Evaluation of nucleoside hydrolase inhibitors for the treatment of African trypanosomiasis

AUTHORS

Maya Berg¹, Linda Kohl², Pieter Van der Veken¹, Jurgen Joossens¹, Mohammed I. Al-Salabi³, Valeria Castagna⁴, Francesca Giannese⁴, Paul Cos⁵, Wim Versées⁶, Jan Steyaert⁶, Philippe Grellier², Achiel Haemers¹, Massimo Degano⁴, Louis Maes⁵, Harry P. de Koning³ and Koen Augustyns¹,*

ADDRESS OF INSTITUTIONS, AUTHOR’S AFFILIATION

¹ Laboratory of Medicinal Chemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium
² Muséum National d'Histoire Naturelle, USM 504-EA3335, Biologie Fonctionnelle des Protozoaires, 61 Rue Buffon, CP52, Paris Cedex 05, 75231, France
³ Institute of Biomedical and Life Sciences, Division of Infection and Immunity, Glasgow Biomedical Research Centre, University of Glasgow, 120 University Place, Glasgow G12 8TA, UK

AAC Accepts, published online ahead of print on 1 March 2010
Copyright © 2010, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.
Biocrystallography Unit and Mass Spectrometry Unit, DIBIT San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy

Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

Structural Biology Brussels, Vrije Universiteit Brussel and Department of Molecular and Cellular Interactions, VIB, Pleinlaan 2, B-1050, Brussels, Belgium.

(CORRESPONDING AUTHOR)

* Corresponding author. Laboratory of Medicinal Chemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium. Tel: +32 (0)3 265 27 17, fax: +32 (0)3 265 27 33

39. E-mail: koen.augustyns@ua.ac.be.
Abstract

In this paper we present the biochemical and biological evaluation of N-arylmethyl-substituted iminoribitol derivatives as potential chemotherapeutic agents against trypanosomiasis. Previously, a library of 52 compounds was designed and synthesized as potent and selective inhibitors of Trypanosoma vivax IAG-NH. However, when tested against bloodstream form Trypanosoma brucei brucei, only one inhibitor, N-(9-deaza-adenin-9-yl)methyl-1,4-dideoxy-1,4-imino-D-ribitol (UAMC-00363), displayed a significant activity (IC$_{50}$ = 0.49 ± 0.31 µM). A validation in an in vivo model of African trypanosomiasis showed promising results for this compound. Several experiments were performed to investigate why only UAMC-00363 showed antiparasitic activity. First, the compound library was screened against T. b. brucei IAG-NH and IG-NH to confirm the inhibitory effect of the compounds previously demonstrated on T. vivax IAG-NH. Second, to verify the uptake of these compounds by T. b. brucei, their affinity for the nucleoside P1 and nucleoside/nucleobase P2 transporter of T. b. brucei was tested. Only UAMC-00363 displayed a significant affinity towards the P2 transporter. It was also shown that UAMC-00363 is concentrated into the cell via at least one additional transporter, since it displayed no resistance against P2 knockout mutants of T. b. brucei. Consequently, no cross-resistance with the diamidine or the melaminophenyl arsenical classes of trypanocides is expected. Third, three enzymes of the purine salvage pathway of procyclic T. b. brucei (IAG-NH, IG-NH and MTAP) were investigated using RNAi knockdown. All these studies showed that it is probably not sufficient to target only the nucleoside hydrolase activity to block the purine salvage pathway of T. b. brucei and therefore it is possible that UAMC-00363 acts on an additional target.

Key words

inhibitors, nucleoside hydrolase, trypanosomiasis, Trypanosoma brucei brucei, transporters
Introduction

In the search for new selective trypanocidal drugs, it has been proposed that the purine metabolism of *Trypanosoma brucei* provides a valuable target. In contrast to mammals, all parasites are unable to synthesize purines *de novo*, and rely instead on the purine salvage pathway (PSP) to obtain purines which are essential for their survival. The PSP is essential for all stages of *T. brucei*. The following enzymes of the purine salvage of *T. b. brucei* are described in literature (Fig. 1): inosine-adenosine-guanosine nucleoside hydrolase (IAG-NH, EC.3.2.2.1), inosine guanosine nucleoside hydrolase (IG-NH, EC. 3.2.2.1), methyl thioadenosine phosphorylase (MTAP, E.C. 2.4.2.28), adenine phosphoribosyltransferase (APRT, E.C.2.4.2.7), hypoxanthine-guanine phosphoribosyltransferase (HGPRT, E.C.2.4.2.8), and adenosine kinase (AK, E.C. 2.7.1.20). Key enzymes in the purine salvage pathway of *Trypanosoma brucei* are the nucleoside hydrolases (NH, E.C. 3.2.2.1). In these parasites, purine bases are obtained by cleavage of the N-glycosidic bond of nucleosides by NH. In *T. b. brucei* two types of NH are present: the purine nucleoside-specific IAG-NH (*TbbIAG-NH*) which prefers inosine, adenosine and guanosine as substrates and the 6-oxopurines-specific IG-NH (*TbbIG-NH*) with high affinity towards inosine and guanosine. It should be noted that NH-activity is absent in mammalian cells. Therefore, NH might provide a good target for the development of new anti-trypanosomal compounds. Consequently, this target has been investigated during the past decade and potent inhibitors of NH have been developed. The immucillins and *N*-arylmethyl-substituted iminoribitol derivatives (Fig. 2) are the strongest inhibitors of *TvIAG-NH* and *TbbIAG-NH* known to date with *K*<sub>i</sub> values in the low nanomolar range. Additionally, many of these inhibitors show selectivity towards the human nucleoside cleaving enzyme, the purine nucleoside phosphorylase (hPNP) (4). So far, no antiparasitic data on *T. b. brucei* have ever been published for these classes of inhibitors. We
hereby reveal antiparasitic activities of the N-arylmethyl-substituted iminoribitol derivatives towards *T. b. brucei*. Interestingly, only one compound in a series of 52 compounds (see supplemental material) shows a remarkable antiparasitic activity *in vitro* against bloodstream form (BSF) *T. b. brucei* and *T. b. rhodesiense*. This activity was confirmed in an *in vivo* model of African trypanosomiasis. The lack of activity for all other compounds can be due to several factors. First, since enzymatic screening was performed on *T. vivax* IAG-NH as this enzyme was easily accessible at the time, differences between the NHs of *T. vivax* and *T. b. brucei* might be the cause of the lack of biological activity observed in *T. b. brucei*. The percent sequence identity for the protein IAG-NH between the orthologous organisms *T. vivax*, *T. b. brucei*, *T. congolense* and *Leishmania major* was determined with a shotgun alignment study (Mac Molly Tetra® software). The amino acid sequence of IAG-NH in *T. vivax* showed 68% of sequence identity with *T. b. brucei*, 64% with *T. congolense* and 56% with *L. major*. Second, absence of antiparasite activity can be a reflection of difficulties with uptake of the compounds. All protozoan parasites studied to date are auxotrophic for purines and depend on transport of various natural nucleosides and nucleobases (12). For *T. brucei*, the multiplicity of transporters is puzzling and it remains unclear why this unicellular parasite requires so many of them. Examination of the *T. brucei* genome revealed 12 distinct equilibrative nucleoside transporters (ENT) family members, most of which have now been shown to be involved in the uptake of nucleosides and/or nucleobases (31). N-arylmethyl-substituted iminoribitol derivatives might be transported into the cell via P1 and P2 transporters (Fig. 1). *TbAT1*, the first ENT gene to be identified in trypanosomes, encodes the P2 adenosine/adenine transporter (27). In addition to its role in purine salvage, it has been shown to be the main transporter for the melaminophenyl arsenicals (e.g. melarsoprol) and diamidine drugs (e.g. pentamidine) that constitute the first-line treatment for trypanosomiasis (7, 28). This transporter is only expressed in BSF parasites (16).
cluster of genes on chromosome two, TbNT2-7, encode a number of purine nucleoside transporters, collectively known as P1 transporters, several of which are expressed in bloodstream forms (35). They mediate the uptake of adenosine, inosine, guanosine and in some cases hypoxanthine (7, 16, 35). Two additional P1-type transporters, designated TbNT10/AT-B and TbNT9/AT-D, are located on chromosome 6 and 9 (2). Third, the target enzyme NH might not be crucial for the survival of the parasite through the existence of bypass mechanisms in the PSP (Fig. 1). To investigate these different hypotheses several experiments were performed. The most potent compounds of the library (Fig. 2) were screened against TbbIAG-NH and TbbIG-NH to confirm the inhibitory effect of the compounds previously demonstrated on TvIAG-NH. A set of transporter studies was also performed. Uptake of these compounds was verified by affinity measurements for the nucleoside P1 and nucleoside/nucleobase P2 transporter of T. b. brucei. In order to examine the importance of the purine salvage enzymes for growth and survival of procyclic T. b. brucei the protein expression of a set of enzymes (IAG-NH, IG-NH and MTAP) was knocked down by RNA interference (RNAi).
**Materials and Methods**

**Synthesis of \( N\)-(9-deaza-adenin-9-yl)methyl-1,4-dideoxy-1,4-imino-D-ribitol (UAMC-00363)**

The synthesis of compound UAMC-00363 has been described previously (22) but minor changes were made in the last reaction step and purification: iminoribitol·HCl (0.15 g, 0.887 mmol), NaOAc (0.08 g, 0.975 mmol) and 37% aq. formaldehyde (0.073 ml, 0.975 mmol) were dissolved in \( H_2O/dioxane \) (4:1, 2 ml/mmol). The reaction mixture was stirred at 95°C for 1.5 h. 9-deazaadenine (0.242 g, 0.975 mmol) was added to the solution and stirred at 95°C for an additional hour. After cooling down to room temperature, the crude mixture was converted to its HCl salt with 1N HCl. Excess HCl was removed by evaporation under reduced pressure and the crude was adsorbed on silica gel, which was then evaporated to dryness under reduced pressure. Purification by column chromatography (DCM:MeOH:N\( \text{H}_2\text{O} \) 80:20:1 to 80:20:5) yielded the product which was again converted to its HCl salt (0.045 g, 15%). Nuclear magnetic resonance data, mass measurements and HPLC data can be found in the supplemental material.

**Enzyme assays**

Enzyme inhibition studies on *T. vivax* IAG-NH: details of the assay and experimental determination of \( K_i \) have been described (22).

Enzyme inhibition studies on *T. b. brucei* IAG-NH and IG-NH: enzymatic assays were performed on recombinant, purified enzymes (details to be published). All measurements were carried out at 37°C in 50 mM Hepes pH 7.3. The \( \Delta\varepsilon \) values (mM\(^{-1}\)·cm\(^{-1}\)) used were: -4.0 at 260 nm for guanosine, and -0.82 at 280 nm for inosine. Progress curves were recorded on an UltroSpec2100 (GE Healthcare). For the IG-NH enzyme, the inhibition constants for the compounds (\( K_i \)) were determined by measuring the initial velocities for the enzymatic hydrolysis of a fixed guanosine
concentration nearing $K_M$ with at least three inhibitor concentrations. Competitive inhibition was assumed based on the similarity between substrate and inhibitor. Experimental data were fitted to the equation:

$$\frac{v_i}{v_0} = \frac{K_M + [S]}{K_M(1 + \frac{[I]}{K_I}) + [S]}$$

where $v_i$ and $v_0$ represent the initial reaction rates in presence and absence of inhibitor, $[S]$ and $[I]$ are the substrate and inhibitor concentrations, respectively. For the IAG-NH enzyme, inosine was used as substrate and inhibition constants were determined by full kinetic analysis, measuring the apparent Michaelis constant $K_M^*$ in presence of a fixed inhibitor concentration. Initial velocities measured at different substrate concentrations were fitted to the Michaelis-Menten equation using the program Graphpad Prism (GraphPad Software, Inc). Inhibition constants were calculated through the following equation for competitive inhibition:

$$K_M^* = K_M \cdot (1 + \frac{[I]}{K_I})$$

Enzyme inhibition studies on human PNP: details of the assay and experimental determination of $K_i$ have been described (4).

Quantification of in vitro antiparasitic activity

The *Trypanosoma brucei brucei* Lister 427 strain (suramin-sensitive) or *T. b. rhodesiense* (strain STIB-900) were used for screening at the University of Antwerp (UA). The strains were maintained in Hirumi (HMI-9) medium, supplemented with 10% inactivated fetal calf serum. All cultures and assays were conducted at 37°C under an atmosphere of 5% CO$_2$. Assays were performed in sterile 96-well microtiter plates, each well containing 200 µl of medium containing $1.5 \times 10^4$ parasites/well and the test compound at appropriate dilution. Compounds were tested at
5 concentrations (64, 16, 4, 1 and 0.25 µM or mg/ml). Parasite growth was compared to untreated-infected (100% parasite growth) and uninfected controls (0% growth). After 3 days of incubation, parasite growth was assessed fluorimetrically after addition of 50 µl resazurin (10µg/ml) (Alamar blue) per well. After 15 hrs (T. b. rhodesiense) or 24 hrs (T. b. brucei) at 37°C, fluorescence was measured (λex 550 nm, λem 590 nm). The results were expressed as % reduction in parasite growth/viability compared to control wells and an IC50 (50% inhibitory concentration) was calculated. When IC50 < 1 µM, a secondary screening is performed on T. b. brucei and T. b. rhodesiense and IC50-values were determined using an extended dose range (2-fold compound dilutions). Compounds were also tested against a nifurtimox-sensitive T. cruzi Tulahuen CL2 β-galactosidase strain, Leishmania infantum MHOM/MA/67/ITMAP263 intracellular amastigotes, and a chloroquine-sensitive (GHA) strain of Plasmodium falciparum (ring stage and schizont). Suramin or melarsoprol, nifurtimox, miltefosin and chloroquine respectively were included as the reference drugs. Cytotoxicity was tested on human simian virus 40-immortalized lung fibroblasts (MRC-5 SV2; ECACC, UK) (11). IC50 values were also obtained at the University of Glasgow (UG) using a very similar protocol, with essentially identical results. Minor differences included an incubation time of 48 hrs in the presence of 2-fold dilutions of test compound starting at 100 µM, followed by a further 24 hrs in the presence of resazurin (Alamar blue, Sigma; 12.5 µg/ml). The UG experiments, like those at UA, used T. b. brucei strain 427 as standard drug sensitive control, assayed in parallel with the TbAT1-KO (28) and B48 (6) drug resistant strains. Diminazene aceturate and pentamidine (both Sigma) were used as positive controls for the resistance phenotypes.
Real time assay for trypanosome viability and mobility

This assay was performed exactly as described previously (6). The inhibition curves of trypanosomes were determined by absorption at 750 nm using a UV/Visible spectrophotometer (HP-8453, Hewlett Packard). Cultured bloodstream form *T. b. brucei* of strain 427 were used, at a cell density of $10^7$ cells per ml in HMI-9. Phenylarsine oxide (Sigma) was used as a positive control.

In vivo assay

Female Swiss mice (4/group) were treated 50 mg/kg orally with UAMC-00363 and were intraperitoneally infected with $1.10^4$ trypanosomal bloodforms (*T. b. brucei* Lister strain 427) per mice (0.25 ml), collected from an infected donor animal, 1 hour later. Intraperitoneal (IP) treatment was continued the next 4 days at 50 mg/kg. The reference compound suramin (1 x 10 mg/kg IP/day) was included. Untreated infected controls generally die between 6 days post infection (dpi) and 9 dpi. To obtain information about the parasitaemia of survivors, on 10 dpi, 14 dpi and 21 dpi, 10 µl of blood was obtained from the tail vein for microscopic estimation of the levels of parasitaemia. The animals were observed for the occurrence/presence of clinical and adverse effects during the course of the experiment. The occurrence of mortality was monitored daily.

Transport studies

Transport of $[^3]$H-adenosine (Amersham; 23 Ci/mmol) and $[^3]$H-hypoxanthine (Amersham; 19 Ci/mmol) was performed exactly as described previously (2, 40). Briefly, bloodstream *T. b. brucei* strain 427 were taken from infected Wistar rats, collected at peak parasitaemia by cardiac
puncture under terminal anesthesia, and separated from blood cells on a DEAE-52 (Whatman) anion exchange column. Trypanosomes were washed twice with 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 and 14 mM glucose, pH 7.3) and resuspended in the same buffer at a concentration of 2x10⁸/ml. 100 µl of this suspension was incubated for 30 s with the same volume of radiolabel at room temperature, in the presence or absence of test compounds. Incubations were stopped by the addition of 1 ml ice-cold unlabelled 1 mM adenosine (P1 or P2) or hypoxanthine (H2) and immediate centrifugation through an oil layer. Radioactivity was determined using a scintillation counter. Kᵢ values were calculated by non-linear regression using the Prism 4 and 5 (Graphpad) packages and the Cheng-Prusoff equation Kᵢ = IC₅₀/[1 + [L]/Kₘ], in which L is the permeant concentration used and Kₘ the Michaelis-Menten constant for the transporter-permeant combination.

RNAi knockdown studies

Trypanosome cell lines and cultures: all cell lines were derivatives of T. b. brucei strain 427 and cultured in SDM79 medium with hemin and 10% fetal calf serum. Cell lines TblAG-NHRNAi, TbIG-NHRNAi, TbMTAPRNAi and TbMTAP-IAGNH-IGNHRNAi express double-stranded RNA from two tetracycline-inducible T7 promoters facing each other in the pZJM vector (41) transformed in 29-13 T. brucei procyclic cells that express the T7 RNA polymerase and the tetracycline repressor (42). The plasmid was electroporated in 29-13 cells and resistant clones were selected by addition of a selection medium (15 µg/ml G418, 20 µg/ml hygromycin, 2 µg/ml phleomycin). RNAi was induced by addition of 1 µg tetracycline per ml of medium and fresh tetracycline was added at each cell dilution. GeneDB database was searched for IAG-NH, IG-NH and MTAP genes. Primer sequences were designed using RNAit primer design software available on
TrypanoFAN (http://trypanofan.path.cam.ac.uk/software/RNAit.html). Primer sequences are available upon request.

RT-PCR: total RNA was extracted from cells grown with or without tetracycline for 48 hrs and RT-PCR was performed as described by Absalon et al., 2007 (1).

Fluorescence assay: this was performed as described previously (1). Slides were observed with a DMR Leica microscope and images were captured with a Cool Snap HQ camera (Roper Scientific).
**Results**

**Enzyme assays**

The design and synthesis of the N-arylmethyl-substituted iminoribitol derivatives has been extensively discussed elsewhere (4, 22, 23). These compounds are potent inhibitors of TvIAG-NH and discriminate against hPNP (table 1). Since biological assays were performed on a *T. b. brucei* strain, compounds (Fig. 2) were screened against TbbIAG-NH to confirm this inhibitory effect (table 1). All compounds are nanomolar inhibitors of the enzyme. The most potent compounds were **UAMC-00363** (*K*<sub>i</sub> = 18 nM) and **UAMC-00109** (*K*<sub>i</sub> = 30 nM), bearing a hydrophobic aglycone that mimics purine nucleosides substrates adenosine and inosine, respectively. Replacing the deazapurine moiety by a thienopyrimidine lowered the efficacy of the inhibitors, as judged from the increase of *K*<sub>i</sub> to 56 nM (**UAMC-00375**) and 190 nM (**UAMC-00312**). The efficacy of inhibition further decreased when the purine ring was substituted by an aminobenzothiazole (**UAMC-00311**, *K*<sub>i</sub> = 560 nM) or a quinoline ring (**UAMC-00115**, *K*<sub>i</sub> = 446 nM). Taken together, these results suggest that, with respect to the aglycone part, structure-activity relationships for TbbIAG-NH inhibition seem to differ substantially from what was observed earlier for TvIAG-NH (4, 22). The same set of compounds was also tested on the *T. b. brucei* IG-NH. Surprisingly, the inhibition of this enzyme with respect to IAG-NH was less efficient, with *K*<sub>i</sub> values in the micromolar range. For IG-NH the most efficient compounds were the quinoline derivative **UAMC-00115** (*K*<sub>i</sub> = 11 µM) and **UAMC-00311** (*K*<sub>i</sub> = 14 µM). Instead the activity of the adenosine-mimicking **UAMC-00363** (*K*<sub>i</sub> = 32 µM) and its analogue **UAMC-00375** (*K*<sub>i</sub> = 31 µM) was three-fold lower. Inhibitory activity of inosine mimics **UAMC-00109** (*K*<sub>i</sub> = 138 µM) and **UAMC-00312** (*K*<sub>i</sub>=120 µM) was 10-fold lower. Thus, the IAG-NH and IG-NH enzymes clearly differ in the active site features, allowing an isozyme-selective inhibition. The fact that the TbbIG-NH remained unaffected and hence fully functional in the PSP (Fig. 1)
might be a first indication for the observed lack of antiparasitic activity. It will be of interest to experimentally identify the active site features that influence the affinity of the IAG-NH and IG-NH towards the compounds here tested.

**In vitro activity against *T. b. brucei***

Nearly all of the 52 compounds (see supplemental material) were tested *in vitro* against *T. b. brucei* (BSF, strain 427). Most compounds did not display IC_{50} values below 5 µM and, accordingly, were considered inactive against this parasite. Additionally, no activity was found against *T. cruzi*, *L. infantum* and *P. falciparum*. Only one compound, **UAMC-00363**, showed high and selective activity against *T. b. brucei* (IC_{50} = 0.49 ± 0.31 µM) in the absence of cytotoxicity to a human cell line (Table 1). In a secondary screening this compound also showed to be active against *T. b. rhodesiense* (IC_{50} = 0.26 ± 0.15 µM). This compound was further tested in a mouse model of African trypanosomiasis.

A deeper investigation of the trypanostatic and trypanocidal mode of action of **UAMC-00363** was performed. The compounds were relatively slow-acting, apparently inducing growth arrest and only becoming trypanocidal at high concentrations (> 5 µM). This was apparent from the biphasic inhibition curve displayed in the Alamar Blue assay (Fig. 3A), similar to the trypanostatic adenosine analogue NA-42, where the first IC_{50} value corresponded to the concentration inducing growth arrest, and the second concentration to the parasitocidal effect (34). We thus looked at the rate at which trypanosomes are killed by **UAMC-00363**, using an *in vitro* lysis assay as described, using phenylarsine oxide as a positive control (28). Fig. 3B shows that up to 20 µM of the test compound failed to affect trypanosome viability or motility for over 7 hrs. Cells were thus followed by microscopic counting for a 48 hour period (Fig. 3C).

Phenylarsine oxide (0.25 µM) killed all cells within 5 hrs whereas pentamidine (1 µM) took 24
hrs. Yet, incubation with 10 µM UAMC-00363 reduced the trypanosome population by approximately 75% after 48 hrs of incubation; no trypanocidal effect was observed at 24 hrs (Fig. 3C). To test whether continuous exposure is required for the trypanostatic effect, parallel cultures of *T. b. brucei* were exposed to only 5 hrs of pentamidine or UAMC-00363, after which they were washed into fresh medium without test compound. In both cases the drug effect was only slightly reversed after removal of the drug (Fig. 3C).

**In vivo assay**

UAMC-00363 was assessed in a rodent model of *T. b. brucei* infection at a dose of 50 mg/kg. The vehicle infected controls (VIC) developed severe trypanosomiasis with 100% mortality at 9 dpi. Treatment with suramin at 10 mg/kg IP for 5 days resulted in 100% survival at 21 dpi with total clearance of parasitaemia at 10 dpi. Treatment with compound UAMC-00363 50 mg/kg once daily for 5 days (the dosing on day one was given orally, IP treatment was continued the next four days) totally cleared infection as reflected in negative blood at 10 dpi, 14 dpi and 21 dpi and showed 75% survival at 21 dpi (Fig. 4). Additional experiments are planned to further explore this promising activity.

**Transport studies**

In order to examine the lack of antiprotozoal activity of our *N*-arylmethyl-substituted iminoribitol library (except for UAMC-00363), inhibition of the P1 and P2 transporters of *T. b. brucei* was assessed for a selection of this library (Table 2). The resultant Kᵢ values reflect the affinity of each compound for the transporter being studied, as the inhibitor concentration that gives 50% occupancy of transporter binding sites, but do not necessarily correlate to uptake rates for the inhibitors. For both the P1 and P2 transporters, recognition motifs have been identified. The P1
transporters have previously been shown to interact with their substrate through hydrogen bonds at position 3, 7, 3' and 5' of adenosine (2), whereas P2 binds principally with the N(1)=C(6)-NH$_2$ amidine motif in addition to interactions with N-9 of the aromatic ring (Fig. 5A), the aromaticity of the purine ring itself further contributes to P2 binding (10, 14). The P2 motif is shared with the diamidines and melaminophenyl arsenicals (Fig. 5B) and has been grafted on inhibitors as means of delivery to the cell (3, 9). On the other hand, resistance against these compounds is developed by deletion of P2 without loss of viability to the parasite (15, 28). Based on the P2 recognition motif, compounds UAMC-00311, UAMC-00312, UAMC-00375, UAMC-00109 and UAMC-00363 (Fig. 2) were considered for affinity testing on these transporters, but compounds UAMC-00109, UAMC-00311 and UAMC-00312, lacking the P2 motif, were not tested against this transporter. The respective transporter affinities were consistent with the predictions based on the known motifs and formed a rationale for the activity of the compounds based on cellular penetration. Interestingly, only inhibitor UAMC-00363 showed a significant affinity towards the P2 transporter ($K_i = 8.2$ µM) (Fig. 6A). Its bio-isostere UAMC-00375 showed a 15 fold lower affinity ($K_i = 125$ µM), thereby indicating that the protonated N-7 of UAMC-00363 is crucial for interactions with this class of purine analogues; in adenosine and adenine this residue is not protonated and in this state does not appreciably contribute to binding. The low affinity of UAMC-00363 towards P1 ($K_i = 355$ µM; Fig. 6B) was predictable since the 3' and 5' hydroxyl groups of the ribose moiety contribute strongly to P1 binding, and indeed constitute the basis for its absolute selectivity for nucleosides over nucleobases. The orientation of the ribose ring in the P1 binding pocket is therefore critical and the iminoribitol orientation is substantially altered, not least because of the methylene linker. For P2 binding, the ribose orientation is less strict since binding mainly occurs via the purine ring (14). An in silico model of P2-substrate interactions, developed using Comparative Molecular Field Analysis (CoMFA)
and Comparative Molecular Similarity Indices Analysis (CoMSIA) (10), showed that a positive charge is favoured at position 7, and either a hydrogen bond acceptor or donor; furthermore the computational analysis identified no steric barriers to the protonation of N-7. The combination of these factors sufficiently explained the relatively low affinity of UAMC-00375 towards P2. In addition to the nucleoside transporters, we tested UAMC-00363 for inhibition of the H2 hypoxanthine transporter, as this nucleobase transporter also displays some affinity for adenosine (Kᵢ = 590 µM) (13). UAMC-00363 did affect H2-mediated hypoxanthine uptake similar to adenosine (Fig. 6C) but it is unlikely that this low affinity flux significantly contributes to its uptake at submicromolar concentration. Since UAMC-00363 showed great selectivity towards *T. b. brucei* and *T. b. rhodensiense* (inactive against *L. infantum*, *T. cruzi* and *P. falciparum*), and the only compound of the series to display high affinity for any of the *T. b. brucei* purine transporters, it is tempting to assume that its selective activity is dependent on accumulation through the *T. b. brucei* specific P2 transporter. This model would predict loss of activity for the compound in a *T. b. brucei* line not expressing P2. Therefore, both UAMC-00363 and UAMC-00375 were tested on the TbAT1-KO clonal line (derived from strain 427 but with the gene coding for TbAT1/P2 experimentally deleted (28) and on the B48 line (generated from TbAT1-KO by in vitro adaptation to pentamidine) (6). These lines are strongly resistant to diminazene or to all diamidines and melaminophenyl arsenicals, respectively(6, 28). Interestingly, no resistance was observed for this compound and no cross-resistance with the amidines nor the melaminophenyl arsenical compounds was observed. We conclude that, besides P2, UAMC-00363 enters the cell via at least one other transporter or via passive membrane permeability although the latter is unlikely. The data summarised in Table 2 suggest that this would not be one of the known *T. b. brucei* purine transporters. It can therefore not be excluded that the other
inhibitors might also be taken up by the cell via this unidentified transport mechanism and that their lack of uptake by P1 or P2 is not the only reason for the absence of antiparasitic activity. If the main target of UAMC-00363 is the NH, it would be expected that the cells would be more sensitive to the compound when offered inosine as sole purine source, than when offered hypoxanthine. We thus determined the IC$_{50}$ values of UAMC-00363 against $T. b. brucei$ strain 427 after several passages in media that contained either hypoxanthine or inosine at 0.1 or 1 mM concentrations. We found no clear differences in the EC$_{50}$ values at the various conditions (data not shown), indicating the existence of an additional target.

RNAi knockdown

Since the purine salvage machinery is remarkably versatile through the existence of multiple NHs and PRTs and the bypass possibility via MTAP (Fig. 1), we chose to target the two NHs of $T. b. brucei$: IAG-NH (Tb927.3.2960, GeneDB) and IG-NH (Tb927.7.4570, GeneDB), as well as the MTAP (Tb927.7.7040, GeneDB). MTAP phosphorolytically cleaves the glycosidic bond of adenine-containing nucleosides and converts 5'-methylthioadenosine (MTA) into adenine. MTAP is via this route involved in both the purine salvage pathway and polyamine metabolism (25). By providing adenine, MTAP can circumvent IAG-NH function. Previous RNA interference-mediated silencing already showed that $T. b. brucei$ AK, responsible for the phosphorylation of adenosine to its 5’-monophosphate AMP, is nonessential under standard growth conditions (26). Phosphoribosyltransferases (PRTs) were not targeted since multiple genes exist and cross-RNAi could not be avoided in an RNAi experiment. The expression of each enzyme was knocked down separately by RNAi and then all three were targeted together (triple RNAi). Efficiency of RNAi was measured by RT-PCR and showed a significant reduction in total mRNA in all three cases (51.0% for IAG-NH, 64.1% IG-NH, and 83.5% for MTAP).
Protein expression could not be measured since no antibody against NH or MTAP was available at the time. The effect of protein knockdown was evaluated by growth curve analysis and showed a slight slowdown in growth for induced \( \text{TbMTAP}^{\text{RNAi}} \) and \( \text{TbIGNH}^{\text{RNAi}} \) cells after 4-5 days when compared to non-induced controls (Fig. 8A). No growth reduction was seen for \( \text{TbIAGNH}^{\text{RNAi}} \) even after 6 days of induction. Although the reduction in cell growth was very limited upon RNAi induction, we observed abnormal cell types (Fig. 7, multinucleated and anucleated cells) in induced \( \text{TbMTAP}^{\text{RNAi}} \) cultures, indicating problems in cell cycle and cytokinesis. These abnormal cells increase in number during the course of induction.

Since it is possible that the different enzymes complement each other, we constructed and analysed a triple \( \text{TbMTAP-IAGNH-IGNH}^{\text{RNAi}} \) cell line. Upon induction, this triple RNAi cell line displayed a phenotype similar to the one observed with the single IG-NH and MTAP knockdown: slight slowdown in growth (Fig. 8B), accompanied by the appearance of abnormal cells (Fig. 7, similar to \( \text{TbMTAP}^{\text{RNAi}} \)), that increased in importance during longer induction periods (data not shown).
Extensive and interdisciplinary research was performed to clarify the lack of biological activity of the potent \(N\)-arylmethyl-substituted iminoribitols (except for UAMC-00363). The UAMC-00363 compound is a nanomolar inhibitor of the IAG-NH from both \(T. \text{vivax}\) and \(T. \text{b. brucei}\). However, other compounds that were previously shown to have nanomolar \(K_i\)'s for the \(T. \text{vivax}\) IAG-NH (4, 22, 23), are less efficient in inhibiting the \(T. \text{b. brucei}\) enzyme. These findings clearly suggest that the two enzymes, despite sharing the same substrate specificity, differ at specific amino acid residues that interact with the inhibitors. Moreover, compounds with nanomolar inhibition constants for the \(T. \text{b. brucei}\) IAG-NH are less potent against \((10^3)\) the IG-NH isozyme, underscoring the existence of distinct active site features between the two enzymes. The fact that these compounds do not sufficiently inhibit the IG-NH of \(T. \text{b. brucei}\) might be a first indication for their lack of biological activity against this parasite. In addition, it was shown that uptake of these inhibitors does not happen via the P1/P2 transport system of \(T. \text{b. brucei}\), except for UAMC-00363. It can not be excluded that uptake happens via at least one other transporter as demonstrated for UAMC-00363. RNAi results show that procyclic \(T. \text{b. brucei}\) can survive when a nucleoside hydrolase (IAG-NH or IG-NH) has been partially knocked-down (51% and 64%). There are two possible explanations for this observation: either the genes are non essential under our experimental conditions or there is sufficient mRNA present after tetracycline induction to sustain normal cell growth (41). Knockdown of IG-NH and MTAP has a more pronounced effect than IAG-NH without being lethal, although the difference is minor (again under the conditions used here). These observations are confirmed with the triple RNAi experiment. An explanation for this effect might be that MTAP is, besides the purine salvage pathway, also involved in the polyamine biosynthesis (25). Inhibition of this enzyme might therefore have greater consequences for the parasite. The fact that the effect for IG-NH is more pronounced than for
IAG-NH can be explained by the kinetic constants of both enzymes. They suggest that IG-NH is the major NH in the purine salvage pathway, therefore knock-down of this enzyme will have a greater, although not lethal, effect (IG-NH has a 10-fold greater $k_{cat}/K_{M}$ value compared to the IAG-NH from *T. vivax* for inosine substrate) (M. Degano, unpublished results). Although preliminary, these results are consistent with the lack of antiparasitic activity observed for our NH-inhibitors. Only inhibitor **UAMC-00363** shows remarkable biochemical an biological activities. It is a nanomolar inhibitor of both TvIAG-NH and TbbIAG-NH, discriminating against hPNP and TbbIG-NH. It shows selective antiparasitic activity against *T. b. brucei* and *T. b. rhodesiense* and was further characterized having an initially trypanostatic effect, only becoming rapidly trypanocidal at high concentrations (> 5 µM). While treatment with low concentrations of **UAMC-00363** clears trypanosome populations only slowly (> 48 hrs), we demonstrated that the inhibitor triggers an irreversible process in the parasite within 5 hrs of incubation, after which the drug could be removed. This process appears to act rapidly to block cell division and growth, leading eventually to cell death, and is reminiscent of observations with inhibitors of *T. b. brucei* phosphodiesterases, which induce a rapid rise in the concentration of the second messenger cAMP (M. K. Gould and H. P. De Koning, unpublished results). The irreversibility of a brief exposure to **UAMC-00363** is potentially of great therapeutic advantage, allowing shorter treatment which in turn reduce the risk of side effects. *In vivo* studies in a rodent model show complete blood clearance after 10 dpi and a survival rate of 75% at 21 dpi. **UAMC-00363** is concentrated into the cell via P2 and at least one other transporter. Interestingly, no resistance is observed and thus no cross-resistance with the amidines nor the melaminophenyl arsenical compounds is expected. As resistance to melarsoprol and to diamidines such as diminazene has become a severe problem in the treatment of human and veterinary trypanosomiasis, the efficacy of any new compound to resistant strains is a prerequisite for its clinical consideration (17). We
conclude that from a series of 52 N-arylmethyl- substituted iminoribitols, UAMC-00363 shows interesting *in vitro* and *in vivo* activities against *T. b. brucei*. This compound is taken up in the parasite by the P2 transporter and at least one other transport mechanism. Besides its inhibition of the IAG-NH, its antiparasitic activity is explained by unknown other targets. It has excellent selectivity towards hPNP and no cross-resistance with existing trypanocidal drugs. We believe that single inhibition of *T. b. brucei* IAG-NH is insufficient and that inhibition of multiple enzymes of the purine salvage pathway is a prerequisite to obtain a trypanocidal effect. Further *in vivo* studies and optimisation of UAMC-00363 are planned.
Abbreviations

AK: adenosine kinase; APRT: adenine phosphoribosyl transferase; BSF: bloodstream form; DCM: dichloromethane; dpi: days post infection; ENT: equilibrative nucleoside transporters; HGPRT: hypoxanthine guanine phosphoribosyl transferase; hPNP: human purine nucleoside phosphorylase; IAG-NH: inosine-adenosine-guanosine nucleoside hydrolase; IG-NH: inosine-guanosine nucleoside hydrolase, IP: intraperitoneal; L. major: Leishmania major; MeOH: methanol; MTAP: 5’-deoxy-5’-methylothioadenosine phosphorylase; NaOAc: sodium acetate; NH: nucleoside hydrolase; NH\textsubscript{4}OH: ammonium hydroxide; P. falciparum: Plasmodium falciparum; PSP: purine salvage pathway; RNAi: ribonucleic acid interference; T. b. brucei (Tbb): Trypanosoma brucei brucei, T. vivax (Tv): Trypanosoma vivax; UAMC: University of Antwerp Medicinal Chemistry; VIC: vehicle infected controls.

Acknowledgments

This work was supported by a research project from the Research Foundation Flanders (FWO-Vlaanderen), the Institute for the promotion of Innovation through Science and Technology in Flanders (IWT-GBOU), the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and a scholarship from the Ministry of the Flemish Community (INSERM2006/2007-Project 1.2006/2007.2). M. Berg has a Ph.D. grant from the Research Foundation Flanders (FWO-Vlaanderen). P. Van der Veken and P. Cos have a postdoctoral grant from the Research Foundation Flanders (FWO-Vlaanderen). The laboratory of Medicinal Chemistry and LMPH are partners of the Antwerp Drug Discovery Network (ADDN, www.addn.be). A. Matheeussen performed the technical laboratory work for the in vitro assay. The excellent technical assistance of W. Bollaert was greatly appreciated.


Rabbinowitsch, M.-A. Rajandream, C. Reitter, S. L. Salzberg, M. Sanders, S. Schobel, 
Tivey, S. Van Aken, D. Walker, D. Wanless, S. Wang, B. White, O. White, S. 
D. Barry, H. Fairlamb, F. Opperdoes, B. G. Barrell, J. E. Donelson, N. Hall, C. M. 
Fraser, S. E. Melville, and N. M. El-Sayed. 2005. The genome of the African trypanosome 

Bridges, D., M. K. Gould, B. Nerima, P. Mäser, R. J. S. Burchmore, and H. P. De 
Koning. 2007. Loss of the high affinity pentamidine transporter is responsible for high levels 
of cross-resistance between arsenical and diamidine drugs in African trypanosomes. Mol. 
Pharmacol. 71:1098-1108.


Cheng, Y.-C., and W. H. Prusoff. 1973. Relationship between the inhibition constant (K_i) 
and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic 

delivery of compounds to Trypanosoma brucei using the melamine motif. Bioorg. Med. 
Chem. 17:2512-2523.

Koning. 2009. Predictive computational models of substrate binding by a nucleoside 


Koning. 2003. The mechanisms of arsenical and diamidine uptake and resistance in 

Schramm. 1999. Iminoribito:1 transition state analogue inhibitors of protozoan nucleoside 
ydrolases. Biochemistry **38**:13147-13154.

2006. New insights into the mechanism of nucleoside hydrolases from the crystal structure 
of the *Escherichia coli* YbeK protein bound to the reaction product. Biochemistry **45**:773-782.

**163**:67-76.


purine-specific *N*-ribohydrolase from *Trypanosoma brucei brucei*. Sequence, expression, and 

De Koning, and G. J. Koomen. 2007. 2, *N*-Disubstituted adenosine analogues with
antitrypanosomal and antimalarial activity. Synthesis, uptake studies and *in vivo* evaluation.


**Fig. 1:** Purine salvage pathway of *T. brucei* depicted with key enzymes and transporters. 1. S-AMP lyase 2. S-AMP synthetase 3. IMP dehydrogenase 4. GMP synthetase 5. guanine deaminase 6. AMP deaminase.

**Fig. 2:** Structure of Immucillins and N-arylmethyl-substituted iminoribitolis.
Fig. 3: Effect of UAMC-00363 on T. b. brucei cultures. A. Alamar Blue assay with T. b. brucei strain 427. ○, UAMC-00363; ▲, UAMC-00109; ■, Pentamidine; □, Diminazene aceturate. B. Effect of UAMC-00363 on viability and motility of T. b. brucei. Trypanosomes ($10^7$ in 1 ml assay buffer) were placed in a cuvette and absorbance was measured at 750 nm for approx. 7.5 h. The assay is based on light scatter by the highly mobile flagellated trypanosomes; reduction in either cell viability or motility will thus reduce absorbance. (a) negative control, no test compound added; (b) UAMC-00363 at 20 µM; (c) UAMC-00363 at 10 µM; (d) positive control, phenylarsine oxide at 1 µM. C. Cultures of $2 \times 10^5$ bloodstream T. b. brucei of strain 427 were incubated with 0.25 µM phenylarsine oxide (□), 1 µM pentamidine (▲), 10 µM UAMC-00363 (○) or no test compound (control; ■) for up to 48 hours (solid lines, or for only 5 hours, after which the drug was washed out by centrifugation (twice) and resuspended in fresh medium (dashed lines). Cell counts were made in duplicate with a haemocytometer under a phase-contrast microscope; average counts are shown.
Fig. 4 Kaplan-Meier survival curve of the *in vivo* assay. Untreated mice (VIC), UAMC-00363-treated mice and suramin-treated mice were followed for 21 dpi. In each group 4 mice were included.

Fig. 5: Graphic representation of the recognition motifs for the P1 and P2 transporters on adenosine (A). Structures of pentamidine and melarsoprol (B); amidine motif is indicated with a dotted line.
Fig. 6: Effect of UAMC-00363 on *T. b. brucei* purine transporters. Purine uptake by (A) the P2 transporter, (B) the P1 transporter and (C) the H2 transporter was measured using (A) 0.05 μM [³H]-adenosine in the presence of a fixed concentration of 1 mM inosine to block the P1 transporter, (B) 0.05 μM [³H]-adenosine in the presence of a fixed concentration of 100 μM adenine to block P2-mediated transport, or (C) 0.1 μM [³H]-hypoxanthine. Variable concentrations of test compounds were added as indicated; ■, UAMC-00363; □, UAMC-00375; Δ, 2,6-diaminopurine 2′-deoxyriboside; [open down triangle], adenosine; ○, hypoxanthine. Data are expressed as pmol(10⁷ cells)^⁻¹^s⁻¹^ and shown as the average of triplicate determinations and SEM, this experiment is representative of at least three similar experiments.
Fig. 7: Fluorescence analysis of non-induced 29-13 cells (non, left), $TbMTAP^{RNAi}$ (middle) and $TbMTAP-IAGNH-IGNH^{RNAi}$ (right) induced mutant cell lines. The top panels show combined phase/Dapi images, illustrating cell shape as well as DNA staining. The bottom panels illustrate the DAPI staining of the nuclei and kinetoplasts.

Fig. 8: (A) Growth rates followed over six days for three mutant cell lines $TbIAGNH^{RNAi}$, $TbIGNH^{RNAi}$, $TbMTAP^{RNAi}$ (single RNAi). A control cell line $TbKIF9B^{RNAi}$ non induced was used (18). (B) Growth rate followed over seven days for the $TbMTAP^{RNAi}$ (single
RNAi) and TbMTAP-IAGNH-IGNH RNAi mutant cell line (triple RNAi). Cumulative cell density takes into account the dilution factors of the cells during the experiment. For each mutant, the experiment was repeated with a second transfectant and similar results were obtained.
Table 1: Biochemical and biological activities of Immucilli ns and N-arylmethyl-substituted iminoribitols. Inhibition of *T. vivax* IAG-NH (TvIAG-NH, $K_{i}$), *T. b. brucei* IAG-NH (TbbIAG-NH, $K_{i}$), *T. b. brucei* IG-NH (TbbIG-NH, $K_{i}$) and human PNP (hPNP, $K_{i}$). Inhibition of *T. b. brucei* (IC$_{50}$). Cytotoxicity was measured against human fibroblast (MRC-5, IC$_{50}$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{i}$, TvIAG-NH (µM) (4, 22, 37)</th>
<th>$K_{i}$, TbbIAG-NH (µM) (21, 29)</th>
<th>$K_{i}$, TbbIG-NH (µM)</th>
<th>$K_{i}$, hPNP (µM) (4, 21, 24)</th>
<th>IC$_{50}$ T. b. brucei (µM)</th>
<th>MRC-5 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmA</td>
<td>(6.2 ± 0.3)$\times 10^{-3}$</td>
<td>0.9$\times 10^{-3}$ (K$_{i}^{*}$)</td>
<td>(4.4 ± 0.9)$\times 10^{-3}$</td>
<td>&gt; 10 (K$_{i}^{*}$)</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>ImmH</td>
<td>(6.2 ± 0.7)$\times 10^{-3}$</td>
<td>24$\times 10^{-3}$ (K$_{i}^{*}$)</td>
<td>(9 ± 2)$\times 10^{-3}$</td>
<td>0.015$\times 10^{-3}$ (K$_{i}^{*}$)</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00363</td>
<td>(4.1 ± 0.7)$\times 10^{-3}$</td>
<td>(18 ± 2)$\times 10^{-3}$</td>
<td>32 ± 5</td>
<td>22.5 ± 5.5</td>
<td>0.49 ± 0.31$^{a}$</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00109</td>
<td>(4.4 ± 0.5)$\times 10^{-3}$</td>
<td>(30 ± 3)$\times 10^{-3}$</td>
<td>138 ± 40</td>
<td>(5.8 ± 1.5)$\times 10^{-3}$</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00375</td>
<td>(19 ± 4)$\times 10^{-3}$</td>
<td>(56 ± 6)$\times 10^{-3}$</td>
<td>31 ± 5</td>
<td>&gt; 10$^{l}$</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00312</td>
<td>(20 ± 3)$\times 10^{-3}$</td>
<td>(190 ± 20)$\times 10^{-3}$</td>
<td>120 ± 27</td>
<td>77.6 ± 18.8</td>
<td>34</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00115</td>
<td>(11 ± 1)$\times 10^{-3}$</td>
<td>(446 ± 36)$\times 10^{-3}$</td>
<td>11 ± 2</td>
<td>&gt; 10$^{l}$</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00346</td>
<td>(58 ± 10)$\times 10^{-3}$</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt; 10$^{l}$</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00218</td>
<td>(29 ± 6)$\times 10^{-3}$</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt; 10$^{l}$</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>UAMC-00311</td>
<td>(40 ± 6)$\times 10^{-3}$</td>
<td>(560 ± 55)$\times 10^{-3}$</td>
<td>14 ± 2</td>
<td>&gt; 10$^{l}$</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
</tbody>
</table>

For $K_{i}$ values standard errors on the fit are provided. $K_{i}^{*}$ for slow-onset inhibition. $^{a}$ average of eight independent determinations. n.d.: not determined.
Table 2: Inhibition constants (Kᵢ) of nucleoside hydrolase inhibitors for the *T. b. brucei* P1, P2 and H2 transporters. n.d. = not determined

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ P1 (µM)</th>
<th>Kᵢ P2 (µM)</th>
<th>Kᵢ H2 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAMC-00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>109</td>
<td>&gt; 1000</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>311</td>
<td>810 ± 220</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>312</td>
<td>300 ± 80</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>363</td>
<td>360 ± 30</td>
<td>8.2 ± 0.6</td>
<td>590</td>
</tr>
<tr>
<td>375</td>
<td>540 ± 120</td>
<td>125 ± 13</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
A

[Chemical structures with annotations]

B

[Chemical structures with annotations]

- adenosine recognition motifs for P1
- adenosine recognition motifs for P2
- pentamidine
- melarsoprol