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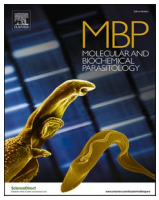

Mapping the transporter-substrate interactions of the *Trypanosoma cruzi* NB1 nucleobase transporter reveals the basis for its high affinity and selectivity for hypoxanthine and guanine and lack of nucleoside uptake

Mustafa M. Aldfer [a], Fabian Hulpia [b,1], Serge van Calenbergh [b], Harry P. De Koning [a,*]

[a] School of Infection and Immunity, Sir Graeme Davies Building, 120 University Place, University of Glasgow, GlasgowG12 8TA, UK
[b] Laboratory for Medicinal Chemistry (Campus Heymans), Ghent University, Ottergemsesteenweg 460, 9000 Gent, Belgium

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ABSTRACT

Trypanosoma cruzi is a protozoan parasite and the etiological agent of Chagas disease, a debilitating and sometimes fatal disease that continues to spread to new areas. Yet, Chagas disease is still only treated with two related nitro compounds that are insufficiently effective and cause severe side effects. Nucleotide metabolism is one of the known vulnerabilities of *T. cruzi*, as they are auxotrophic for purines, and nucleoside analogues have been shown to have genuine promise against this parasite in vitro and in vivo. Since purine antimetabolites require efficient uptake through transporters, we here report a detailed characterisation of the *T. cruzi* NB1 nucleobase transporter with the aim of elucidating the interactions between TcrNB1 and its substrates and finding the positions that can be altered in the design of novel antimetabolites without losing transportability. Systematically determining the inhibition constants (K_i) of purine analogues for TcrNB1 yielded their Gibbs free energy of interaction, ΔG^0. Pairwise comparisons of substrate (hypoxanthine, guanine, adenine) and analogues allowed us to determine that optimal binding affinity by TcrNB1 requires interactions with all four nitrogen residues of the purine ring, with N1 and N9, in protonation state, functioning as presumed hydrogen bond donors and unprotonated N3 and N7 as hydrogen bond acceptors. This is the same interaction pattern as we previously described for the main nucleobase transporters of *Trypanosoma brucei* spp. and *Leishmania major* and makes it the first of the ENT-family genes that is functionally as well as genetically conserved between the three main kinetoplast pathogens.

1. Introduction

Chagas disease, or American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*. It is an endemic disease and has been found mainly in Latin American countries, where the triatomine vectors, commonly known as 'kissing bugs', are endemic [1]. More recently, the disease has encroached on the Southern United States [2,3], as a result of the continued spread of the vector as well as human migration of symptomatic and asymptomatic carriers [4,5]. Incidence in other parts of the world is also linked to migration [6,7]. The *T. cruzi* parasite affects an estimated 6–7 million people, and the infection has high morbidity and mortality rates, that are estimated to lead to more than 7000 deaths annually, based on estimates of the World Health Organisation [1].

Although much effort over decades has reduced transmission by direct vector contact in much of Latin America [3,7], other transmission routes including ingestion of food and drink contaminated with triatomine faeces, vertical transmission and blood transfusion, continue to add to the case load [8–10]. Treatment of Chagas disease remains problematic, especially when it has progressed from the acute to the chronic phase, with only two old, related drugs available for the clinical treatment of Chagas disease, benznidazole and nifurtimox [11]. Both drugs exhibit relatively low cure rates, particularly in the chronic phase [12,13]. In addition, these drugs have potentially severe side effects that may lead to discontinuation of treatment [14]. There are some developments towards new drugs [11,15–18] but to the best of our knowledge none is close to clinical practice.

T. cruzi is a mandatory intracellular parasite capable of infecting most types of nucleated cells of the mammalian host as amastigotes [12].

* Corresponding author.
E-mail address: Harry.de-Koning@glasgow.ac.uk (H.P. De Koning).
[1] Present address: Janssen Pharmaceutica NV, Turnhoutseweg 30, 2340 Beerse, Belgium.

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These parasites acquire the nutrients from their host cell and modulate the metabolism to facilitate their acquisition. During *in vivo* and *in vitro* assay studies, infected cells showed uncommon metabolic features especially regarding energy metabolism – increasing glucose uptake and glycolysis, synthesis of amino acids and fatty acids, and mitochondrial respiration and mitochondrial biogenesis; production of reactive oxygen species was also greatly increased in infected heart tissue [19–22]. However, the nutritional requirements of the parasite are not limited to energy metabolism, but also include the need for purines, for which they are known to be auxotrophic [23], but more surprisingly also preformed pyrimidines [22], for which they are not [23]. This is consistent with studies with purine and pyrimidine inhibitors on intracellular amastigotes, both of which were effective [24]. Cardiomyocytes isolated from infected mice showed lower levels of purine nucleosides and hypoxanthine than in uninfected controls, and adenine was almost undetectable in the infected cells [20]. This may have been the result of increased purine degradation but certainly also of purine salvage by the *T. cruzi* amastigotes, which has been reported to be more efficient for the nucleobases than the nucleosides [23].

The nucleobase transporters of *T. cruzi* are therefore of considerable interest as they would likely be essential for optimal growth of, particularly, the intracellular amastigotes. However, nucleobase transport has been much more studied in *T. brucei* [25–27] and in *Leishmania* species [28–31]. Where the encoding has been identified, protozoan nucleobase transporters have always been of the Equilibrative Nucleoside Transporter (ENT) family [32–37], but it has been speculated that, because all the ENT genes of *T. brucei* and *Leishmania* have been cloned and characterised without identifying the uracil-specific carriers of either species [38–40], or the recently reported, highly specific hypoxanthine- and adenine-specific transporters of *T. brucei* [41], an as-yet to be identified gene family may contribute to purine salvage in the kinetoplastidae [42]. The importance of nucleobase transporters in intracellular kinetoplastids is shown from the reduced parasite burden with *Leishmania major* amastigotes after NT4 knockdown with RNAi in vitro and reduced lesion size in a mouse model [43]. Moreover, deletion of NT4 was also shown to impair intra-macrophage survival, and all attempts to delete both *Leishmania* nucleobase transporter genes, NT3 and NT4, were unsuccessful [31].

In *T. cruzi*, we have previously identified and heterologously expressed four ENT genes: TcrNT1 was found to be an inosine/guanosine transporter [44]; TcrNT2 is thymidine-specific [45]; TcrNB1 was shown to have highest affinity for hypoxanthine and guanine [44]; and TcrNB2 was specific for adenine [35]. Of these, TcrNB1 was only partly characterised, as part of the original survey of the four TcrENTs, and in the current report we present a full characterisation and binding mode of this carrier, making it the most comprehensive description yet of a nucleobase transporter from an intracellular protozoan.

2. Materials and methods

2.1. Materials

Radiolabelled hypoxanthine was obtained from Perkin-Elmer ([^{3}H (G)-hypoxanthine, 16.1 Ci/mmol). Nucleobases and nucleosides used in this study were obtained from Sigma-Aldrich unless specified below. 1-Deazaadenine (FH14885), 9-methyl,1-deazahypoxanthine (FH14872), 1-deazahypoxanthine (FH14875), 7-Br-allopurinol (JBMAM034) and 9-deazaguanine (JBMAM002) were synthesised in-house at Ghent University; all synthesises were previously described, most in [37]. 9-Deazahypoxanthine was from Carbosynth. 7-Deazahypoxanthine was from Alfa Aesar. 1-Methylhypoxanthine was from AKOS. 9-Deazaxanthine was a gift from Dr Howard B. Cottam, University of California, San Diego. 3-Deazahypoxanthine was from Fluorochem UK.

2.2. Culture of T. b. brucei procyclics

The cell line utilised here was a procyclic *T. b. brucei* cell line (Lister strain 427) created by the deletion of the TbNT8.1/8.2/8.3 nucleobase locus [41] and subsequently transfected with the TcrNB1 gene in the plasmid pHD1336 [44]. The cells were cultured in SDM-79 medium (Life Technologies), supplemented with 7.5 g/mL of hemin and 10% of Foetal Bovine Serum (FBS, Biosera, Kansas City, MO, USA) in non-vented plastic bottles at 27 °C.

2.3. Transport assays

All transport assays used 50 nM [3 H]-hypoxanthine as radiolabel and were performed exactly as described previously (Wallace et al., 2002). Briefly, cells were grown to late log-stage, harvested by centrifugation and washed into the assay buffer (AB; 33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.5 mM $CaCl_2$, 0.07 mM $MgSO_4$, 5.8 mM NaH_2PO_4, 0.03 mM $MgCl_2$, 23 mM $NaHCO_3$, 14 mM D-glucose, pH 7.3). Test compounds (nucleosides and nucleobases) were serially diluted in AB and mixed with the radiolabel, both at 2× the final concentration, and layered over an oil layer consisting of 7:1 (v/v) of di-n-butylphthalate and mineral oil (Sigma) in a 1.5 mL microfuge tube. An equal volume (100 μL) of cell suspension, containing 10^7 procyclics, was added, incubating with the radiolabel and test compound for 3 s before 1 mL of 'stop solution' (ice-cold 1 mM hypoxanthine in AB) was added and the cells centrifuged through the oil layer for 1 minute at maximum speed in a microfuge. The tubes were then flash-frozen in liquid nitrogen; the tip containing the cell pellet clipped off and collected in a scintillation tube; the cells were solubilised in 2% SDS for one hour on a rocking platform after which 3 mL of Scintilogic U scintillation fluid (Lablogic) was added. Radiation was quantified in a 300SL Hidex scintillation counter. DPM values were converted to pmol of hypoxanthine using standards and plotted to a sigmoid curve with variable slope using Prism 9 (GraphPad), yielding EC_{50}s, from which the K_i values using the Cheng-Prusoff equation [46]. The Gibbs free energy was calculated using the equation $\Delta G^0 = -RT\ln(K_i)$, in which R is the gas constant and T the absolute temperature [27].

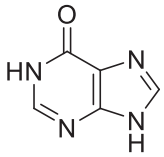
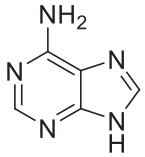
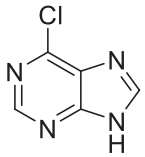
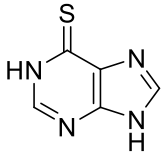
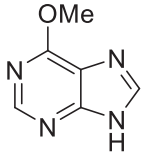
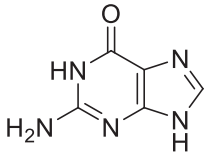
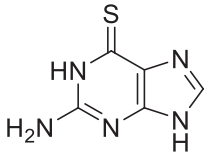
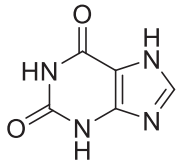
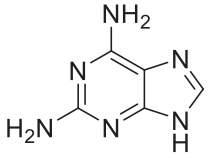
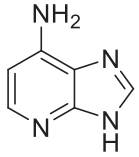
3. Results

The previous work with TcrNB1 was conducted through heterologous expression in the procyclic *T. brucei* cell line NBT-KO [44]. This cell line, from which a locus of three nucleobase transporters (TbNT8.1/8.2/8.3) has been deleted, served very well for the characterisation of TcrNB1, although it retained a just-measurable uptake rate of both hypoxanthine and adenine, through highly specific carriers called HXT1 and ADET1, respectively [41]. This work showed that TcrNB1 must be assayed with a very low radiolabel concentration, maximal 50 nM [^{3}H]-hypoxanthine, due to its extremely low K_m value for this nucleobase, 94.7 ± 3.6 nM [44]; all K_m and K_i values are listed in Table 1. The K_i value for guanine was similar, at 122 ± 22 nM but that of the aminopurine nucleobase adenine was substantially higher, with a K_i of 3.7 ± 0.5 μM, which represents a loss of 9.1 kJ/mol in the Gibbs free energy of interaction (expressed as $\delta(\Delta G^0)$) compared to hypoxanthine. Substantially lower affinity was found for the corresponding nucleosides, with adenosine not inhibiting 50% of 50 nM hypoxanthine uptake at 1 mM – a 20,000-fold excess (Table 1). Pyrimidine nucleosides and nucleobases were also very poor inhibitors of TcrNB1, with only cytidine inhibiting hypoxanthine uptake by more than 50% at 1 mM (57.7 ± 6.1%, n=3, $P < 0.01$) [44].

Here, we aim to provide the rationale for the observed pattern of substrate affinity by probing the contribution of each part of the purine ring to the total ΔG^0 of interaction, as described for other protozoan and human transporters [27,47–49].

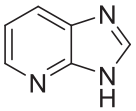
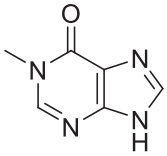
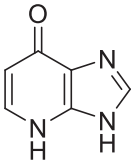
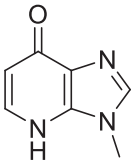
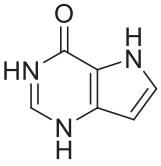
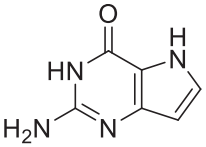
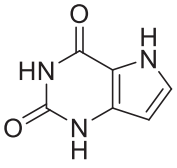
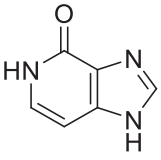
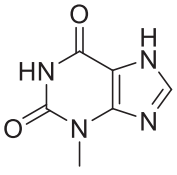
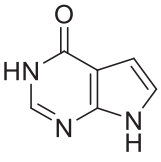
First, we explored the contribution of substituents at position 6 of the purine ring to binding. The lower affinity of adenine versus

Table 1
K_m and Ki values for TcrNB1.

	Substrate	K_i or K_m value (μM)	n	ΔG^0 (KJ/mol)	δ (ΔG^0) (KJ/mol)	relative to
	Hypoxanthine[a,b]	0.093 ± 0.004	4	-40.1		
	Adenine[b]	3.73 ± 0.5	3	-31.0	9.1	hypoxanthine
	6-chloropurine	3.65 ± 0.24	3	-31.0	-0.1	adenine
	6-Mercaptopurine	0.42 ± 0.02	3	-36.4	3.7	hypoxanthine
	6-Methoxypurine	1.49 ± 0.02	3	-33.24	6.86	hypoxanthine
	Guanine[b]	0.122 ± 0.022	3	-38.4	1.7	hypoxanthine
	6-thioguanine	0.36 ± 0.04	4	-36.8	1.6	guanine
	Xanthine	18.4 ± 1.8	4	-27.0	13.1	hypoxanthine
	2,6-Diaminopurine	12.9 ± 1.0	3	-27.9	3.1	adenine
	1-Deazaadenine (FH14885)	36.3 ± 5.1	3	-25.3	5.6	adenine

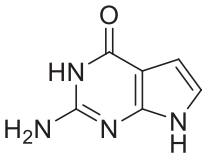
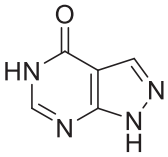
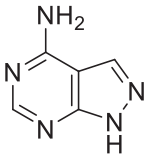
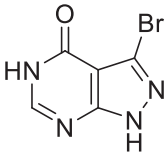
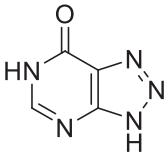
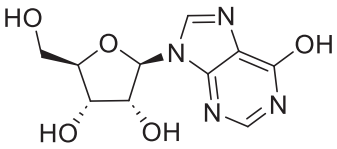
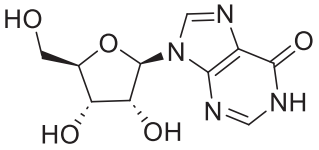
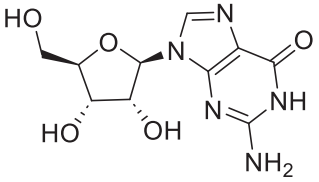
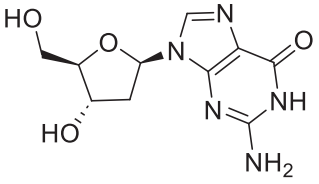
(*continued on next page*)

Table 1 (*continued*)

	Substrate	K_i or K_m value (μM)	n	ΔG^0 (KJ/mol)	δ (ΔG^0) (KJ/mol)	relative to
	1-Deazapurine	12.1 ± 1.6	4	-28.1	2.9	adenine
	1-Methylhypoxanthine	0.93 ± 0.13	3	-34.4	5.6	hypoxanthine
	1-Deazahypoxanthine (FH14875)	203 ± 33	4	-21.1	19.0	hypoxanthine
	9-Methyl,1-deazahypoxanthine (FH14872)	>1000	2	>−17.1	23.0	hypoxanthine
	9-Deazahypoxanthine	33.3 ± 4.9	4	-25.55	14.5	hypoxanthine
	9-deazaguanine (JBMAM002)	53.5 ± 7.6	3	-24.4	14.0	guanine
	9-deazaxanthine	>1000	3	>17.1	>10	Xanthine
	3-Deazahypoxanthine	45.7 ± 3.7	3	-24.8	15.3	hypoxanthine
	3-methylxanthine	NE,1000	3	>17.1	9.9	xanthine
	7-Deazahypoxanthine	>1000		>−17.1	>23	hypoxanthine

(*continued on next page*)

Table 1 (*continued*)

	Substrate	K_i or K_m value (μM)	n	ΔG^0 (KJ/mol)	$\delta(\Delta G^0)$ (KJ/mol)	relative to
	7-Deazaguanine	982 ± 161	3	-17.2	21.2	guanine
	Allopurinol	82.1 ± 9.6	4	-23.3	17.8	hypoxanthine
	Aminopurinol	753 ± 20	3	-17.8	13.1	Adenine
	7-Br-allopurinol (JBMAM034)	219 ± 38	4	-20.9	2.4	allopurinol
	8-Azahypoxanthine	770 ± 2	2	-17.8	22.3	hypoxanthine
	Inosine[b]	316 ± 24	3	-20.0	20.1	hypoxanthine
	Adenosine[b]	N.E., 1000	3			
	Guanosine	17.1 ± 5.1	5	-27.2	11.2	guanine
	2'-deoxyguanosine	53.0 ± 2.6	3	-24.4	2.79	guanosine

a, K_m value; [b], from reference [44]. NE, no effect at the indicated concentration.

hypoxanthine indicates that the 6-amino group of the former does not positively contribute. This was confirmed by the observation that 6-chloropurine displayed the same K_i value as adenine (3.65 ± 0.24 μM; Fig. 1, Table 1), ruling out that the amine group acts as a hydrogen bond donor.

Substitution of a thio group for the 6-keto group of hypoxanthine and guanine, afforded good inhibitors of the hypoxanthine transport, although with somewhat higher K_i values of 0.42 ± 0.02 μM ($P < 0.0001$; $\delta(\Delta G^0) = 3.7$ KJ/mol) and 0.36 ± 0.04 μM ($P < 0.05$; $\delta(\Delta G^0) = 1.59$ KJ/mol) for 6-mercaptopurine and 6-thioguanine, respectively (Fig. 2, Table 1). The 6-thio for 6-oxo substitution disfavours H-bond formation, while retaining protonation of N1. The small difference in binding energy between the oxo and thio substrates should be attributed to either the larger size of the sulphur atom compared to oxygen (steric

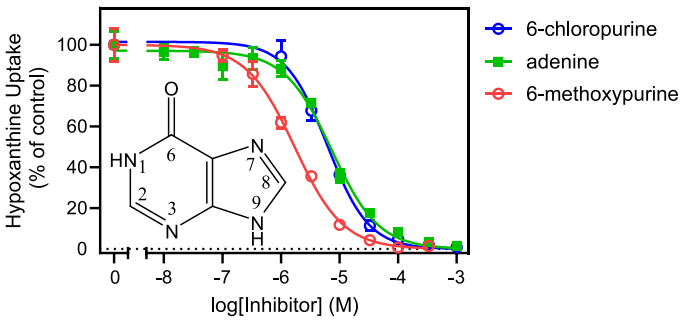


Fig. 1. Transport of 50 nM [^{3}H]-hypoxanthine over 3 s by TcrNB1 expressed in *T. b. brucei* NB-KO procyclics, inhibited by various concentrations of 6-substituted purine nucleobases: 6-aminopurine (adenine), 6-chloropurine and 6-methoxypurine, showing that the methoxy substitution provides superior affinity to the transporter compared to other small single-bond substitutions. Inset: structure of hypoxanthine with purine ring numbering. All symbols are the average of triplicates and error bars are SEM (when not shown error bars fall within the symbol). Hill slopes were consistently near −1, indicating uptake by a single transporter. Each inhibitor experiment is representative of at least three identical experiments with very similar outcomes.

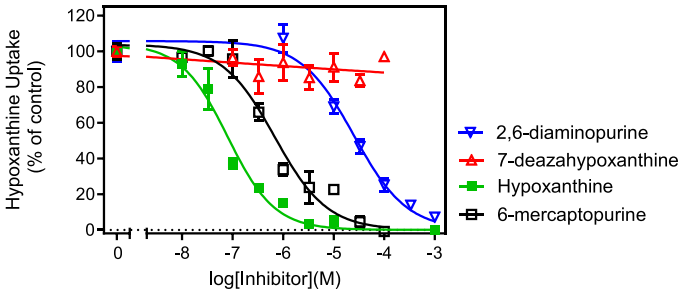


Fig. 2. Effect of several hypoxanthine and guanine analogues on the transport of 50 nM [^{3}H]-hypoxanthine by Tbb NB-KO procyclics. Hypoxanthine is included as the high affinity benchmark, against which can be seen that substitution of N7 for carbon or 6-oxo for thio (6-mercaptopurine) or amine (2,6-diaminopurine) is deleterious for binding. Symbols are the average of triplicate and SEM.

hindrance) or a difference in the tautomeric equilibrium of protonation states at N1 and N3. The former can be ruled out by the observation that TcrNB1 actually has somewhat higher affinity for 6-methoxypurine than for adenine (6-aminopurine; $P < 0.05$; $\delta(\Delta G^0) = 2.5$ KJ/mol)(Fig. 1) although the methoxy group is larger than the amine. We thus conclude that substitutions at position 6 are not severely size-limited and do not directly contribute to binding. However, the preference of 6-keto and -thio substituents to form amide rather than imide tautomers, resulting in a protonated N1, confers the strong preference of TcrNB1 to bind these bases over adenine.

The contribution of N1(H) to binding, then, can be most directly derived from pairwise comparisons between hypoxanthine or guanine with their respective analogues that have small single-bond substitutions at position 6. Hypoxanthine versus adenine or 6-chloropurine yields a $\delta(\Delta G^0)$ of 9.1 KJ/mol in both cases and the comparison of guanine with 2,6-diaminopurine shows a $\delta(\Delta G^0)$ of 10.5 KJ/mol (Fig. 2, Table 1) – yielding an average of −9.6 KJ/mol in binding energy for N1(H), which presumably acts as a hydrogen bond donor. The unprotonated N1 probably does not contribute directly to the binding of adenine, although differences in charge distribution around the ring, and/or tautomeric equilibrium shifted 1-deazapurine ($\delta(\Delta G^0) = 2.9$ KJ/mol versus adenine; $P < 0.05$) and 1-deazaadenine ($\delta(\Delta G^0) = 5.6$ KJ/mol versus adenine; $P < 0.01$) to lower affinity.

Further evidence of a contribution by N1(H) comes from the 10-fold higher K_i value for 1-methylhypoxanthine compared to hypoxanthine ($\delta(\Delta G^0) = 5.6$ KJ/mol) (Fig. 3, Table 1), although it cannot be excluded that steric hindrance could be part of this energy difference. 1-Deazahypoxanthine displayed a strongly diminished affinity with a K_i value of 202 ± 33 μM (Fig. 3), representing a loss of 19 KJ/mol in binding energy, which is greater than the loss of the hydrogen bond at N1(H) only, apparently due to a tautomeric state affecting the protonation of the other purine ring nitrogens, specifically N3.

9-Methyl,1-deazahypoxanthine did not inhibit uptake of 50 nM [^{3}H]-hypoxanthine at all ($K_i > 1$ mM), reflecting a loss of at least 23 KJ/mol in a side-by-side comparison with hypoxanthine and approximately 14 KJ/mol relative to adenine (N9 is protonated in both hypoxanthine and adenine). The importance of N9(H) in the binding of nucleobases by TcrNB1 was further compared by pairwise comparisons of 9-deazahypoxanthine versus hypoxanthine ($\delta(\Delta G^0) = 14.5$ KJ/mol), 9-deazaguanine versus guanine ($\delta(\Delta G^0)$ =14.0; Fig. 4, Table 1) and 9-deazaxanthine, which did not inhibit at 1 mM, versus xanthine ($\delta(\Delta G^0) > 10$). These observations are all consistent with a strong interaction to N9(H), likely a H-bond. The requirement of a hydrogen bond to N9(H) of course necessitates the proximity of an H-bond acceptor in the TcrNB1 binding pocket and provides a clear rationale for the strong selectivity of nucleobases over nucleosides.

The involvement of an unprotonated N3 as an apparent hydrogen bond acceptor is evident from the comparison of hypoxanthine and 3-deazahypoxanthine, which displayed a K_i value of 45.7 ± 3.7 μM ($P < 0.0001$; $\delta(\Delta G^0) = 15.3$ KJ/mol; Fig. 4). Xanthine, which differs from hypoxanthine by the 2-keto and consequent protonation of N3, yielded a similar loss of binding energy in pairwise comparison with hypoxanthine ($P < 0.001$; $\delta(\Delta G^0) = 13.1$ KJ/mol), consistent with the need for an unprotonated N3, although a possible steric or other effect of the 2-keto group makes this value less certain. The observation that 1 mM of 3-methylxanthine was unable to inhibit hypoxanthine uptake (Fig. 4), however, lends further credibility to a prominent role of N3 in substrate binding.

The nitrogen residue at position 7 is unprotonated in the purine bases hypoxanthine, guanine and adenine, and contributes 12.5 KJ/mol to the binding of nucleobases by the *T. b. brucei* H2 transporter [27] and the *Leishmania major* NT3 carrier [28]. For the TcrNB1 carrier, we again found that the 7-deaza analogues of hypoxanthine and guanine displayed much lower affinity than their respective nucleobases – in fact we were unable to obtain >50% inhibition at 1 mM of 7-deazahypoxanthine or 7-deazaguanine (Fig. 2), making it impossible to put a specific value on the Gibbs free energy of the interaction. The antiprotozoal drug allopurinol [50,51], in which N7 has shifted to position 8 of the purine ring, also displayed quite low affinity, with a K_i of 82.1 ± 9.6 μM (Fig. 3) although this result appears to indicate that the new N8 in part compensates for the loss of N7. Aminopurinol displayed lower affinity than

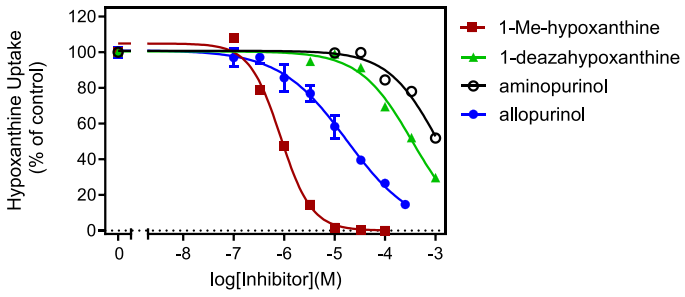


Fig. 3. Effects of 1-substited hypoxanthine analogues and pyrazolopyrimidines on the transport of 50 nM [^{3}H]-hypoxanthine by Tbb NB-KO procyclics. The carbon-substitution of N1, giving rise to different tautomeric states, resulted in much lower affinity than the methylation of N1, which retains the tautomeric state with N3, N7 and N9(H). The 6-oxo pyrazolopyrimidine displayed substantially higher affinity than the 6-amine analogue, reflecting a similar shift for hypoxanthine and adenine. All points were determined in triplicate; averages and SEM are shown.

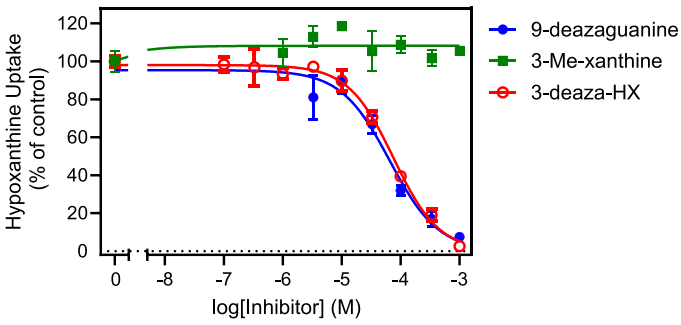


Fig. 4. Examples of the low affinity for TcrNB1 of purine nucleobase analogues that lack an unprotonated N3 (3-methylxanthine, 3-deazahypoxanthine) or a protonated N9 (9-deazaguanine). The experiments shown are representative of 3 – 4 identical experiments measuring transport of 50 nM [^{3}H]-hypoxanthine by TcrNB1 expressed in *T. b. brucei* NB-KO procyclics. Cells were incubated with radiolabel, in the presence and absence of inhibitor, for 3 seconds before addition of ice-cold stop solution and centrifugation through oil. Symbols are average and SEM of triplicate determinations.

allopurinol for TcrNB1 (K_i = 753 ± 20 μM; $\delta(\Delta G^0)$ = 5.5 KJ/mol; Fig. 3), in line with the similar difference between hypoxanthine and adenine, which shows that these pyrazolopyrimidines very likely assume the same binding pocket orientation as the corresponding purine nucleobases. A steric restriction at position 7 is therefore a likely conclusion from the comparison of allopurinol and 7-bromoallopurinol (K_i = 219 ± 38 μM; $\delta(\Delta G^0)$ = 2.4 KJ/mol).

The above analysis leads to a model of preferential binding of oxopurine nucleobases by TcrNB1 through strong interactions with N1(H), N3, N7 and N9(H). However, complexities such as shifting tautomeric equilibrium, charge distribution around the ring, and the limits of solubility for some of the analogues, make the quantitative assignment of exact amounts of interaction energy to each of those positions uncertain.

4. Discussion

Purine and pyrimidine analogues have been clinically used for decades as antiviral [52,53] and anticancer agents [54,55] and have equally been identified as possessing promising activity against a broad range of protozoa [56–59]. Recent developments towards the implementation of a nucleoside-based therapy for antiprotozoal infections have included a class of substituted 7-deaza-adenosine and -inosine analogues [60–64], including against *T. cruzi* [17,65,66]. There is abundant evidence that the antiprotozoal activity requires suitable transporters for the nucleoside/nucleobase analogues and that this is often a defining issue for activity and resistance [49,61]. For instance, the antibiotic nucleosides cordycepin (3'-deoxyadenosine) and tubercidin (7-deazaadenosine) rely on the TbAT1/P2 transporter of *T. b. brucei* for their trypanocidal activity, and resistance to either is observed upon deletion of the TbAT1 gene [67]. This reliance on specific transporters limits the modifications that can be made to an antiprotozoal nucleoside, as 2-F-cordycepin retained almost all trypanocidal activity and affinity for TbAT1/P2, but 2-Cl-cordycepin lost most affinity for this transporter, and its trypanocidal activity [68]. Moreover, the specific transporter for an anti-infective nucleoside drug may be restricted to certain (sub)-species, resulting in narrow activity of the compound. To remain with the same example, whereas *brucei*-group trypanosomes (*T. b. brucei*, *T. evansi*, *T. equiperdum*) have a TbAT1/P2 transporter, *T. congolense*, *T. vivax* and *T. cruzi* do not [35,64,69]. To counteract this disadvantage and identify broad-spectrum trypanocides we have recently focussed on the P1-type nucleoside transporters that are shared by all the main African trypanosome species and identified analogues with strong activity against all these species [49].

However, *T. cruzi*, the 'American trypanosome', does not express a classical P1-type nucleoside transporter (i.e. high affinity for adenosine, inosine and guanosine and their respective 2'-deoxynucleosides [70]) and, compared with many other kinetoplastid parasites, the nucleoside/nucleobase carriers of *T. cruzi* have not been investigated in great detail. However, this species appears to have 4 ENT genes, that encode carriers for inosine/guanosine (TcrNT1), for thymidine/tubercidin (TcrNT2), for adenine (TcrNB2) and for hypoxanthine/guanine (TcrNB1) [35,44,45]. A thorough understanding of the basis for selectivity of these carriers is essential for any pharmacological targeting of the parasite's nucleoside metabolism and here we present the in-depth analysis of the TcrNB1 transporter.

In our previous report [44] we already described the very high affinity for hypoxanthine and guanine, the more modest affinity for adenine and low affinity for nucleosides. Selectivity of nucleobases over nucleosides was shown to be the result, at least in part, of a strong interaction between N9(H) of the purine substrate with the TcrNB1 binding pocket. The 9-deaza analogues of hypoxanthine, guanine and xanthine all displayed a much-reduced affinity, translating into a loss of ~14 KJ/mol in Gibbs free energy. TcrNB1 does not appear to significantly interact with substitutions at position 6 and allows at least some size flexibility there, judging from the quite high affinity for 6-methoxypurine (and this could be further explored), and thus does not meaningfully contribute to the preference for oxopurine bases. This leaves the protonation state of N1 as the only other difference between adenine and hypoxanthine, and the very high K_i values for 1-deazahypoxanthine and 9-methyl,1-deazahypoxanthine confirmed the importance of a protonated N1. Similarly, 3-deaza and 7-deaza analogues of hypoxanthine and/or guanine displayed very low affinity for TcrNB1 and further evidence (xanthine, 3-methylxanthine, allopurinol, aminopurinol) was all consistent with both of these positions being involved in binding, in their unprotonated state.

The optimal binding of purine bases by TcrNB1 thus requires N1(H), N3, N7 and N9(H). Interestingly, this is very different from the binding motif of the human facilitative nucleobase transporter (hFNT1) that we previously characterised in erythrocytes, and requires interactions with a 6-oxo or -amine, unprotonated N1 and N3 and 2-amino in addition to π-orbital interactions [27]. However, the binding motif of TcrNB1 is identical to that of the *T. b. brucei* H2 nucleobase transporter [27] and the equivalent transporter NBT1 of *L. major* [28]. It thus emerges that the TcrNB1 binding motif is absolutely conserved with the other main kinetoplastid pathogens, *T. brucei* spp and *Leishmania* spp, but very different from the human transporter binding mode – a difference that can easily be exploited to specifically target nucleobase analogues to the parasites while limiting penetration into human cells, as discussed by Wallace et al. [27]. A programme of screening of nucleobase analogues based on these findings would be a logical next step.

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Author declaration

The authors declare that all experimental procedures were performed in accordance with applicable laws in the respective countries where they were performed. No ethical permissions were required for the experimental work undertaken.

CRediT authorship contribution statement

Serge Van Calebergh: Writing – review & editing, Supervision, Funding acquisition. **Harry P. De Koning:** Writing – review & editing, Writing – original draft, Validation, Supervision, Data curation, Conceptualization. **Mustafa A Aldfer:** Writing – original draft,

Investigation. **Fabian Hulpia:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] W.H.O, Chagas disease in Latin America: an epidemiological update based on 2010 estimates, Wkly Epidemiol. Rec. 90 (2015) 33–44.
[2] M.N. Garcia, D. Aguilar, R. Gorchakov, S.N. Rossmann, S.P. Montgomery, H. Rivera, L. Woc-Colburn, P.J. Hotez, K.O. Murray, Case report: evidence of autochthonous Chagas disease in southeastern Texas, Am. J. Trop. Med. Hyg. 92 (2015) 325.
[3] R.M. Mills, Chagas disease: epidemiology and barriers to treatment, Am. J. Med. 133 (2020) 1262–1265.
[4] C. Bern, L.A. Messenger, J.D. Whitman, J.H. Maguire, Chagas disease in the united states: a public health approach, Clin. Microbiol. Rev. 33 (2019) e00023–19.
[5] E.E. Conners, T.L. Ordoñez, C. Cordon-Rosales, C.F. Casanueva, S.M. Miranda, K. C. Brouwer, Chagas disease infection among migrants at the Mexico/Guatemala border, Am. J. Trop. Med. Hyg. 97 (2017) 1134–1140.
[6] S. Antinori, L. Galimberti, R. Bianco, R. Grande, M. Galli, M. Corbellino, Chagas disease in Europe: a review for the internist in the globalized world, Eur. J. Intern. Med. 43 (2017) 6–15.
[7] K.C.F. Lidani, F.A. Andrade, L. Bavia, F.S. Damasceno, M.H. Beltrame, I.J. Messias-Reason, T.L. Sandri, Chagas disease: from discovery to a worldwide health problem, Front. Public Health 7 (2019) 166.
[8] C. Barona-Vilar, M.J. Giménez-Martí, T. Fraile, C. González-Steinbauer, C. Parada, A. Gil-Brusola, D. Bravo, et al., Prevalence of *Trypanosoma cruzi* infection in pregnant Latin American women and congenital transmission rate in a non-endemic area: the experience of the Valencian health programme (Spain), Epidemiol. Infect. 140 (2012) 1896–1903.
[9] J. Guarner, Chagas disease as example of a reemerging parasite, Semin. Diagn. Pathol. 36 (2019) 164–169.
[10] B.L. Timm, A.N.S. da Gama, M.M. Batista, Dd.G.J. Batista, D.W. Boykin, H.P. De Koning, Soeiro Correia, MdN, Arylimidamides have potential for chemoprophylaxis against blood-transmitted Chagas disease, Pathogens 12 (2023) 701.
[11] P. García-Huertas, N. Cardona-Castro, Advances in the treatment of Chagas disease: promising new drugs, plants and targets, Biomed. Pharmacother. 142 (2021) 112020.
[12] C. Bern, Chagas' disease, N. Engl. J. Med. 373 (2015) 456–466.
[13] J.A.M. Pérez-Molina, I. Molina, Chagas disease, Lancet 391 (2018) 82–94.
[14] P.A. Sales Junior, I. Molina, S.M. Fonseca Murta, A. Sánchez-Montalvá, F. Salvador, R. Corrêa-Oliveira, C.M. Carneiro, Experimental and clinical treatment of Chagas disease: a review, Am. J. Trop. Med. Hyg. 97 (2017) 1289–1303.
[15] J.S. De Araújo, P.B. da Silva, M.M. Batista, R.B. Peres, C. Cardoso-Santos, T. D. Kalejaiye, J.C. Munday, E. De Heuvel, G.J. Sterk, K. Augustyns, I.G. Salado, A. Matheeussen, I. De Esch, H.P. De Koning, R. Leurs, L. Maes, M.N.C. Soeiro, Evaluation of phthalazinone phosphodiesterase inhibitors with improved activity and selectivity against *Trypanosoma cruzi*, J. Antimicrob. Chemother. 75 (2020) 958–967.
[16] J.S. De Araújo, C. França da Silva, D. da Gama Jaén Batista, A.N. Silva da Gama, L. Ferreira de Almeida Fiuza, C.R. Fonseca-Berzal, P. Bernardino da Silva, M. Meuser Batista, M. Sijm, T.D. Kalejaiye, H.P. De Koning, L. Maes, G.J. Sterk, R. Leurs, M.N. Correia Soeiro, Efficacy of novel pyrazolone phosphodiesterase inhibitors in experimental mouse models of *Trypanosoma cruzi*, Antimicrob. Agents Chemother. 64 (2020) e00414-20.
[17] L.F. De Almeida Fiuza, D.G.J. Batista, R.D. Girão, F. Hulpia, P. Finamore, M. M. Aldfer, E.K. Elmahallawy, H.P. De Koning, O.C. Moreira, S. Van Calenbergh, M. N. Soeiro, Phenotypic evaluation of nucleoside analogues against *Trypanosoma cruzi* infection: *in vitro* and *in vivo* approaches, Molecules 27 (2022) 8087.
[18] J. González-Bacerio, I. Arocha, M.E. Aguado, Y. Méndez, S. Marsiccobetre, M. Izquierdo, D.G. Rivera, K. Figarella, N.L. Uzcátegui, KBE009: A bestatin-like inhibitor of the *Trypanosoma cruzi* acidic M17 aminopeptidase with in vitro anti-trypanosomal activity, Life 11 (2021) 1037.
[19] J.J. Wen, P.C. Yachelini, A. Sembaj, R.E. Manzur, N.J. Garg, Increased oxidative stress is correlated with mitochondrial dysfunction in chagasic patients, Free Radic. Biol. Med. 41 (2006) 270–276.
[20] N. Gironès, S. Carbajosa, N.A. Guerrero, C. Poveda, C. Chillon-Marinas, M. Fresno, Global metabolomic profiling of acute myocarditis caused by *Trypanosoma cruzi* infection, PLoS Negl. Trop. Dis. 8 (2014) e3337.
[21] S. Shah-Simpson, G. Lentini, P.C. Dumoulin, B.A. Burleigh, Modulation of host central carbon metabolism and in situ glucose uptake by intracellular Trypanosoma cruzi amastigotes, PLoS Pathog. 13 (2017) e1006747.
[22] K.L. Caradonna, J.C. Engel, D. Jacobi, C.H. Lee, B.A. Burleigh, Host metabolism regulates intracellular growth of *Trypanosoma cruzi*, Cell Host Microbe 13 (2013) 108–117.
[23] W.E. Gutteridge, M. Gaborak, A re-examination of purine and pyrimidine synthesis in the three main forms of *Trypanosoma cruzi*, Int. J. Biochem 10 (1979) 415–422.
[24] J. Nakajima-Shimada, Y. Hirota, T. Aoki, Inhibition of *Trypanosoma cruzi* growth in mammalian cells by purine and pyrimidine analogs, Antimicrob. Agents Chemother. 40 (1996) 2455–2458.
[25] H.P. De Koning, S.M. Jarvis, Hypoxanthine uptake through a purine-selective nucleobase transporter in *Trypanosoma brucei brucei* procyclics is driven by protonmotive force, Eur. J. Biochem. 247 (1997) 1102–1110.
[26] H.P. De Koning, S.M. Jarvis, Purine nucleobase transport in bloodstream forms of *Trypanosoma brucei brucei* is mediated by two novel transporters, Mol. Biochem. Parasitol. 89 (1997) 245–258.
[27] L.J.M. Wallace, D. Candlish, H.P. De Koning, Different substrate recognition motifs of human and trypanosome nucleobase transporters: selective uptake of purine antimetabolites, J. Biol. Chem. 277 (2002) 26149–26156.
[28] M.I. Al-Salabi, L.J.M. Wallace, H.P. De Koning, A *Leishmania major* nucleobase transporter responsible for allopurinol uptake is a functional homologue of the *Trypanosoma brucei* H2 transporter, Mol. Pharmacol. 63 (2003) 814–820.
[29] M.I. Al-Salabi, H.P. De Koning, Purine nucleobase transport in amastigotes of Leishmania mexicana: involvement in allopurinol uptake, Antimicrob. Agents Chemother. 49 (2005) 3682–3689.
[30] D. Ortiz, M.A. Sanchez, H.P. Koch, H.P. Larsson, S.M. Landfear, An acid-activated nucleobase transporter from Leishmania major, J. Biol. Chem. 284 (2009) 16164–16169.
[31] D. Ortiz, M.A. Sanchez, S. Pierce, T. Herrmann, N. Kimblin, H.G. Archie Bouwer, S. M. Landfear, Molecular genetic analysis of purine nucleobase transport in *Leishmania major*, Mol. Microbiol. 64 (2007) 1228–1243.
[32] M.A. Sanchez, R. Tryon, S. Pierce, G. Vasudevan, S.M. Landfear, Functional expression and characterization of a purine nucleobase transporter gene from Leishmania major, Mol. Membr. Biol. 21 (2004) 11–18.
[33] R. Burchmore, L.J.M. Wallace, D. Candlish, M.I. Al-Salabi, P. Beal, M.P. Barrett, S. A. Baldwin, H.P. De Koning, Cloning, heterologous expression, and in situ characterization of the first high affinity nucleobase transporter from a protozoan, J. Biol. Chem. 278 (2003) 23502–23507.
[34] C. Henriques, M.A. Sanchez, R. Tryon, S.M. Landfear, Molecular and functional characterization of the first nucleobase transporter gene from African trypanosomes, Mol. Biochem. Parasitol. 130 (2003) 101–110.
[35] M.M. Aldfer, T.A. AlSiari, H.A.A. Elati, M.J. Natto, I.A. Alfayez, G.D. Campagnaro, B. Sani, R.J.S. Burchmore, G. Diallinas, H.P. de Koning, Nucleoside transport and nucleobase uptake null mutants in *Leishmania mexicana* for the routine expression and characterisation of purine and pyrimidine transporters, Int. J. Mol. Sci. 23 (2022) 8139.
[36] N.B. Quashie, D. Dorin-Semblat, P.G. Bray, G.A. Biagini, C. Doerig, L.C. Ranford-Cartwright, H.P. De Koning, A comprehensive model of purine uptake by the malaria parasite *Plasmodium falciparum*: identification of four purine transport activities in intraerythrocytic parasites, Biochem. J. 411 (2008) 287–295.
[37] G.D. Campagnaro, H.A.A. Elati, S. Balaska, M.E. Martin Abril, M.J. Natto, F. Hulpia, K. Lee, L. Sheiner, S. Van Calenbergh, H.P. De Koning, A *Toxoplasma gondii* oxopurine transporter binds nucleobases and nucleosides using different binding modes, Int. J. Mol. Sci. 23 (2022) 710.
[38] H.P. De Koning, S.M. Jarvis, A highly selective, high affinity transporter for uracil in *Trypanosoma brucei brucei*; evidence for proton-dependent transport, Biochem. Cell Biol. 76 (1998) 853–858.
[39] I.G. Papageorgiou, L. Yakob, M.I. Al-Salabi, G. Diallinas, K. Soteriadou, H.P. De Koning, Identification of the first pyrimidine nucleobase transporter in Leishmania: similarities with the *Trypanosoma brucei* U1 transporter and antileishmanial activity of uracil analogues, Parasitology 130 (2005) 275–283.
[40] S. Gudin, N.B. Quashie, D. Candlish, M.I. Al-Salabi, S.M. Jarvis, L.C. Ranford-Cartwright, H.P. De Koning, *Trypanosoma brucei*: a survey of pyrimidine transport activities, Exp. Parasitol. 114 (2006) 103–108.
[41] G.D. Campagnaro, K.J.H. Alzahrani, J.C. Munday, H.P. De Koning, *Trypanosoma brucei* bloodstream forms express highly specific and separate transporters for adenine and hypoxanthine; evidence for a new protozoan purine transporter family? Mol. Biochem. Parasitol. 220 (2018) 46–56.
[42] H.P. De Koning, Pyrimidine transporters of protozoa – A class apart? Trends Parasitol. 23 (2007) 190.
[43] F. Tohidi, Z. Babaei, B. Kazemi, M. Bandehpour, I. Sharifi, M.R. Rabiei, E.S. Dezaki, Transient knockdown of Nucleoside transporter 4 gene expression as a therapeutic target in *Leishmania major* by antisense RNA: In vitro and in vivo studies, J. Vector Borne Dis. 56 (2019) 98–104.
[44] G.D. Campagnaro, J. de Freitas Nascimento, R.B.M. Girard, A.M. Silber, H.P. De Koning, Cloning and characterisation of the equilibrative nucleoside transporter family of *Trypanosoma cruzi*: ultra-high affinity and selectivity to survive in the intracellular niche, Biochim. Biophys. Acta Gen. Subj. 1862 (2018) 2750–2763.
[45] M. Aldfer, I.A. Alfayez, H.A.A. Elati, N. Gayen, E.K. Elmahallawy, A.M. Murillo, S. Marsiccobetre, S. Van Calenbergh, A.M. Silber, H.P. De Koning, The *Trypanosoma cruzi* nucleoside transporter TcrNT2 is a conduit for the uptake of 5-F-2'deoxyuridine and tubercidin analogues, Molecules 27 (2022) 8045.
[46] Y.-C. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction, Biochem Pharmacol. 22 (1973) 3099–3108.
[47] H.P. De Koning, C.J. Watson, S.M. Jarvis, Characterisation of a nucleoside/proton symporter in procyclic *Trypanosoma brucei brucei*, J. Biol. Chem. 273 (1998) 9486–9494.
[48] M.I. Al-Salabi, L.J.M. Wallace, A. Lüscher, P. Mäser, D. Candlish, B. Rodenko, M. K. Gould, I. Jabeen, S.N. Ajith, H.P. De Koning, Molecular interactions underlying the unusually high adenosine affinity of a novel *Trypanosoma brucei* nucleoside transporter. Mol. Pharmacol. 71 (2007) 921–929.

[49] M.A. Ungogo, M.M. Aldfer, M.J. Natto, H. Zhang, R. Chisholm, K. Walsh, M. C. McGee, K. Ilbeigi, J.I. Asseri, R.J.S. Burchmore, G. Caljon, S. Van Calenbergh, H. P. De Koning, Cloning and characterisation of Trypanosoma congolense and T. vivax nucleoside transporters reveal the potential of P1-type carriers for the discovery of broad-spectrum nucleoside-based therapeutics against animal African Trypanosomiasis, Int. J. Mol. Sci. 24 (2023) 3144.
[50] W. Apt, A. Arribada, I. Zulantay, A. Solari, G. Sánchez, K. Mundaca, X. Coronado, J. Rodríguez, L.C. Gil, A. Osuna, Itraconazole or allopurinol in the treatment of chronic American trypanosomiasis: the results of clinical and parasitological examinations 11 years post-treatment, Ann. Trop. Med. Parasitol. 99 (2005) 733–741.
[51] S. Martinez, J.J. Marr, Allopurinol in the treatment of American cutaneous leishmaniasis, N. Engl. J. Med. 326 (1992) 741–744.
[52] R.J. Geraghty, M.T. Aliota, L.F. Bonnac, Broad-spectrum antiviral strategies and nucleoside analogues, Viruses 13 (2021) 667.
[53] F. Amblard, D. Patel, E. Michailidis, S.J. Coats, M. Kasthuri, N. Biteau, Z. Tber, M. Ehteshami, R.F. Schinazi, HIV nucleoside reverse transcriptase inhibitors, Eur. J. Med. Chem. 240 (2022) 114554.
[54] L. Hruba, V. Das, M. Hajduch, P. Dzubak, Nucleoside-based anticancer drugs: mechanism of action and drug resistance, Biochem. Pharmacol. 215 (2023) 115741.
[55] C. Stresemann, F. Lyko, Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine, Int. J. Cancer 123 (2008) 8–13.
[56] B. Rodenko, A.M. Van der Burg, M.J. Wanner, M. Kaiser, R. Brun, M.K. Gould, H. P. De Koning, G.J. Koomen, 2,N^6-Disubstituted adenosine analogues with antitrypanosomal and antimalarial activity. Synthesis, uptake studies and in vivo evaluation, Antimicrob. Agents Chemother. 51 (2007) 3796–3802.
[57] P. Lawton, Purine analogues as antiparasitic agents, Expert Opin. Ther. Pat. 15 (2005) 987–994.
[58] M.H. El Kouni, Potential chemotherapeutic targets in the purine metabolism of parasites, Pharm. Ther. 99 (2003) 283–309.
[59] F. Ranjbarian, M. Vodnala, K.J.H. Alzahrani, G.U. Ebiloma, H.P. De Koning, A. Hofer, 9-(2-Deoxy-2-fluoro-ß-D-arabinofuranosyl) is a potent antitrypanosomal adenosine analogue that circumvents transport-related drug resistance, Antimicrob. Agents Chemother. 61 (2017) e02719–16.
[60] F. Hulpia, G.D. Campagnaro, M. Scortichini, K. Van Hecke, L. Maes, H.P. De Koning, G. Caljon, S. Van Calenbergh, Revisiting tubercidin against kinetoplastid parasites: aromatic substitutions at position 7 improve activity and reduce toxicity, Eur. J. Med. Chem. 164 (2019) 689–705.
[61] F. Hulpia, D. Mabille, G.D. Campagnaro, G. Schumann, L. Maes, I. Roditi, A. Hofer, H.P. De Koning, G. Galjon, S. Van Calenbergh, Combining tubercidin and cordycepin scaffolds results in highly active candidates to treat late-stage sleeping sickness, Nat. Commun. 10 (2019) 5564.
[62] F. Hulpia, J. Bouton, G.D. Campagnaro, I.A. Alfayez, D. Mabille, L. Maes, H.P. De Koning, G. Caljon, S. Van Calenbergh, C6-*O*-Alkylated 7-deazainosine nucleoside analogues: discovery of potent and selective anti-sleeping sickness agents, Eur. J. Med. Chem. 188 (2020) 112018.
[63] M.J. Natto, F. Hulpia, E.R. Kalkman, S. Baillie, A. Alhejeli, Y. Miyamoto, L. Eckmann, S. Van Calenbergh, H.P. De Koning, Deazapurine nucleoside analogues for the treatment of *Trichomonas vaginalis*, ACS Infect. Dis. 7 (2021) 1752–1764.
[64] D. Mabille, K. Ilbeigi, S. Hendrickx, M.A. Ungogo, F. Hulpia, C. Lin, L. Maes, H. P. De Koning, S. Van Calenbergh, G. Caljon, Nucleoside analogues for the treatment of animal African trypanosomiasis, Int. J. Parasitol. Drugs Drug Resist 19 (2022) 21–30.
[65] C. Lin, F. Hulpia, C.F. da Silva, D.D.G.J. Batista, K. Van Hecke, L. Maes, G. Caljon, M.N.C. Soeiro, S. Van Calenbergh, Discovery of Pyrrolo[2,3-b]pyridine (1,7-Dideazapurine) nucleoside analogues as anti-*Trypanosoma cruzi* agents, J. Med. Chem. 62 (2019) 8847–8865.
[66] C. Lin, F. Hulpia, I. Karalic, L. De Schepper, L. Maes, G. Caljon, S. Van Calenbergh, 6-Methyl-7-deazapurine nucleoside analogues as broad-spectrum antikinetoplastid agents, Int J. Parasitol. Drugs Drug Resist 17 (2021) 57–66.
[67] F. Geiser, A. Lüscher, H.P. De Koning, T. Seebeck, P. Mäser, Molecular pharmacology of adenosine transport in *Trypanosoma brucei*: P1/P2 revisited, Mol. Pharmacol. 68 (2005) 589–595.
[68] S.K. Vodnala, T. Lundbäck, E. Yeheskieli, B. Sjöberg, A.L. Gustavsson, R. Svensson, G.C. Olivera, A.A. Eze, H.P. De Koning, L.G.J. Hammarström, M.E. Rottenberg, Structure-activity relationships of synthetic cordycepin analogues as experimental therapeutics for African trypanosomiasis, J. Med. Chem. 56 (2013) 9861–9873.
[69] J.C. Munday, K.E. Rojas López, A.A. Eze, V. Delespaux, J. Van Den Abbeele, T. Rowan, M.P. Barrett, L.J. Morrison, H.P. De Koning, Functional expression of TcoAT1 reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake, Int J. Parasitol. Drugs Drug Resist 3 (2013) 69–76.
[70] H.P. De Koning, S.M. Jarvis, Adenosine transporters in bloodstream forms of *T. b. brucei*: substrate recognition motifs and affinity for trypanocidal drugs, Mol. Pharmacol. 56 (1999) 1162–1170.