiently assimilated by one-step reactions of phosphoribosyltransferases for adenine, hypoxanthine-guanine, and xanthine (8). The current study therefore focuses on the main T. brucei brucei and human nucleobase transporters. This approach will allow the identification of groups that determine high affinity uptake by either the trypanosome or the cells of its mammalian host. The escalating epidemic of African trypanosomiasis (9) and the onset of drug resistance (10) necessitate the development of a new generation of trypanocides, and the parasite salvage pathways are potential targets for chemotherapeutic intervention.

Purine nucleobase transport in bloodstream T. b. brucei has been previously characterized, and two distinct carriers, H2 and H3, were identified. The H2 displayed a much higher affinity and a broader specificity than H3 (4). The almost 100-fold difference in \( K_m \) for these transporters allows the study of the H2 transporter separately at a very low \(^{3}H\)hypoxanthine concentration. We have now determined the substrate recognition motif for H2 and compared it with that of the human facilitative diffusion nucleobase transporter (hFNT1), which is the only nucleobase transporter in erythrocytes (11, 12) but has also been described in various other tissues (13–17) and been shown to mediate the uptake of several chemotherapeutic purine analogues (17–20). This approach to comparing transporters is all the more relevant because molecular approaches are unavailable: no protozoan or metazoan nucleobase transporters have yet been cloned.

Substrate recognition motifs have been shown to accurately predict the uptake of chemotherapeutic agents by T. b. brucei. Such models were developed for the T. b. brucei P1 and P2 adenosine transporters (21) and showed that the recognition motif of P2, but not of P1, was present in trypanocides as different as diamidines, melaminophenyl arsenicals, and isometamidium (a phenanthridine-amidine hybrid). All these have now been shown to be transported by P2 or at least to bind to the transporter with sub-micromolar affinity (2, 22–25).

We now show, on the basis of the H2 and hFNT1 substrate recognition motifs, that selective uptake of subversive nucleobase analogues by trypanosomes is possible and provide a structural rationale for the selection of such compounds. We also show that selective uptake alone is an insufficient requirement for trypanocidal action and that further studies of purine metabolism in trypanosomes will be required for rational design of antimetabolites.

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‡ The abbreviations used are: hFNT1, human facilitative nucleobase transporter 1; AraA, adenine-9-β-D-arabinoside.
**Substrate Recognition in Nucleobase Transporters**

**EXPERIMENTAL PROCEDURES**

**Purine Analogues and Radiolabeled Compounds**—[8-3H]Hypoxanthine (1.18 TBq/mmol) was purchased from Amersham Pharmacia Biotech, UK. [2,8-3H]Adenine (1.2 TBq/mmol) was obtained from PerkinElmer Life Sciences. Natural purines, pyrimidines, and purine analogues were purchased from Sigma Chemical Co., Fluka (9-methyguanine), Aldrich (aminopurin, 6-benzoylcytosine, 6-chloropurine, 1-deazapurine, 2,6-dichloropurine, 6-dimethylaminopurine, and 6-(3H)-pyrimidine), and ICN (7-azaindole). SN2635 was from Nanosyn (Mountain View, CA). 3-Deazaadenine and 6-methylguanine were kindly donated by the Department of Health & Human Services of the National Institutes of Health. 9-Deazaadenine was a kind gift from John Secrist III of the Southern Research Institute (Birmingham, AL). 9-Deazaadenine was generously provided by Howard Cottam of the University of California, San Diego.

**Trypanosomes**—Adult female Wistar rats were infected with *T. b. brucei* strain 427 by intra-peritoneal injection. At peak parasitemia, blood was collected by cardiac puncture under terminal anesthesia. The parasites were separated from the blood cells using a DE52 (Whatman) anion exchange column (26), washed twice in the assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl2, 0.07 mM MgSO4, 5.8 mM NaH2PO4, 0.3 mM MgCl2, 23 mM NaHCO3, 14 mM glucose, pH 7.3), and resuspended at 109 cells/ml. Cell viability and motility were checked under a phase-contrast microscope at the conclusion of each experiment.

**Human Erythrocytes**—Whole blood from healthy human volunteers was centrifuged, and red cells were washed twice in erythrocyte buffer (140 mM NaCl, 5 mM KCl, 20 mM Tris, 2 mM MgCl2, 0.1 mM EDTA, pH 7.4) and resuspended at a hematocrit ratio of ~25%; the typical density was 2.3–10×1011 cells/ml.

**Uptake Assays**—Uptake of 0.1 μM radiolabeled hypoxanthine in *T. b. brucei* was determined as previously described (3, 5), with small modifications. Briefly, 100 μl of the trypanosome suspension (~1011 cells) was mixed with 100 μl of assay buffer containing [3H]hypoxanthine and test inhibitor. Uptake was stopped after 30 s by addition of an ice-cold 4× solution of unlabeled hypoxanthine and centrifugation through an oil layer (7:1 (v/v) mix of di- n-butyolphthalate and light mineral oil (Sigma)). The microcentrifuge tubes were then flash-frozen in liquid nitrogen, and the bottom containing the cell pellet was cut off. The cells were then solubilized in 2% SDS and mixed with scintillation fluid, and the radioactivity was determined in a liquid scintillation counter. Uptake of 1 μM [2,8-3H]Adenine by human erythrocytes was similarly measured by an oil-stop method, using 100% dibutylphthalate for the oil layer and 2.3×1012 erythrocytes per assay point. Cells were solubilized in 250 μl of 1% Triton X-100, and hemoglobin was precipitated with 0.5 ml of 5% trichloroacetic acid. After centrifugation (10 min, 15,000×) the supernatant was transferred to scintillation vials and mixed with 3 ml of scintillation fluid, and the radioactivity was counted. All uptake data presented are presumed to represent “mediated uptake,” defined as total uptake minus diffusion, taken to be uptake in the presence of saturating concentrations of unlabeled permeant. Some test compounds were used from stock solutions in 100% Me2SO. Final concentration ranges of unlabeled permeant varied from 10−100 μM, with 100 μM of test compound (usually at 250 μM) to 100 μM of HMI9 medium (730 μl/liter Isco’s modified Dulbecco’s medium (product number 31980; Invitrogen) 1 mM adenosine, 50 μM bathocuproine disulfonic acid, 160 mM thymidine, 1 mM pyruvate, 2 mM 2-mercaptoethanol, 1 mM cysteine (all from Sigma), 50,000 units/liter penicillin, 50 mg/liter streptomycin, 30 mg/liter kanamycin, 20% fetal bovine serum (all from Invitrogen)) (30). Negative control values were obtained from wells with 100 μl of medium without test compound, whereas positive controls included dilution series of known trypanocides such as dimazene aceturate (Sigma). To each well, 100 μl of medium, containing 16 culture-adapted bloodstream *T. b. brucei*, was added. The plates were incubated at 37°C for 48 h after which 20 μl of alamarBlue reagent (Bio-Source, Camarillo, CA) was added. After a further 24-h incubation, fluorescence was determined in a PerkinElmer Life Sciences LS55B Fluorometer (λex = 480 nm, λem = 530 nm).

**RESULTS**

**Substrate Recognition by Trypanosome and Human Erythrocyte Nucleobase Transporters**—Natural purines and a variety of purine analogues were assayed for their ability to inhibit trypanosome or human erythrocyte nucleobase transport activities. By calculating the free energies involved in the interaction between the transporters and the various molecules, structure-activity relationships can be modeled and compared. The Ki values were determined from dose-response curves, and ∆G° values for the interaction between transporter and permeant were calculated, subject to the assumptions outlined under “Experimental Procedures.” Table I lists the Ki values and binding energies for both transporters.

**Hypoxanthine Uptake in T. b. brucei**—The kinetic parameters of the H2 hypoxanthine transporter have been described previously: hypoxanthine uptake is saturable, rapid, and linear over at least 50 s, with a Km of 123 ± 15 nM and a Vmax of 1.1 ± 0.2 pmol (107 cells)−1 s−1 (4). Here we describe its substrate recognition profile and present a model for the interactions between purine ring and transporter binding site.

**The T. b. brucei H2 transporter has some preference for oxopurines, as illustrated by the 30-fold lower Ki value for hypoxanthine than for adenine (Fig. 1A). Yet, there is an apparent interaction between the H2 binding pocket and the position 6 amino group, as indicated by the difference of 4.3 kJ/mol in ∆G° between adenine and pyrimidine (Fig. 1A). This indicates that, at position 6, a hydrogen bond donor is favored.**

**Data Analysis**—IC50 values (minimum of six points in triplicate) were determined from the inhibition profiles for the various inhibitors using the Fig.P. software package (Biosoft). The Cheng-Prusoff equation (27) was then used to give the Ki values for the analogues,

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

(Eq. 1)

in which L is the permeant concentration. Gibbs free energy, ∆G°, was calculated from,

\[
\Delta G° = -RT \ln(K_i)
\]

(Eq. 2)

in which R is the gas constant and T is the absolute temperature. It should be pointed out that these equations are only valid for competitive inhibition. The observation that inhibition profiles for both H2 and hFNT1 were consistently near ~1 is consistent with competitive inhibition of a single transporter. Whereas bloodstream trypanosomes do express two distinct purine nucleobase transporters, the chosen [3H]hypoxanthine concentration allows for >95% uptake by H2 (4). To date, inhibition of *T. b. brucei* purine transporters by analogues has always been shown to be competitive (2, 5, 23, 24), and hypoxanthine and adenine were shown to use the same transporter in human erythrocytes (28). All the available evidence therefore suggests that a simple model of competition with the radiolabeled permeant for interaction with the transporter binding site is applicable.

**In Vitro Drug Sensitivity Assays**—Drug sensitivity in cultured bloodstream trypanosomes was performed using the method of Ráz et al. (29). Serial dilutions of test compounds were made in 96-well plates by serial dilution (29). All the available evidence therefore suggests that a simple model of competition with the radiolabeled permeant for interaction with the transporter binding site is applicable.
TABLE I
Inhibition constants and Gibbs free energy for purine and pyrimidine inhibitors of TbH2 and hFNT1

| Compound                  | \( K_i \) \( \mu M \) | \( \Delta G^0 \) kJ/mol | \( \mu M \) | \( \Delta G^0 \) kJ/mol |
|---------------------------|-------------------------|--------------------------|-------------------------|--------------------------|
| H2                        |                         |                          | hRBC                    |                          |
| Purines                   |                         |                          |                         |                          |
| Hypoxanthine              | 0.12 ± 0.02<sup>a</sup> | -39.5                    | 290 ± 21                 | -20.2                    |
| Guanine                   | 0.56 ± 0.18<sup>a</sup> | -36.8                    | 28 ± 2.0                 | -26.0                    |
| Adenine                   | 3.2 ± 1.1<sup>a</sup>   | -31.4                    | 16 ± 4.5                 | -27.3                    |
| Xanthine                  | 8.8 ± 3.9<sup>a</sup>   | -28.9                    | >150                     |                          |
| Allopurinol               | 4.0 ± 2.2<sup>a</sup>   | -30.8                    | 150 ± 9                  | -21.8                    |
| Oxyapurinol               | 16 ± 3.5                | -27.4                    | ND                       |                          |
| Aminopurinol              | 240 ± 50<sup>b</sup>    | -20.7                    | 120 ± 30                 | -22.4                    |
| 2-Thioaminopurinol        | ND                      |                          | 20 ± 6.2                 | -26.8                    |
| Purine                    | 18 ± 3.7                | -27.0                    | 86 ± 20                  | -23.2                    |
| 1-Methyladenine           | 84 ± 14<sup>b</sup>     | -23.3                    | ND                       |                          |
| 1-Deazapurine             | 22 ± 8.2                | -26.6                    | 680 ± 17<sup>b</sup>     | -18.1                    |
| 1,3-Dimethylxanthine      | NE, 1000<sup>a</sup>    | ND                       |                          |                          |
| 1,7-Dideazapurine (7-azaindole) | NE, 1000         | ND                       |                          |                          |
| 2,6-Diaminopurine         | 25 ± 5.7                | -26.3                    | 43 ± 10                  | -24.9                    |
| 2,6-Dichloropurine        | 200 ± 38                | -21.1                    | 59 ± 3.9                 | -24.1                    |
| 3-Methyladenine           | NE, 250                 | NE                       | 520 ± 52<sup>b</sup>     | -18.7                    |
| 3-Deazaguanine            | 10 ± 2.0                | -28.5                    | 520 ± 24<sup>b</sup>     | -18.7                    |
| O6-Methylguanine          | 3.4 ± 0.6               | -31.2                    | ND                       |                          |
| 6-Thioguanine             | 0.43 ± 0.03             | -36.3                    | 95 ± 8                   | -23.0                    |
| 6-Thiouridine             | 13.3 ± 0.4              | -33.5                    | ND                       |                          |
| 6-Methoxypurine           | 6.9 ± 1.9               | -29.5                    | 240 ± 66<sup>b</sup>     | -20.6                    |
| 6-Benzylxanthine          | 150 ± 33                | -21.5                    | ND                       |                          |
| 6-Chloropurine            | 17 ± 3.3                | -27.2                    | 45 ± 5.8                 | -24.8                    |
| 6-Dimethylaminopurine     | 190 ± 36                | -21.2                    | ND                       |                          |
| 7-Deazaguanine            | 55 ± 12                 | -24.3                    | 15 ± 4.8                 | -27.6                    |
| 8-Azaguanine              | 7.2 ± 0.9               | -29.4                    | ND                       |                          |
| 8-Azaadenine              | 110 ± 14                | -22.7                    | ND                       |                          |
| 8-Azaglyoxanthine         | 27 ± 6                  | -26.0                    | ND                       |                          |
| Uric acid (8-hydroxyxanthine) | NE, 250              | NE                       | 250                      | -29.3                    |
| 9-Methylguanine           | 70 ± 16                 | -23.7                    | 7.4 ± 2                   | -29.3                   |
| 9-Deazadencine            | 70 ± 13                 | -23.7                    | ND                       |                          |
| 9-Deazaquinine            | 8.0 ± 1.3               | -29.1                    | 8.0 ± 2.5                | -29.1                    |
| NS20635                   | 4100 ± 1800<sup>b</sup> | -13.6                    | 1100 ± 47<sup>b</sup>    | -16.9                    |
| Pyrimidines               |                         |                          |                         |                          |
| 4-(3H)-Pyrimidone         | 900 ± 290               | -17.4                    | 7740 ± 1900<sup>b</sup>  | -12.1                    |
| Thymine                   | 82 ± 25°                | -23.3                    | 1000°<sup>b</sup>        |                          |
| Cytosine                  | >500°                   | NE                       | 1000°<sup>b</sup>        |                          |
| Uracil                    | 60 ± 14°                | -24.1                    | NE, 1000                 |                          |
| Pyrimidine                | >ND                     |                          | >10,000                  |                          |
| Nucleosides               |                         |                          |                         |                          |
| Guanosine                 | 11 ± 1.8<sup>a</sup>    | -28.3                    | NE, 250                  |                          |
| Inosine                   | 70.2 ± 9.2              | -23.7                    | NE, 1000                 |                          |
| Adenosine                 | 580 ± 180<sup>a</sup>   | -12.9                    | NE, 1000                 |                          |
| Acycloguanosine           | 62 ± 5                  | -24                      | ND                       |                          |
| Ara-hypoxanthine          | 300 ± 47                | -20.1                    | ND                       |                          |
| Thymidine                 | >1000°<sup>a</sup>      | ND                       |                          |                          |
| Cytidine                  | NE, 1000<sup>a</sup>    | ND                       |                          |                          |
| Uridine                   | 500°                    | ND                       |                          |                          |
| Others                    |                         |                          |                         |                          |
| Lumazine                  | 370 ± 28                | -19.6                    | ND                       |                          |
| Ascorbate                 | NE, 2500                | ND                       |                          |                          |

<sup>a</sup> Value taken from De Koning and Jarvis (4).
<sup>b</sup> Estimated number, extrapolated from incomplete inhibition curves.
<sup>c</sup> ND, not determined.
<sup>d</sup> NE, no effect at the indicated concentration.
<sup>e</sup> 42 ± 2.2% inhibition at a concentration of 1 mM thymine.

The slightly lower affinity for guanine than for hypoxanthine (\( \Delta G^0 = 2.7 \) kJ/mol) shows that substitutions at position 2 may be unfavorable for binding to H2. Two other comparisons (2,6-diaminopurine versus adenine and 6-thioguanine versus 6-thiopurine) give an estimate of 3.5 ± 0.6 kJ/mol loss of binding energy from the 2-NH\(_2\) substitution (Table I). The slightly bulkier chloride substitution was even more detrimental to binding (\( \Delta G^0 = 6.1 \) kJ/mol, 2,6-dichloropurine versus 6-chloropurine, Table I), suggesting the lower affinity could be the result of steric effects, although these substitutions would also reduce the partial negative charge on N3, making this residue a less effective hydrogen bond acceptor. Evidence that N3 does act in this way is provided by the 8.3 kJ/mol energy difference between guanine and 3-deazaguanine (Fig. 1B). Fur-
thermore, methylation of N3 leads to a dramatic loss in affinity, because 250 μM 3-methyladenine completely failed to inhibit H2-mediated [3H]hypoxanthine uptake (Table I). Alternatively, the methylation of N3 could physically prevent optimal positioning of the substrate in the binding pocket.

The pyrimidine ring thus contributes 20.8 kJ/mol to the total of 39.4 kJ/mol Gibbs free energy for the interactions between the hypoxanthine molecule and the transporter, indicating a 9-deazaadenine (Fig. 1D) had no effect on [3H]hypoxanthine uptake (Table I). Alternately, the methylation of N3 could physically prevent optimal positioning of the substrate in the binding pocket.

Fig. 2), displayed a similar contribution from the imidazole half of this purine. Consistent with this prediction, 4-(3H)-pyrimidone, consisting of the hypoxanthine molecule and the transporter, indicating a Gibbs bond acceptor, follows most directly from the involvement of N7 in binding, most probably as a hydrogen bond donor. Given the higher difference in ΔG° between most nucleobases and their respective nucleosides (adenine-adenosine, 12.9 kJ/mol; hypoxanthine-inosine, 15.8 kJ/mol), it is likely that steric factors equally contribute to the low affinity for nucleosides.

The H2 transporter therefore employs four interactions to bind either oxopurines or aminopurines. The observed ΔG° values of hypoxanthine (−39.5 kJ/mol) and adenine (−31.3 kJ/mol), based on their K_m values (Table I), are in close agreement of the total of the four predicted interactions (−41.1 and −32.8 kJ/mol, respectively).

Human Erythrocyte Facilitative Nucleobase Transporter—Transport of 1 μM [3H]adenine by human erythrocytes was rapid and linear over 20 s (Fig. 3) with a rate of 0.056 pmol (10^7 cells)^−1 s^−1. This rate was reduced by 92% in the presence of 1 mM unlabeled permeant (Fig. 3 and inset), showing that transport was saturable. In all experiments, [3H]adenine uptake in human erythrocytes was monophasic, with a Hill coefficient near −1, consistent with a single transport entity for adenine, as reported by previous investigators (12, 28). The transport conformed to Michaelis-Menten kinetics (Fig. 4A) with an apparent K_m of 16.2 ± 4.5 μM and a V_max of 1.92 ± 0.89 pmol (10^7 cells)^−1 s^−1 (n = 3). Subsequent inhibition profiles were ob-
in the presence (○) or absence (■) of 1 mM unlabeled adenine. Numbering for purines, pyrimidines, and indoles is indicated.

The human nucleobase transporter displayed a clear preference for aminopurines over oxopurines, with a $K_i$ for hypoxanthine of 288 ± 21 μM ($n = 3$), over 10-fold higher than the $K_m$ value for adenine. This seems to derive from transporter-permeant interactions with unprotonated N1 and 6NH$_2$ but not with N1(H). The importance of N1 clearly follows from the 8ΔG$^0$ between purine and 1-deazapurine of –5.1 kJ/mol (Fig. 4B). Similarly, the energy difference between purine and adenine is only –7.1 kJ/mol, indicating that either the 6-keto group or the proton on N1 contributes to some extent to its binding by hFNT1. The binding energy for 6-thioguanine is 3.1 kJ/mol lower than for guanine (Table I). It is therefore most likely that the keto group acts as an acceptor for a weak hydrogen bond, because 6-thioguanine exists predominantly in the thione (–S) rather than thiol (–SH) tautomeric form, and the thione group is much less capable of forming an H-bond than is the keto group. The difference of 3 kJ/mol in binding energy between purine and hypoxanthine confirms that the H-bond with N1 is stronger than the one to 6-keto. It also indicates that there is no contribution from N1(H) to the binding of hypoxanthine, because this would have significantly increased its observed low affinity. The binding energy for analogues with other substitutions at position 6 (such as chloride or methoxy) that do not change the protonation state of N1 was not significantly different from the 8ΔG$^0$ for purine ($p > 0.05$ in Student t-test, Table I).

Other sites of interaction of purines with hFNT include N(3) and amine substitutions at position 7. The former can be concluded from the 8ΔG$^0$ of 7.3 kJ/mol between guanine and 3-deazaguanine (Fig. 4D) or 8.6 kJ/mol between adenine and 3-methyladenine (Fig. 4C), whereas the latter is evident from the higher affinity for guanine than for hypoxanthine (8ΔG$^0$ = 5.8 kJ/mol). The inability of xanthine and uric acid to inhibit [3H]adenine uptake at the limit of their solubility is consistent with the importance of an H-bond donor at position 2 and an H-bond acceptor at position 3 (Table I). The estimated 8ΔG$^0$ for these interactions brings the total binding energy for adenine, hypoxanthine, and guanine through interactions with the pyrimidine half of the molecule to –16.5, –8.9, and –16.2 kJ/mol, respectively, which is consistently ~10 kJ/mol less than the observed value (Table I). However, neither N7 nor N9 play a direct role in the interaction with hFNT1, because 7-deazaguanine, 9-deazaguanine, and 9-methylguanine all have slightly higher affinity than guanine (Table I). Yet, 4-(3H)-pyrimidone, identical to hypoxanthine without the imidazole ring, displayed a very low affinity and a ΔG$^0$ of –12.1 kJ/mol, 8.6 kJ/mol higher than hypoxanthine (Table I). Similarly, the compound NS20635 (Fig. 4C), lacking both nitrogen atoms and π-orbitals in the 5-membered ring, displayed a 68-fold lower affinity than adenine (8ΔG$^0$ = –10.5 kJ/mol). Pyrimidine had little effect on hFNT1 transport at concentrations as high as 10 mM and has consequently a ΔG$^0$ > 10 kJ/mol compared with purine. The above data are all consistent with attributing this energy to interactions between π-orbitals of the purine ring, and in
performed in triplicate and representative of three or four identical, independent experiments.

In this report we have addressed purine nucleobase transport in human erythrocytes and T. brucei parasites that live freely in the mammalian bloodstream and therefore share the same environment, with their transporters competing for the same permeants. For the T. b. brucei H2 transporter, we build on our previously published limited characterization (4). The Michaelis-Menten constants and limited substrate specificity data for the facilitative nucleobase transporter in human erythrocytes have also been published previously (12, 31, 32), and the current findings, using the oil-stop technique developed by Domin et al. (31), are in close agreement with previous studies. Our $K_m$ and $K_i$ values for adenine, guanine, and hypoxanthine, the main permeants of hFNT1, are very similar to those previously reported (31), and the $K_i$ value for allopurinol of $121 \pm 29 \mu M$ is comparable to the reported $K_m$ value of $268 \mu M$ (33). We have now extended these studies to a large number of potential inhibitors with the aims of identifying the interactions between permeant and transporter binding pocket that determine substrate specificity and establishing whether selective uptake of purine analogues by either transporter is possible.

Purine antimetabolites have been enormously successful in chemotherapy of a wide variety of diseases and infections. Examples of the many nucleobases and nucleosides with therapeutic qualities include 6-thioguanine, 5-fluorouracil, and 6-mercaptopurine in leukemia and cancer therapy; AraA, acyclovir, and ganciclovir against DNA viruses; ribavirin and 3-deazaadenosine against RNA viruses; 3'-azido-3'-deoxythymidine (AZT) and 3'-thia-2',3'-dideoxythymidine (3TC) against retroviruses; and allopurinol and pyrimethamine against protozoan infections (34). The efficacy and selectivity of these drugs is at least in part dependent on the expression of specific transporters by the target cell (22, 35). Recent studies have shown clear differences in the expression of specific purine transporters by untransformed and malignant cells (36-38), highlighting the need for an approach to chemotherapy that takes into account transporter selectivity as well as the regulation of transporter expression. The mechanism of uptake of selected individual antimetabolites such as ribavirin (39), acyclovir (19), 2',3'-dideoxyguanosine (17), or allopurinol (33) has also been reported. However, the modeling of the transporter recognition motifs of the purine transporters reported here allows semi-quantitative predictions as to the efficiency of uptake for a wide range of purine analogues.

![Transport of $[^3H]$adenine by human erythrocytes](image-url)
affinities for adenosine (molecule. Even though these transporters display very similar permeant by binding to completely different parts of the purine species (55% identity at amino acid level) may bind the same transporters, even closely related transporters of the same species are speculative and for presentation purposes only. Estimations of the Gibbs free energy of these interactions are given in kJ/mol. The depiction of π-orbitals indicates binding by π-π stacking.

As we have previously shown for the T. b. brucei P1 and P2 transporters, even closely related transporters of the same species (55% identity at amino acid level) may bind the same permeant by binding to completely different parts of the purine molecule. Even though these transporters display very similar affinities for adenosine (K_m values of 0.38 and 0.92 μM, respectively), the different interactions involved in the binding lead to very different substrate selectivity profiles (21). The substrate recognition models explained the prominent role of the P2 transporter in the uptake of the main trypanocidal drugs (2, 21, 40), whereas P1 displayed 50- to 100-fold lower affinity for these compounds (21) and has not been implicated in the uptake of trypanocides.

The present study shows that H2 and hFNT1 also interact very differently with the purine ring. Both transporters are adequately described as purine-specific high affinity nucleobase transporters, but although the H2 transporter displayed highest affinity for hypoxanthine, hFNT1 bound hypoxanthine with >2000-fold lower affinity, its preferred substrate being adenosine. The ability of H2 to display very high affinity to both aminopurines and oxopurines appears to be linked to its potential to form H-bonds with either the 6-amine group or the N1 proton of the predominant lactam tautomeric form of oxopurines. In contrast, optimal binding by hFNT1 requires an unprotonated “pyrimidine type” N1 as present in aminopurines. Yet, hFNT1 displays relatively high affinity for guanine due to a weak hydrogen bond to the 6-keto group and a stronger one to the amine at position two. Both these interactions appear to be absent in H2 binding pocket, and this transporter displays 3-fold lower affinity for guanine than for hypoxanthine, presumably for steric reasons.

The one feature shared by the two base transporters is a strong interaction, presumably by H-bonding, with N3, which is unprotonated in all natural purines except xanthine and uric acid. This is consistent with the δ(ΔG°) value of 10.6 kJ/mol for xanthine compared with hypoxanthine binding by H2, which consists of the loss of the N3 H-bond (8.3 kJ/mol) and the steric effect of the substitution at position 2. For hFNT1 the affinity of xanthine would also be expected to be lower than for hypoxanthine, particularly because an H-bond donor such as an amine appears to be favored in position 2, but we were unable to establish a K_i value due to limitations of solubility. Similarly, the hFNT1 transporter was not inhibited by up to 250 μM uric acid, but H2 also failed to bind this purine, in which N7 is protonated. The total lack of affinity of these transporters for uric acid, which exists at up to 400 μM in human plasma, is an essential adaptation to the bloodstream environment, because the high urate levels would compete with low levels of other purine bases, and urate cannot be utilized for the synthesis of nucleotides. Purine nucleobase transporters in a number of other organisms, including Aspergillus nidulans (41, 42), Neurospora crassa (43), and Candida, do transport urate.

Both transporters derive roughly half of their binding energy from interactions with the imidazole part of the purine ring, explaining their preference of purines over pyrimidines. However, whereas H2 forms strong H-bonds with N7 and N(9)H, neither residue was directly involved in interactions with hFNT1. The conclusion that π-π stacking of the aromatic purine ring and one or more aromatic residues in the transporter binding pocket plays an important role in stabilizing the binding of the permeant is not without precedent. Structural studies have shown that π-orbital stacking plays a major role in the active site of many purine-metabolizing enzymes (44–47) and in the stability of nucleic acids (48). Our own studies also showed that this mechanism is in large part responsible for the very high affinity of the T. b. brucei P2 transporter (21). The combined electrostatic and Van der Waals interactions between the π-orbitals of aromatic amino acids can exceed 10 kJ/mol at optimal orientation (49).

The models for the substrate recognition motif for H2 and hFNT1 presented here show that there is ample scope for selective uptake of purine analogues by either transporter. Examples are the 220- and 50-fold higher affinities for 6-thioguanine and 3-deazaguanine, respectively, by H2, whereas hFNT1 displays much higher affinity for 9-methylguanine and 7-deazaguanine. Higher levels of selectivity could be achieved with rationally designed hypoxanthine analogues, because these display the greatest differential affinity between the two carriers (K_i(hFNT1)/K_i(H2) is 2400). However, although the hFNT1 transporter, or closely related members of the same transporter family, appears to be the most ubiquitous human nucleobase transporter and the only one characterized in blood cells, other nucleobase transporters have been characterized in specialized cells of the kidney, intestine, choroid plexus, and placenta (12). These sodium-dependent nucleobase transporters usually display high affinity for their substrates and seem to be expressed at the interface of the bloodstream with other environments. The importance of this class of transporters for the distribution, side effects, and pharmacokinetics of therapeutic nucleobase analogues would argue for intensified efforts for their cloning and characterization.

It is not argued here that selective uptake alone would be sufficient for selective therapeutic effects. As important as the accumulation of the analogue is the cell’s ability to metabolize and incorporate the analogue in a way that compromises the cell’s survival. This truism is graphically illustrated in the current study with the lack of in vitro trypanocidal action of several antimetabolites, including 6-thioguanine and 6-mercaptopurine, which displayed very high affinity for H2 and were most probably efficiently taken up by the parasite. Yet

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2 G. Daillinas, personal communication.
these compounds are effective against leukemia and other malignancies (50, 51). Although the current study did not aim to identify novel antiprotozoan purine analogues, several of the compounds used in the main structural study, notably amino-purinol and AraA, did display some trypanocidal action in *vitro*. However, these compounds were not high affinity substrates of H2 but, based on the known substrate recognition motifs (21), of the P2 aminopurine transporter.

In summary, we have developed quantitative models for substrate recognition by the main human and *T. b. brucei* nucleobase transporters and demonstrated that these can predict affinities of the respective transporters for purine analogues with therapeutic potential. The very different mechanisms by which these transporters interact with their permeants indicate that the geometry of their binding domains is likely to be quite different. The kinetic methodology used in this study provides a more direct approach to elucidating key transporter-permeant interactions than site-directed mutagenesis or X-ray crystallography and will be complementary to such studies once these become possible through the cloning of the transporters involved.

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