Cloning, Heterologous Expression, and in Situ Characterization of the First High Affinity Nucleobase Transporter from a Protozoan*

Richard J. S. Burchmore‡, Lynsey J. M. Wallace‡, Denise Candlish§, Mohammed I. Al-Salabi‡, Paul R. Beal§, Michael P. Barrett‡, Stephen A. Baldwin§, and Harry P. de Koning‡

From the Institute of Biomedical and Life Sciences, Division of Infection and Immunity, University of Glasgow, Glasgow G12 8QQ, United Kingdom and the §School of Biochemistry & Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

While multiple nucleoside transporters, some of which can also transport nucleobases, have been cloned in recent years from many different organisms, no sequence information is available for the high affinity, nucleobase-selective transporters of metazoa, parazoa, or protozoa. We have identified a gene, TbNBT1, from Trypanosoma brucei that encodes a 435-residue protein of the equilibrative nucleobase transporter superfAMILY. The gene was expressed in both the procyclic and bloodstream forms of the organism. Expression of TbNBT1 in a Saccharomyces cerevisiae strain lacking an endogenous purine transporter allowed growth on adenine as sole purine source and introduced a high affinity transport activity for adenine and hypoxanthine, with $K_m$ values of 2.1 ± 0.6 and 0.86 ± 0.22 μM, respectively, as well as high affinity for xanthine, guanine, guanosine, and allopurinol and moderate affinity for inosine. A transporter with an indistinguishable kinetic profile was identified in T. brucei procyclics and designated H4. RNA interference of TbNBT1 in procyclics reduced cognate mRNA levels by ~80% and H4 transport activity by ~90%. Expression of TbNBT1 in Xenopus oocytes further confirmed that this gene encodes the first high affinity nucleobase transporter from protozoa or animals to be identified at the molecular level.

In the last few years, major progress has been made in the identification of the genes encoding nucleobase and nucleobase transporters. In particular, nucleobase transporters from a large number of species, ranging from bacteria to humans, have been cloned and characterized (1, 2). Some of these have been shown also to transport nucleobases, but usually with much lower affinity than the corresponding nucleosides. Prominent examples are the human and rat ENT family members (3–5) and the Plasmodium falciparum nucleobase transporter PfNT1 (6, 7), with $K_m$ values for the nucleobases above or near millimolar levels. The Trypanosoma brucei ThbAT1 adenine transporter (8) is exceptional in that it has a high affinity for adenine ($K_m$ = 0.38 μM), slightly higher than for adenosine (0.59 μM) (9). However, transport of adenine by TbAT1 has not been demonstrated.

Nucleobase-selective transport activities have also been identified in many species, but sequence information for the encoding genes is available only for bacteria, yeast, fungi, and the plant Arabidopsis thaliana (10). On the basis of gene alignments and identifiable motifs, three families of nucleobase transporters have been identified to date: nucleobase-ascorbate transporters, purine-related transporters, and Arabidopsis purine permeases (10). The only mammalian members in these families are the human and rat ascorbate transporters (11–13), which align with nucleobase transporters from Aspergillus nidulans (14, 15), maize (16), and bacteria (10) to form the nucleobase-ascorbate transporter gene family.

Purine transporters of protozoan parasites have attracted much attention because of their role in salvage of essential nutrients (10, 17) and both their actual and potential roles in the accumulation of chemotherapeutic agents within the pathogen (9, 18–21). The protozoan nucleobase transporter genes identified to date are all members of an extensive family of proteins designated the equilibrative nucleobase transporters (ENT)* (6–8, 19, 20, 22, 23), although some in fact appear to be proton symporters (24).

None of the genes reported so far encode high affinity nucleobase transporters like those that have been described previously for T. brucei (25–27) or recently for Leishmania major (21) and Toxoplasma gondii (28). However, the substantially completed T. brucei genome sequence reveals the existence of a considerable number of ENT family members. A locus with six similar genes (the TbNT family) has recently been described (23), with members that encode purine nucleobase transporters. One member of this family (TbNT5) transports the nucleobase hypoxanthine with a $K_m$ of ~50 μM, in addition to transport of the nucleobases adenosine and inosine with high affinity ($K_m$ <5 μM).

Here, we report the identification and cloning of a novel member of the ENT gene family, with substantial homology to both the TbNT family and TbAT1. This transporter was expressed in a nucleobase transporter-deficient yeast mutant (29) to screen for any nucleobase or nucleobase transport activity. By this approach, we identified a high affinity nucleobase transporter, TbNBT1, which has identical substrate specificity to a high affinity nucleobase transport activity that we have characterized in parallel in T. brucei. We thus propose that the multiple ENT family members that are encoded by trypano-

* The abbreviations used are: ENT, equilibrative nucleoside transporter; NBT, nucleobase transporter; ORF, open reading frame; RNAi, RNA interference.
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Experimental Procedures

Cloning of TbNBT1—An ENT homologue was identified from T. brucei sequence data bases (W. Sanger Institute T. brucei sequence data bases). Multiple overlapping GSS sequences were identified and assembled using Con- tigExpress (VectorNTI Suite, Informax) to generate an open reading frame (ORF) that encoded a putative protein with ~30% amino acid identity to TbAT1 or to the TbNT family. Oligonucleotide primers, designed from regions flanking the ORF, were employed in a polymer- ase chain reaction (PCR) using the proofreading DNA polymerase Pfu to amplify a DNA fragment of the expected size from T. brucei genomic DNA. Products from two independent PCR reactions were cloned into the PCR cloning vector pGEMTeasy (Promega) and completely sequenced on both strands (MBSU sequencing service, Glasgow, Scotland, UK).

Southern and Northern Blotting—Performed by standard tech- niques, using the complete TbNBT1 ORF as probe.

RNA Interference (RNAi)—A fragment (~600 bp) of the TbNBT1 ORF was amplified by PCR and cloned into the BamHI and HindIII restriction sites of the double-stranded RNAi vector p2TT (30). The resulting construct, p2TTbNBT1, was integrated into T. brucei procyclic strain 29-13, as described previously (30). Double-stranded RNAi transcript was subjected to high throughput sequencing in yeast. The phenotype of induced and uninduced cells was compared by Northern blot and assayed for [3H]hypoxanthine transport.

Expression in Yeast—The complete TbNBT1 ORF was excised from pGEMTeasy using NotI and subcloned into the NotI site of the yeast expression vector pDR195 (31). A uracil auxotrophic fcy2 mutant MG887-1 (29) was transformed with the resultant construct (TbNBT1: pDR195) and selected on uracil-free medium. Transformed yeast were plated onto yeast nitrogen base without ammonium salts and amino acids, supplemented with 4 mM hypoxanthine as the sole nitrogen source.

Nucleobase and Nucleoside Transport Assays—Transport assays for trypanosomes were performed as described previously (32). Yeast were grown at 30°C in complete minimal medium lacking uracil, to a density of 1–2 OD units at 600 nm. Yeast transport assays were performed as described for T. brucei transport assays.

Expression in Xenopus Oocytes and Transport Assays—The complete TbNBT1 ORF was excised from pGEMTeasy using EcoRI fragment and subcloned into the corresponding sites of the Xenopus expression vector pGEMHE (33). The result constructs were linearized by digestion with NheI and then transcribed with T7 polymerase in the presence of 100 GppG cap using the mMESSAGE MACHINE™ (Ambion) transcription system. Preparation of Xenopus laevis oocytes, injection (with 10–20 mg RNA transcripts or with water alone), subsequent incubations and assays of the transport of radiolabeled nucleobases and nucleosides were performed essentially as described previously by Huang et al. (34). Transport assays were performed at 20°C over a period of 30 min using a permeant concentration of 100 μM, and each transport value shown represents the mean ± S.E. of 10–12 oocytes.

Results

TbNBT1 Sequence and Homology—ENT family members were identified by searches of the T. brucei genome data base with the amino acid sequence of the characterized TbAT1 aden- osine transporter (8). In addition to the recently described TbNT family (22, 23), encoding six closely related ENT hom- ologues, a number of other ENT family members were identified in the incomplete genome data base. An open reading frame (Fig. 1) with 32% identity and 52% similarity to TbAT1 was selected for functional characterization and was cloned by PCR amplification from T. brucei 927 and 427 genomic DNA. DNA sequencing confirmed that the cloned ORFs were identical and encoded an ENT homologue. Hydropathy profile analysis sug- gested a protein with 11 putative hydrophobic membrane-span- ning α helices, consistent with a membrane transport function. Based on functional characterization (see below) this open reading frame was designated T. brucei nucleobase transporter 1 (TbNBT1).

Gene Arrangement and Developmental Expression—Three almost identical homologues of TbNBT1 are described in the incomplete T. brucei genome project. TbNBT1 is most similar to a single predicted ORF that is assigned to chromosome 10 and that differs from TbNBT1 at two amino acids. Two other ORFs, both encoding putative ENT family members that are greater than 95% identical to TbNBT1, are assigned to chro- mosome 11. One of these ORFs has been submitted to Gen- Bank™ by other workers (TbNT8.1, GenBank™ accession number AF516605). However, the incomplete sequence information for both these regions do not preclude the possibility that other related ORFs are encoded at these loci, and indeed, arrays of tandemly repeated ORFs are common in the trypano- some genome. To investigate the possibility that TbNBT1 is a member of a multigene family, Southern blots of T. brucei 927 genomic DNA were hybridized with the TbNBT1 open reading frame (Fig. 2A). High stringency hybridization conditions re- vealed three bands in several restriction digests, a pattern that is consistent with two or more similar genes. However, partial restriction digests did not indicate the presence of an extensive tandem repeat. Southern blot data did not support the possibility that TbNBT1 homologues are encoded on separate chromo- somes, although Southern blots hybridized with TbNBT1 under lower stringency conditions (not shown) revealed additional bands that may contain more divergent ENT family members. Northern blotting of total RNA isolated from procyclic and bloodstream form trypanosomes revealed a single trans- script at ~2.5 kb and indicated that similar levels of TbNBT1 mRNA accumulate in these stages (Fig. 2B).

Functional Expression of TbNBT1 in Yeast—Transport ass- says were performed on S. cerevisiae strain MG887-1, transformed with empty vector pDR195, did not accumulate [3H]hypoxan- thine, [3H]adenine, [3H]inosine, or [3H]adenosine with time (Fig. 3). However, MG887-1 expressing TbNBT1:pDR195 ac- quired the ability to accumulate these purines (Fig. 3). In particular, [3H]adenine and [3H]hypoxanthine were accumulated rapidly and in a saturable manner, as transport in the presence of 1 mM unlabeled permeant was not significantly different from zero (Fig. 3, A and B). In contrast, transport of [3H]inosine and [3H]adenosine was not fully inhibited by 1 mM or even 2.5 mM unlabeled permeant (Fig. 3, C and D), indicating that the affinity of TbNBT1 for nucleosides was considerably less than for nucleobases. The transport in the presence of unlabeled nucleobase could not be explained by diffusion or endogenous transport of the yeast, since no uptake of nucleo- bases was observed in transformants with empty vector pDR195.

Further characterization of [3H]adenine and [3H]hypoxan- thine transport by TbNBT1 in MG887-1 revealed high affinity for purine nucleobases and guanosine, with Kᵅ or Kᵅᵠ values between 0.66 and 5.3 μM (Fig. 4 and Table I). As expected from

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the results in Fig. 3, C and D, inosine and, particularly, adenosine displayed much lower affinity (Table I). Even the surprisingly high affinity for guanosine is some 4-fold lower than for the corresponding nucleobase, guanine. A similar trend of relatively high affinity for guanosine on a purine nucleobase transporter was earlier reported for the T. b. brucei H2 (25) and Leishmania major LmNBT1 (21) carriers. This may be related to the formation of an internal hydrogen bond between the two-amine group on the purine ring and the 5'-hydroxyl group of the ribose moiety, stabilizing the molecule in a favorable configuration (21).

Expression of TbNBT1 in Xenopus Oocytes—To further validate the results of heterologous expression in yeast, we also performed a limited expression study in Xenopus laevis oocytes. Four groups of oocytes were injected in parallel with either water or cRNA encoding TbNBT1 or the human equilibrative nucleoside transporter hENT1 (35). Consistent with previously reported findings (4, 35), hENT1 expressed in oocytes efficiently transported both [3H]uridine and [3H]adenosine, but not the corresponding nucleobases, both at pH 7.5 (Fig. 5) and at pH 6.0 (not shown).

Oocytes expressing TbNBT1 rapidly accumulated [3H]adenine and, to a lesser extent, [3H]uracil, but no significant mediated transport of [3H]adenosine or [3H]uridine was observed at pH 7.5 (Fig. 5). However, at pH 6.0, the rate of uptake of adenosine in oocytes injected with TbNBT1 transcripts was significantly (3.4-fold) greater than in water-injected control oocytes (p < 0.01; data not shown), possibly indicating a more efficient transport as a result of the increased proton gradient over the oocyte plasma membrane.

Characterization of Two Hypoxanthine Transporters in Procyclic T. b. brucei—Transport assays using very low concentrations of [3H]hypoxanthine revealed the presence of two distinct hypoxanthine transporters in T. b. brucei procyclic cells (Fig. 6). A previously reported lower affinity transporter, H1 (27), was observed alongside a higher affinity hypoxanthine transport activity, which we designated H4. In the current set of experiments, conducted at 30 nM [3H]hypoxanthine, H1 and H4 activities were separated by fitting inhibition plots to an equation for two-site competition and to Eadie-Hofstee plots, which produced kinetic constants in good agreement with each other. Under these conditions, the kinetic constants for H1 were very similar to those previously reported (27) (Km = 15.2 ± 2.3 μM; Vmax = 2.4 ± 0.7 pmol (107 cells)⁻¹ s⁻¹ (n = 7)). Although the H4 transporter has a lower Vmax (0.27 ± 0.08 pmol (107 cells)⁻¹ s⁻¹ (n = 7)), it was generally responsible for...
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During the cloning experiments of TbNBT1, the radiolabeled permeant was 30 nM of label, due to its much higher affinity for hypoxanthine ($K_m = 0.55 \pm 0.07 \mu M$ ($n = 7$)). The presence of the two transporters was further illustrated with the aid of selective inhibitors of H4. Uracil and guanosine do not inhibit H1-mediated $[^3H]$hypoxanthine transport up to 1 mM (27), but dose-dependently inhibited H4 activity (Fig. 7). However, affinities for adenine, guanine, xanthine, and allopurinol were similar for H1 and H4 (Table I and Ref. 27). These results establish that H4 is a previously fold, respectively (32), but had little or no effect on affinity for nine) have been shown to reduce affinity for H2 28- and 150-

or N7 in guanine for CH (3-deaza- and 7-deazaguanine) have been shown to reduce affinity for H2 28- and 150-fold, respectively (32), but had little or no effect on affinity for H4 (Table I). These results establish that H4 is a previously unknown transporter, distinct from either H1 and H2, and indeed from H3, which is a relatively low affinity hypoxanthine transporter in T. b. brucei bloodstream forms (25).

Table I also shows the kinetic parameters for TbNBT1, determined by expression in S. cerevisiae, alongside those for the H4 transporter in procyclins. For none of the inhibitors was a significant difference in $K_i$ or $K_m$ value observed ($p > 0.05$), providing strong evidence that the NBT1 gene encodes the H4 transporter activity. It also validates the S. cerevisiae expression system used in our study as appropriate for this protozoan transporter, producing accurate and reproducible results.

Further evidence that TbNBT1 encodes the H4 transport activity derives from experiments with RNAi. T. b. brucei procyclins were transformed with the vector p2T7 (30), containing a 0.6-kb fragment of the TbNBT1 ORF under the control of the tetracycline-inducible promoter. Northern blots revealed that TbNBT1 mRNA levels in these cells were ~80% reduced after 5 days incubation with 1 μg/ml tetracycline (Fig. 2C). Incubation with tetracycline also greatly reduced the transport rate of the high affinity hypoxanthine transporter, and while the $K_m$ did not significantly differ between tetracycline exposed and control cells (0.24 ± 0.06 versus 0.17 ± 0.04 μM), the $V_{max}$ was reduced from 3.2 ± 0.2 to 0.20 ± 0.04 pmol (10^7 cells)⁻¹ s⁻¹ (Fig. 7).

Discussion

Nucleobase transport processes have been described in archea, bacteria, fungi, plants, protozoa, and vertebrates, including humans and, alongside nucleoside transporters, play an essential role in the transport of purines and pyrimidines across biomembranes (10). Moreover, these transporters mediate the internalization of many purine and pyrimidine antitumor agents as well as anticancer and antiviral agents, in chemotherapy against protozoan infections, the treatment of gout or in the prevention of organ rejection (10, 36, 37).

The study of nucleobase transport has been complicated, however, by the fact that it is often problematical to study transport of solutes in the cells or tissues expressing the transporters or under physiological conditions. In addition, many cell types express multiple nucleoside and/or nucleobase transporters, and it is frequently difficult to separate and identify the contribution of individual transporters to overall flux of radiolabeled permeant. The conclusive characterization of a single transporter (subtype therefore relies on the cloning of the gene encoding it and its expression in a suitable heterologous system. It is essential, however, that the results with the expression system can be verified, as it is possible that transporters have different characteristics in the original cell.

We have expressed a T. b. brucei gene, TbNBT1, with strong homology to the TbAT1 adenosine transporter, in S. cerevisiae, and characterized the induced transport activity in detail. The resulting kinetic profile was indistinguishable from the H4 hypoxanthine transporter profile in T. b. brucei procyclins.

![Fig. 4](image)

Affinity constants determined for TbNBT1 expressed in yeast and for the H4 transport activity in procyclic T. b. brucei

| $K_m$ or $K_i$ in yeast | $K_m$ or $K_i$ in procyclics |
|-------------------------|-----------------------------|
| Hypoxanthine* 0.66 ± 0.22 | 0.55 ± 0.07 |
| Adenine* 2.1 ± 0.6 | 2.6 ± 0.4 |
| Guanine 1.4 ± 0.3 | 2.6 ± 0.6 |
| Xanthine 4.3 ± 11 | 5.0 ± 0.5 |
| Allopurinol 5.4 ± 11 | 2.5 ± 0.4 |
| Uricil 68 ± 6.5 | 95 ± 27 |
| Adenosine 1900 ± 980 | 860 ± 170 |
| Inosine 47 ± 17 | 20 ± 7 |
| Guanosine 5.3 ± 1.5 | 4.7 ± 1.4 |
| 3-Deazaguanine ND* | 2.0 ± 0.3 |
| 7-Deazaguanine ND* | 4.9 ± 0.4 |

* $K_m$ value.

ND, not done.
characterized in parallel. Positive identification of H4 as the TbNBT1 gene product was provided by specific knockdown with RNAi of TbNBT1 fragments. The results of TbNBT1 expression in Xenopus oocytes were also entirely consistent with the expression in yeast. These observations confirm the suitability of our S. cerevisiae expression system and the accuracy of the results obtained with it.

The results show that TbNBT1 is primarily a nucleobase transporter, with highest affinity for hypoxanthine and adenosine, which are transported efficiently. High affinity was also displayed for the other naturally occurring purine nucleobases, guanine and xanthine. The relatively high affinity for guanosine was nevertheless 3–4-fold lower than for the corresponding base, and the affinity for other nucleosides was much lower. Rates of transport for nucleosides were also much lower than for nucleobases. The only other potential nucleobase transporter from a protozoan that has been cloned to date is the T. b. brucei P2 aminopurine transporter encoded by TbAT1 (8), which has a similar affinity for adenine and adenosine (9, 38). However, it is not known how well TbAT1 transports adenine, as all functional studies on TbAT1 have used [3H]adenosine. The assessment of TbAT1-mediated [3H]adenine uptake by bloodstream forms of T. b. brucei is complicated by the fact that these cells express multiple adenine transporters, including H2, H3 (25), NBT1, and possibly others.

Thus TbNBT1 is the first ENT family member so far identified that is primarily a high affinity nucleobase transporter, as the microbial and plant nucleobase transporters cloned to date align into different families (10) and the other known ENT transporters are nucleoside transporters with at best a secondary capacity to transport nucleobases (see Introduction). A number of ENT homologues can be readily identified in the T. brucei genome data base. These include close homologues of TbNBT1, such as TbNT8.13 and more divergent ORFs that nevertheless have significant homology to TbNBT1 as well as to the nucleoside transporters of the TbNT family (23) and TbAT1 (8). More trypanosomal ENT family members are likely to come to light as the genome sequence project is completed. Such a plethora of nucleoside transporters was surprising, but the discovery that a trypanosomal ENT family member encodes a high affinity nucleobase transporter raises the possibility that the complex, developmentally regulated nucleobase transport capacity of trypanosomes (39) is mediated by ENT family transporters. This observation represents important progress toward elucidating the mechanisms of nucleobase acquisition by protozoan parasites but might also advance understanding of nucleobase transport in mammalian cells. In addition to the well characterized mammalian nucleoside transporters ENT1 and ENT2, at least two additional genes of the ENT family have recently been identified in the human genome (1, 40). While these have not yet been fully characterized, it is possible that some of these novel ENTs encode the high affinity nucleobase transporters described in various human tissues and cell lines (10, 41, 42). The current breakthrough could aid the identification of these pharmacologically important transporters, and in the rational design of novel purine antimetabolites with increased selectivity for protozoan over host transporters.

3 C. Henriques, M. A. Sanchez, R. Tryon, and S. M. Landfear, unpublished data.
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REFERENCES
1. Hyde, R. J., Cass, C. E., Young, J. D., and Baldwin, S. A. (2001) Mol. Membr. Biol. 18, 53–63
2. Rittel, M. W. L., Ng, A. M. L., Yao, S. Y. M. Graham, K., Loewen, S. K., Smith, K. M., Hyde, R. J., Karpinski, E., Cass, C. E., Baldwin, S. E., and Young, J. D. (2001) Mol. Membr. Biol. 18, 65–73
3. Crawford, C. R., Patel, D. H., Naeye, C., and Belt, J. A. (1998) J. Biol. Chem. 273, 5288–5293
4. Ward, J. L., Sherali, A., Mo, Z.-P., and Tse, C.-M. (2000) J. Biol. Chem. 275, 8575–8581
5. Yao, S. Y. M., Ng, A. M. L., Vickers, M. F., Sundaram, M., Cass, C. E., Baldwin, S. A., and Young, J. D. (2002) J. Biol. Chem. 277, 24938–24948
6. Parker, M. D., Hyde, R. J., Yao, S. Y. M., McRobert, L., Cass, C. E., Baldwin, J. D., Young, J. D., McConkey, G. A., and Baldwin, S. A. (2000) Biochem. J. 349, 67–75
7. Carter, N. S., Ben Mamoun, C., Lu, W., Silva, E. O., Landfear, S. M., Goldberg, D. E., and Ullman, B. (2000) J. Biol. Chem. 275, 10683–10691
8. Maser, P., Sutterlin, C., Kralli, A., and Kaminisky, R. (1999) Science 285, 242–244
9. Carter, N. S., and Fairlamb, A. H. (1993) Nature 361, 173–175
10. De Koning, H. P., and Diallinas, G. (2000) Mol. Membr. Biol. 17, 75–94
11. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U. V., Chen, X.-Z., Wang, Y., Brubaker, R. F., and Hediger, M. A. (1999) Nature 399, 70–75
12. Rajan, D. P., Huang, W., Dutta, B., Devoe, L. D., Leibach, F. H., Ganapathy, V., and Prasad, P. D. (1999) Biochem. Biophys. Res. Commun. 262, 762–768
13. Daruwala, R., Sanger, J., Koh, W. S., Rumsey, S. C., and Levine, M. (1999) FEBS Lett. 469, 480–484
14. Diallinas, G., and Scanzocchi, C. (1989) Genetics 122, 341–350
15. Diallinas, G., Gerninkiel, L., Arst, H. N., Cecchetto, G., and Scanzocchi, C. (1995) J. Biol. Chem. 270, 8610–8622
16. Argyrou, E., Sophianopoulos, V., Schultze, N., and Diallinas, G. (2001) Plant Cell 13, 953–964
17. Carter, N. S., Landfear, S. M., and Ullman, B. (2001) Trends Parasitol. 17, 142–145
18. De Koning, H. P. (2001) Int. J. Parasitol. 31, 512–522
19. Vasudevan, G., Carter, N. S., Drew, M. E., Beverley, S. M., Sanchez, M. A., Serfage, A., Ullman, B., and Landfear, S. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9873–9878
20. Carter, N. S., Drew, M. E., Sanchez, M., Vasudevan, G., Landfear, S. M., and Ullman, B. (2000) J. Biol. Chem. 275, 20935–20941
21. Al-Salahi, M. I., Wallace, L. J. M., and De Koning, H. P. (2003) Mol. Pharmacol. 63, 814–823
22. Sanchez, M. A., Ullman, B., Landfear, S. M., and Carter, N. S. (1999) J. Biol. Chem. 274, 30244–30249
23. Sanchez, M. A., Tryon, E., Green, J., Boor, J., and Landfear, S. M. (2002) J. Biol. Chem. 277, 21499–21504
24. De Koning, H. P., Watson, C. J., and Jarvis, S. M. (1998) J. Biol. Chem. 273, 9486–9494
25. De Koning, H. P., and Jarvis, S. M. (1997) Mol. Biochem. Parasitol. 89, 245–258
26. De Koning, H. P., and Jarvis, S. M. (1998) Biochem. Cell Biol. 76, 853–858
27. De Koning, H. P., and Jarvis, S. M. (1997) Eur. J. Biochem. 247, 1102–1110
28. De Koning, H. P., Al-Salahi, M. I., Cohen, A. M. Coumbe, G. H., and Watson, J. M. (2003) Int. J. Parasitol., in press
29. Gillissen, B., Birklle, L., Andre, B., Kuhn, C., Rentsch, D., Brandl, B., and Fommers, W. F. (2000) Plant Cell 12, 291–300
30. LaCount, D. J., Bruse, S., Hill, K. L., and Denelson, J. E. (2000) Mol. Biochem. Parasitol. 111, 67–76
31. Rentsch, D., Laloi, M., Bonfara, I., Schmelzter, E., Delrot, S., and Frommer, W. B. (1995) FEBS Lett. 376, 264–268
32. Wallace, L. J. M., Candlish, D., and De Koning, H. P. (2002) J. Biol. Chem. 277, 26149–26156
33. Liman, E. R., Trygat, J., and Hess, P. (1992) Neuron 9, 861–871
34. Huang, Q. Q., Harvey, C. M., Paterson, A. H., Cass, C. E., and Young, J. D. (1993) J. Biol. Chem. 268, 20613–20619
35. Griffiths, M., Beaumont, N., Yao, S. Y. M., Sundaram, M., Boumah, C. E., Davies, A., Kwong, F. Y. P., Coo, I., Cass, C. E., Young, J. D., and Baldwin, S. A. (1997) Nat. Med. 3, 89–93
36. Kuhl, V. M. (1997) Prog. Drug Res. 46, 195–222
37. Cheson, B. D. (2002) Semin. Oncol. 29, 33–45
38. De Koning, H. P., and Jarvis, S. M. (1999) Mol. Pharmacol. 56, 1162–1170
39. De Koning, H. P., Watson, C. J., Sutcliffe, L., and Jarvis, S. M. (2000) Mol. Biochem. Parasitol. 106, 93–107
40. Acimovic, Y., and Coo, I. R. (2002) Mol. Biol. Evol. 19, 2199–2210
41. Plagemann, P. G., Wohlhueter, R. M., and Wolfenden, C. (1988) Biochim. Biophys. Acta 947, 405–443
42. Griffith, D. A., and Jarvis, S. M. (1996) Biochim. Biophys. Acta 1266, 153–181
43. Hofmann, K., and Stoffel, W. (1993) TMbase: A Database of Membrane Spanning Proteins Segments (www.ch.embnet.org/software/TMPRED_form. html)