Combining tubercidin and cordycepin scaffolds results in highly active candidates to treat late-stage sleeping sickness

Fabian Hulpia1, Dorien Mabille2, Gustavo D. Campagnaro3, Gabriela Schumann4, Louis Maes2, Isabel Roditi4, Anders Hofer5, Harry P. de Koning3,6, Guy Caljon2,6 & Serge Van Calenbergh1,6*

African trypanosomiasis is a disease caused by Trypanosoma brucei parasites with limited treatment options. Trypanosoma is unable to synthesize purines de novo and relies solely on their uptake and interconversion from the host, constituting purine nucleoside analogues a potential source of antitrypanosomal agents. Here we combine structural elements from known trypanocidal nucleoside analogues to develop a series of 3’-deoxy-7-deazaadenosine nucleosides, and investigate their effects against African trypanosomes. 3’-Deoxytubercidin is a highly potent trypanocide in vitro and displays curative activity in animal models of acute and CNS-stage disease, even at low doses and oral administration. Whole-genome RNAi screening reveals that the P2 nucleoside transporter and adenosine kinase are involved in the uptake and activation, respectively, of this analogue. This is confirmed by P1 and P2 transporter assays and nucleotide pool analysis. 3’-Deoxytubercidin is a promising lead to treat late-stage sleeping sickness.
Sleeping sickness or human African trypanosomiasis (HAT) is almost always fatal and is endemic in much of sub-Saharan Africa, coinciding with the geographical localization of the tsetse fly vector. Its causative agent is the haemoflagellate protozoan parasite Trypanosoma brucei spp., of which T. b. gambiense and T. b. rhodesiense are infectious to humans, and prevalent in West and Central Africa, and in East and Southern Africa, respectively. Patients initially show non-specific symptoms such as fever and general malaise, caused by parasites proliferating in the haemolymphatic system (stage 1 disease), after which the trypanosomes invade the central nervous system (CNS; stage 2 disease), thereby causing severe neurological complications, one of which is the altered sleep/wake cycle that gave this infectious disease its name.

Treatment of HAT is currently based on the following five drugs: pentamidine, suramin, melarsoprol, eflornithine and nifurtimox. A sixth drug, fexinidazole, recently concluded clinical trials successfully. Pentamidine and suramin are the first-line drugs against stage 1 disease caused by T. b. gambiense and T. b. rhodesiense, respectively. The first-line treatment for the second stage of T. b. gambiense HAT is a nifurtimox–eflornithine combination therapy, with eflornithine monotherapy used when nifurtimox is unavailable or contraindicated. Melarsoprol, an organo-arsenic compound, leads to treatment-related death in 2.5 to 5% of cases, and is now restricted to the treatment of stage 2 T. b. rhodesiense HAT, while being almost completely phased out for stage 2 T. b. gambiense HAT. All these drugs suffer from major limitations ranging from stage-specific efficacy (e.g. only active against stage 1 disease) to significant toxicity, as well as the necessity for parenteral administration (intravenous for suramin, melarsoprol and eflornithine and intramuscular for pentamidine), which poses practical challenges in rural Africa. Clinical trial results with orally administered fexinidazole showed it is safe and effective against T. b. gambiense HAT, marking it the first new HAT therapeutic in three decades, as well as the first oral monotherapy against both stage 1 and stage 2 HAT. Nonetheless, resistance is readily induced in vitro and fexinidazole displays cross-resistance with nifurtimox. Additionally, this drug requires a high pill burden treatment regime, underscoring that research efforts for the discovery of new therapeutics to treat this neglected tropical disease remain of significant interest.

Protozoan parasites are incapable of synthesizing purine nucleosides de novo and hence rely on uptake and salvage of exogenous purines. In this context, purine analogues that can act as inhibitors or ‘subversive’ substrates of purine salvage enzymes are a promising source of compounds with activity against protozoan parasites (e.g. cordycepin and tubercidin) and have been shown to exhibit good activity against African trypanosomes. Moreover, nucleoside analogues could have the advantage of a higher likelihood to cross the blood–brain barrier (BBB) and thus be active against stage 2 HAT, owing to the presence of specific (purine) transporters at the BBB. The nucleoside antibiotics cordycepin and tubercidin represent two of the most thoroughly studied antitrypanosomal nucleoside analogues (Fig. 1).

Inspired by the activity of tubercidin against T. brucei spp., we recently explored a series of 7-substituted tubercidin analogues and identified analogues displaying promising in vitro activity against kinetoplastid parasites. In an attempt to further increase the antitrypanosomal activity, we set out to investigate the effect of modifying the sugar part of tubercidin and its 7-substituted analogues.

The present communication reports the identification of a promising adenosine analogue that is highly active in both stage 1 and stage 2 mouse models of HAT. Furthermore, we demonstrate its affinity for T. brucei adenosine transporters, and provide insights into its mechanism of action by applying whole-genome RNA interference (RNAi) screening, and analysis of its metabolism in the parasite through nucleotide pool analysis.

**Results**

Hybrid nucleosides display highly potent in vitro activity. Based on the reported activity of cordycepin and tubercidin, and taking into account our recently reported tubercidin derivatives, a small series of 2′- and 3′-deoxytubercidin analogues was synthesized (Supplementary Methods) and evaluated in vitro against T. brucei spp. (Fig. 2).

Comparison of the in vitro activity profile of the ribofuranose (6 and 8), 2′-deoxy (11 and 12) and 3′-deoxyribofuranose analogues (9 and 10) showed a clear preference for the latter with respect to antitrypanosomal potency, as well as selectivity (Table 1).

Moreover, we observed that the effect of most of the analogues remained almost unchanged (3-fold) when assayed against drug-resistant T. brucei strains (Table 2), except for the 11-fold reduction in sensitivity to 9 observed in trypanosomes lacking the TpAT1 gene encoding the P2 adenosine transporter, that is, TbAT1-KO. Surprisingly, several analogues (6, 8 and 12) displayed reduced activity to the isometamidium-resistant cell line ISMR1, which is not known to have altered nucleoside transport. This may however be related to the fact that these parasites lack kinetoplasts and have reduced mitochondrial membrane potential, resulting in a substantially slower growth rate.

![Purine numbering](https://example.com/purine-numbering.png)

**Fig. 1** Different nucleoside analogues with reported activity against African trypanosomes. [Cordycepin: TCMDC-143080; Formycin B: TCMDC-143083 (codes originating from ref. [6]).]
3′-Deoxytubercidin analogues are trypanocidal. Incubation of wild-type  
*T. brucei* cells with 8, 9 and 10 at 2× and 5× their half-
maximal effective concentration (EC₅₀) showed that derivatives  
9 and 10 display a clear trypanocidal effect against  
*T. brucei* bloodstream forms in vitro, whereas cultures incubated with 8 at 
either 2× and 5× EC₅₀ only displayed growth arrest after a few 
hours, demonstrating a more trypanostatic characteristic
(Supplementary Fig. 1 and Supplementary Fig. 2). We further 
observed that cells treated with adenosine analogues 8, 9 and 10 
at 2× and 5× EC₅₀ showed clear aberrant morphology at 6 h 
and especially at 12 h, with gross distortions of cell shape and the 
apparent formation of intracellular vacuoles. Staining with the 
fluorescent DNA dye DAPI (4′,6-diamidino-2-phenylindole) 
revealed no evidence of inhibition of cytokinesis, which usually

**Table 1 In vitro drug sensitivity of 7-deazapurine nucleoside analogues.**

| Cpd. | T. b. brucei EC₅₀ (µM) | T. b. rhodesiense EC₅₀ (µM) | MRC-5 EC₅₀ (µM) | SI (MRC/T. b. brucei) | SI (MRC/T. b. rhodesiense) |
|------|------------------------|-----------------------------|-----------------|------------------------|-----------------------------|
| 6    | 0.48 ± 0.1             | 0.036 ± 0.001               | 2.2 ± 0.7       | 4                      | 61                          |
| 8    | 1.2 ± 0.3              | 0.12 ± 0.02                 | 12 ± 2          | 10                     | 107                         |
| 9    | 0.048 ± 0.009          | 0.00052 ± 0.0004            | >64             | >1333                  | >123,077                    |
| 10   | 0.0013 ± 0.0003        | 0.00040 ± 0.00009           | 15 ± 3          | 11,462                 | 37,500                      |
| 11   | 48 ± 1                 | >64                         | 6.1 ± 0.7       | 13                     | 50                          |
| 12   | 0.46 ± 0.08            | 0.12 ± 0.01                 | >64             | 13                     | 50                          |

EC₅₀ values, determined with the Alamar blue cell viability assay, are expressed in µM, and represent mean and SEM. The number of independent replicates was n = 5 (8, 9, 10), n = 4 (6) and n = 2 (11 and 12). Cytotoxicity was assessed in human MRC-5 fibroblasts. Source data are provided as a Source Data file.

**Table 2 In vitro drug sensitivity against drug-resistant  
*T. brucei*.**

| Cpd. | Lister 427 EC₅₀ (µM) | TbATT-KO EC₅₀ (µM) | RF | B48 EC₅₀ (µM) | RF | ISMR1 EC₅₀ (µM) | RF |
|------|----------------------|---------------------|----|---------------|----|-----------------|----|
| 6    | 0.15 ± 0.03          | 2.61 ± 0.70         | 17.2**| 4.3 ± 1.3     | 28.7*| 1.7 ± 0.5       | 11.1**|
| 7-BR-TUB | 0.32 ± 0.27      | 0.825 ± 0.0173      | 2.62***| 0.448 ± 0.196 | 1.42| 1.529 ± 0.157   | 4.85***|
| 9    | 3′-Deoxy-TUB 0.033 ± 0.001 | 0.375 ± 0.012 | 11.3***| 0.368 ± 0.013 | 11.0***| 0.324 ± 0.0038 | 0.97|
| 10   | 7-BR-3′-deoxy-TUB 0.0018 ± 0.0003 | 0.0021 ± 0.00025 | 1.2 | 0.0013 ± 0.0003 | 0.75| 0.0011 ± 0.0002 | 0.63|
| 11   | 2′-Deoxy-TUB 96.4 ± 13.8 | 43.7 ± 2.2 | 0.45**| 41.8 ± 1.4    | 0.43**| 77.8 ± 1.7      | 0.81|
| 12   | 7-BR-2′-deoxy-TUB 0.31 ± 0.063 | 0.76 ± 0.18 | 2.37***| 0.83 ± 0.16   | 2.64**| 0.92 ± 0.12     | 2.93**|
| Pentamidine | 0.011 ± 0.001 | 0.018 ± 0.002 | 1.8**| 0.99 ± 0.16  | 94.6***| 0.14 ± 0.04 | 13.8***|
| Dimazene | 0.42 ± 0.064 | 4.5 ± 0.9 | 10.6***| 7.2 ± 1.6 | 16.9***| 2.9 ± 0.4 | 6.9***|
| Isometamidium | 0.65 ± 0.085 | 0.75 ± 0.14 | 1.2 | 0.56 ± 0.13 | 0.85| 3.1 ± 0.5 | 4.8***|

In vitro antitrypanosomal evaluation against drug-resistant  
*T. brucei* cell lines. EC₅₀ values are given in µM and are mean and SEM of three independent determinations (n = 3). RF = Resistance factor, that is, the ratio of EC₅₀ of resistant and reference (Lister 427WT) cell line. TbATT-KO: T. brucei cell line lacking the TbAT1(P2) transporter gene. B48: pentamidine, diminazene and melaminophenyl arsenic resistant  
*T. brucei* line. ISMR1: isometamidium-resistant  
*T. brucei* cell line. TUB, tubercidin. Source data are provided as a Source Data file.

* p < 0.05, ** p < 0.01, *** p < 0.001; Student’s t test (two-sided).
leads to the appearance of large, multi-nucleated cells (Supplementary Fig. 3). The morphology of the nucleus and kinetoplast appears not to have been substantially altered in compound-treated cell populations.

Nucleoside analogue 9 is metabolically stable. Incubations with mouse, rat and human S9 microsomal fractions revealed that 9 is metabolically stable, defined as ≥50% of parent compound remaining after 30 min (Supplementary Table 1). Next, the impact of adenosine deaminase on 7-deazapurine nucleoside analogues 6, 9 and 10 was studied, given that it has been described to greatly affect the antitrypanosomal activity of adenosine analogues17,18,23. Therefore, the in vitro antitrypanosomal activity was re-assessed in the presence of an inhibitor of this enzyme, 2'-deoxycoformycin (Supplementary Table 2). In contrast to cordycepin (3), the EC50 values of the 7-deazapurine analogues (6, 9 and 10) were not significantly affected by the inhibition of adenosine deaminase.

Based on its low cytotoxicity, potent in vitro activity (Fig. 2) and metabolic stability, nucleoside analogue 9 was selected for follow-up evaluation in mouse models of HAT.

Compound 9 shows excellent efficacy in murine HAT models. First, 9 was evaluated in a mouse model of acute HAT. Intraperitoneal administration at 10 mg kg⁻¹ s.i.d. (once a day) for 5 days, or oral dosing at 25, 12.5 and 6.25 mg kg⁻¹ b.i.d. (twice a day) for 5 days resulted in negative blood parasitaemia and survival of all treated mice at the pre-set endpoint of 21 days post infection (dpi) (Supplementary Fig. 4). No adverse reactions have been noted nor was weight loss recorded in any of the treatment schedules, indicating that 9 was well tolerated by the test animals.

Unfortunately, the bromo analogue 10, although more potent in vitro, was not well tolerated at a dose of 6.25 mg kg⁻¹ orally, and was not pursued further.

Next, 9 was evaluated in a CNS mouse model of HAT. Oral dosing at 25 mg kg⁻¹ b.i.d. for 5 days resulted in excellent activity, comparable to the reference drug for CNS clearance, melarsoprol. All treated animals showed negative blood parasitaemia and survival of all treated mice at the pre-set endpoint of 21 days post infection (dpi) (Supplementary Fig. 4). No adverse reactions have been noted nor was weight loss recorded in any of the treatment schedules, indicating that 9 was well tolerated by the test animals.

Unfortunately, the bromo analogue 10, although more potent in vitro, was not well tolerated at a dose of 6.25 mg kg⁻¹ orally, and was not pursued further.
ment of the different inserts in the mode of action of
9
of

Whole-genome RNAi screening. Exposure of a genome-wide
T. b. brucei strain, compared to the BS221 cell line from which it was derived. Results are expressed as the mean IC₅₀
(µM), and error bars represent SEM and are based on two independent experiments (n = 2), each with two biological replicates. + = tetracycline-induced clones; − = non-induced clones. All experiments were performed with two independently generated RNAi clones (a, b). *P < 0.05, **P < 0.001, ***P < 0.0001, Kruskal-Wallis test with Dunn’s multiple comparison test. Source data are provided as a Source Data file.

Out of the five knockdown constructs and the AT1-KO tested, only the knockdown targeting adenosine kinase (ADKIN; Tb927.6.2300) and the adenosine transporter 1 (AT1; Tb927.5.286b) KO cell line decreased the sensitivity for 9: from an EC₅₀ of 0.0077 ± 0.00017 µM (mean and SEM, n = 2) to 0.10 ± 0.005 µM (mean and SEM, n = 2) upon ADKIN knockdown (Fig. 4a) and from 0.0064 ± 0.0004 µM (mean and SEM, n = 2) to 0.15 ± 0.03 µM (mean and SEM, n = 2) upon AT1-KO (Fig. 4b). Notably, despite the ~20-fold reduced susceptibility observed in the ADKIN knockdown cell lines, the in vitro activity of 9 remained in the submicromolar range, suggesting that both ADKIN and AT1 contribute to an important extent but are not essential per se for the antitrypanosomal activity of 9. However, it must also be realized that the RNAi knockdown of ADKIN is unlikely to be 100% and that residual ADKIN activity therefore may be responsible for the remaining activity. For the remaining four constructs, no significant differences in susceptibility could be observed between tetracycline-induced and non-induced clones (Supplementary Fig. 6).

Adenosine transporter assays. The polar nature of the presented nucleoside analogues renders transmembrane translocation through passive diffusion highly unlikely. To confirm the role for the TbAT1/P2 aminopurine transporter in the drug sensitivity of trypanosomes to analogue 9 and to assess the contribution of the P1 purine nucleoside transporter⁴¹ in the uptake of this and structurally related nucleosides, we investigated analogues 8, 9 and 10 for their ability to inhibit P1- and P2-mediated [³H]adenosine uptake (Fig. 5). The Kₘ values for adenosine, obtained as control, were consistent with previously reported values (Table 3)⁴⁰,⁴¹.

Regarding transport via P1, we observed that all three analogues, and especially 9, presented substantially lower affinity than adenosine (Fig. 5 and Table 3), corroborating the lower sensitivity of the TbAT1-KO and B48 strains to 9 (Table 2 and Fig. 4). This indicates a high level of reliance of 9 on P2 transport and is in line with the identification of the encoding gene (TbAT1) in the whole-genome RNAi screening experiment. Interestingly, the addition of a bromine at the 7-position of 9 (analogue 10) improved the affinity for P1 10-fold and recovered

![Graph of susceptibility of ADKIN RNAi-mediated knockdown and TbAT1-KO cell lines to 9.](image)
5.9 kJ mol⁻¹ in Gibbs free energy (Table 3), whereas the presence of the 3′-OH (8 versus 10) only doubled the affinity for P1 (Table 3). The very modest binding energy ΔG° associated with the 3′-OH [δ(ΔG°) = 1.6 kJ mol⁻¹], which has previously been shown to be one of the interaction points of adenosine with P1 having an apparent contribution of 18.4 kJ mol⁻¹ to the binding, appears to indicate that the 7-substituted analogues orient differently in the P1-binding pocket, making the 3′-OH less important.

The 3′-deoxy analogues displayed higher affinity for P2 than adenosine, with cordycepin (3) and 9 displaying almost identical Kᵢ values, roughly 3-fold lower than the Kᵢ value for adenosine, representing an energy advantage of ~3 kJ mol⁻¹, the same as the δ(ΔG°) for 10 compared to 8 (Table 3). This is similar to the energy gain upon removal of the 2′-OH and consistent with P2 being essentially an adenine transporter condoning the ribose moiety.

### Intracellular fate of analogue 9

RNAi experiments revealed the importance of ADKIN for the activity of nucleoside 9. Therefore, the intracellular nucleotide pools of *T. brucei* after exposure to 25 μM of this nucleoside analogue were further analysed. Compound 9 did not cause any change in the balance or total quantity of intracellular purine and pyrimidine nucleotides in comparison to untreated trypanosomes (Fig. 6a, b). However, the analysis unambiguously showed that 9 is internalized and metabolized by *T. brucei* to monoo-, di- and triphosphates (Fig. 6a, Supplementary Figs. 7 and 8). Incubation of analogue 9 with purified recombinant TbADKIN was performed to characterize the kinetic parameters of the first phosphorylation step (Fig. 6c), revealing a
$K_m$ of 195 ± 24 µM (mean and SE, $n = 3$) and $V_{max}$ mg$^{-1}$ of 0.116 ± 0.0048 U (µmol s$^{-1}$) (mean and SE, $n = 3$), similar to what is reported for 2'-deoxyadenosine$^{33}$. In agreement, we got comparable activities with 1 mM 9 and 2'-deoxyadenosine that was used as control (Fig. 6c).

Analogue 9 does not impact *T. brucei* DNA and RNA synthesis. Cell cycle analysis by flow cytometry on *T. brucei* parasites exposed for 24 h to various concentrations of 9 indicated no major impact on DNA synthesis with only a minor effect on relative trypanosome numbers in the S phase (Fig. 7), suggesting

**Fig. 7** Evaluation of the effect of 9 on cell cycle regulation and RNA synthesis. a Gating strategy. b Results of the cell cycle analysis at different concentrations of compound 9. Results of the cell cycle analysis (2N, S phase, 4N) expressed in total cell numbers (c, $n = 2$) and relative cell numbers (d, $n = 2$). e Total RNA yield from trypanosomes exposed to different concentrations of compound 9 ($n = 2$). RNA content quantified using RT-qPCR specifically targeting SL-RNA (f, $n = 2$ with two replicates), 185 rRNA (g, $n = 2$ with two replicates) and TERT mRNA (h, $n = 2$ with two replicates). RNA content and transcript levels are expressed as relative to the non-treated trypanosomes (control). Error bars are SEM. Source data are provided as a Source Data file.
that the formed triphosphate of analogue 9 (9-TP) does not inhibit the parasite’s DNA polymerase. Subsequently, the impact of various concentrations of analogue 9 on RNA synthesis was analysed using standard quantification methods on the total RNA extracts and specific quantitative reverse transcription PCR (RT-qPCR) for the quantitative detection of *T. brucei* SL-RNA, 18S ribosomal RNA (rRNA) and TERT (telomerase reverse transcriptase) transcripts (Fig. 7e–h). The results indicate that neither nucleoside 9 nor any of its derived phosphorylated metabolites act as chain terminators for RNA synthesis given the absence of a major impact on the total RNA pool and the levels of specific transcripts produced by RNA polymerase I and II.

The absence of major perturbations in the nucleotide pool of cells treated with 9 appears to indicate that it exerts its trypanocidal activity by some mechanism other than causing an imbalance in the intracellular nucleotide pool34. Moreover, the absence of DNA fragmentation or disruption of DNA synthesis shows that 9 is not incorporated into DNA, as the 3’-deoxy analogue would be an obligate chain terminator. Additionally, we have shown that 9 (or any of its phosphorylated metabolites) does not interfere with RNA synthesis. The presence of all three phosphorylated metabolites, as shown by high-performance liquid chromatography (HPLC) analysis, further complicates mode-of-action investigations, as it was shown that the triphosphate of tubercidin was responsible for the inhibition of *T. brucei* phosphoglycerate kinase34, an enzyme from the glycolytic pathway.

In summary, we here described the discovery and effects of potent antiparasomal agents having a 3’-deoxy-7-deazaadenosine structure. Analogue 9 represents a potent and orally bioavailable chemotherapeutical candidate for treatment of human and animal African trypanosomiasis, which shows no detrimental level of cross-resistance with currently used drugs.

**Methods**

**Ethics statement for animal models**. The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 1 January 2013, and the Declaration of Helsinki in its latest version) and was approved by the Ethical Committee of the University of Antwerp, Belgium [UA-ECD 2017–04].

**Trypanosoma brucei in vitro drug susceptibility assays.** Drug susceptibility tests with Lister 427WT, BTAT1-KO48, BS221, and 1SMR12 were performed using an assay based on the viability indicator dye resazurin in 96-well plates, each well containing 2 × 10^4 cells. The plates were incubated for 48 h with a doubling dilution series of the test compounds in HMI-9/fetal bovine serum at 37°C/5% CO2 (23 dilutions starting at 100 µM, except for the pentamidine control (50 µM)), after which resazurin (20 µL of 125 µg mL^-1) was added to each well (containing 200 µL of cells and test compound) was added to each well and the plates incubated for another 24 h. Fluorescence was determined using a FLUOstar Optima (BMG Labtech, Durham, NC) and the results fitted to a sigmoid curve with variable slope using Prism 5.0 or Prism 7.04 (GraphPad, San Diego, CA).

Susceptibility assays with *T. brucei* Squib 4279 or *T. b. rhodesiense* STIB-90050 were performed under similar conditions as above, but using 10 concentrations of a 4-fold compound dilution series starting at 64 µM. Trypanosoma brucei Squib 4279 was seeded at 1.5 × 10^4 parasites/well and *T. b. rhodesiense* at 4 × 10^3 parasites per well, followed by the addition of resazurin (final concentration of 10 µg mL^-1) after 24 h (*T. brucei*) or 6 h (*T. b. rhodesiense*).

Susceptibility assays with NY-SM13, BS221, and BTAT1-KO48 (derived from BS221) cells were performed using 10 concentrations of a 4-fold compound dilution series starting at 64 µM. Parasites were seeded at 4 × 10^3 parasites per well and exposed to drug compound for 72 h, after which resazurin (final concentration of 10 µg mL^-1) was added to the plates. Fluorescence intensities were determined after 6 h.

**Cytotoxicity on MRC-5 fibroblasts.** Cytotoxicity assays were performed on MRC-5 V52 (Sigma-Aldrich/ECACC, catalogue number 84100041) human embryonic lung fibroblasts, which were cultured in minimum essential medium with Earle’s salt medium, supplemented with 1-glutamine, NaHCO3, and 5% inactivated foetal calf serum. All cultures and assays were conducted at 37°C with 5% CO2. Ten microliters of the aqueous compound dilutions were added to 190 µL of MRC-5 V52 (3 × 10^5 cells mL^-1). Cell growth was compared to untreated control wells (100% cell growth) and medium control wells (0% cell growth). After a 3-day incubation, cell viability was assessed fluorometrically after the addition of 50 µL resazurin per well (final concentration of 10 µg mL^-1). After 4 h at 37°C, fluorescence was measured (λex 550 nm, λem 590 nm). The results were expressed as percentage reduction in cell growth/viability compared to control wells and an EC50 was determined.

**Mouse model of acute HAT (T. b. brucei Squib 427).** Female Swiss mice (body weight (DW) – 20–24 g; Janvier Labs, France) were allocated randomly to groups of three animals and infected intraperitoneally (i.p.) with 10^7 T. b. brucei Squib 427 derived from a heavily infected donor mouse. Drinking water and food were available ad libitum throughout the experiment. Analogue 9 was formulated in 10%
(v/v) polyethylene glycol 400 in water at 2 mg mL⁻¹ or in 5% (v/v) Tween-80 in water at 4 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was added Chlamydomonas reinhardtii, Sol. Strain, for 5 days at 25 µg mL⁻¹ i.p. i.d. for 5 days at 10 mg kg⁻¹ (all groups n = 3). The reference drug suramin was formulated in phosphate-buffered saline (PBS) at 2.5 mg mL⁻¹ and administrated s. i. p. for 5 days at 10 mg kg⁻¹ (n = 8). All treatments were initiated 30 min prior to the i.p. infection. Animals were observed for the occurrence of clinical or adverse effects during the course of the experiment and the weight was daily. Parasitaemia was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi (pre-set endpoint). As a test of cure, blood samples (250 µL) were collected from treated mice at 21 dpi and were sub-inoculated i.p. in naive Swiss mice (n = 3), followed by monitoring of parasitaemia as follow-up.

Mouse model of stage 2 HAT. Female Swiss mice (BW = 20–24 g; Janvier Labs France) were randomly allocated to groups of 4 animals and infected i.p. with 10⁶ T. brucei ANaTAR11, PYPYRE92 (T. b. brucei in 5% (v/v) polyethylene glycol 400 in water at 2 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was formulated in 5% (v/v) Tween-80 in water at 4 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was administered by oral gavage for 5 days at 25 µg kg⁻¹ i.d. (n = 2), 25 mg kg⁻¹ i.d. (n = 3), 12.5 mg kg⁻¹ i.d. (n = 2) or 6.25 mg kg⁻¹ s. i. d. (n = 3). The reference drug melarsoprol was formulated as a stock solution of 3.6% in propylene glycol. The concentration had a significant effect on the death rate. Parasitocytosis was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi. For a constant, high level of expression, in the background strain of the RNAi library). The transformed NY-SM cells were cloned by limiting dilution. Two clones of each RNAi insert were exposed to tetracycline (1 µg mL⁻¹) for 25 min at 37 °C. Cells were analysed on a MACSQuant iCanto (Becton Dickinson). The background of the RNAi library). The transformed NY-SM cells were cloned by limiting dilution. Two clones of each RNAi insert were exposed to tetracycline to induce the RNAi phenotype and subsequently subjected to different concentrations of 9 to determine the susceptibility of induced versus non-induced clones.

Drug susceptibility assays with induced clones were performed as described above for NY-SM cells, with an initial induction period of 68 h with 1 µg mL⁻¹ tetracycline prior to the start of the drug susceptibility assay.

In vitro adenosine transporter assay. Transport via P1 was measured using B48 cells, which lack the P2 transport system26, using a [3H]adenosine concentration of 0.1 µM, whereas the transport via P2 was assayed in B48 cells transfected with TbAT1(P2) gene (B48 + TbAT1)33 for a constant, high level of expression, in the presence of 100 µM of inosine to block the P1 transporter (0.033 µM [3H]adenosine). The transport of [3H]adenosine (40 Ci mmol⁻¹) was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi (pre-set endpoint). As a test of cure, blood samples (250 µL) were collected from treated mice at 21 dpi and were sub-inoculated i.p. in naive Swiss mice (n = 3), followed by monitoring of parasitaemia as follow-up.

Mouse model of stage 2 HAT. Female Swiss mice (BW = 20–24 g; Janvier Labs France) were randomly allocated to groups of 4 animals and infected i.p. with 10⁶ T. b. brucei ANaTAR11, PYPYRE92 (T. b. brucei in 5% (v/v) polyethylene glycol 400 in water at 2 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was formulated in 5% (v/v) Tween-80 in water at 4 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was administered by oral gavage for 5 days at 25 µg kg⁻¹ i.d. (n = 2), 25 mg kg⁻¹ i.d. (n = 3), 12.5 mg kg⁻¹ i.d. (n = 2) or 6.25 mg kg⁻¹ s. i. d. (n = 3). The reference drug melarsoprol was formulated as a stock solution of 3.6% in propylene glycol. The concentration had a significant effect on the death rate. Parasitocytosis was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi. For a constant, high level of expression, in the background strain of the RNAi library). The transformed NY-SM cells were cloned by limiting dilution. Two clones of each RNAi insert were exposed to tetracycline to induce the RNAi phenotype and subsequently subjected to different concentrations of 9 to determine the susceptibility of induced versus non-induced clones.

Drug susceptibility assays with induced clones were performed as described above for NY-SM cells, with an initial induction period of 68 h with 1 µg mL⁻¹ tetracycline prior to the start of the drug susceptibility assay.

In vitro adenosine transporter assay. Transport via P1 was measured using B48 cells, which lack the P2 transport system26, using a [3H]adenosine concentration of 0.1 µM, whereas the transport via P2 was assayed in B48 cells transfected with TbAT1(P2) gene (B48 + TbAT1)33 for a constant, high level of expression, in the presence of 100 µM of inosine to block the P1 transporter (0.033 µM [3H]adenosine). The transport of [3H]adenosine (40 Ci mmol⁻¹) was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi (pre-set endpoint). As a test of cure, blood samples (250 µL) were collected from treated mice at 21 dpi and were sub-inoculated i.p. in naive Swiss mice (n = 3), followed by monitoring of parasitaemia as follow-up.

Mouse model of stage 2 HAT. Female Swiss mice (BW = 20–24 g; Janvier Labs France) were randomly allocated to groups of 4 animals and infected i.p. with 10⁶ T. b. brucei ANaTAR11, PYPYRE92 (T. b. brucei in 5% (v/v) polyethylene glycol 400 in water at 2 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was formulated in 5% (v/v) Tween-80 in water at 4 mg mL⁻¹ and was freshly prepared at every administration. Analogue 9 was administered by oral gavage for 5 days at 25 µg kg⁻¹ i.d. (n = 2), 25 mg kg⁻¹ i.d. (n = 3), 12.5 mg kg⁻¹ i.d. (n = 2) or 6.25 mg kg⁻¹ s. i. d. (n = 3). The reference drug melarsoprol was formulated as a stock solution of 3.6% in propylene glycol. The concentration had a significant effect on the death rate. Parasitocytosis was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi. For a constant, high level of expression, in the background strain of the RNAi library). The transformed NY-SM cells were cloned by limiting dilution. Two clones of each RNAi insert were exposed to tetracycline to induce the RNAi phenotype and subsequently subjected to different concentrations of 9 to determine the susceptibility of induced versus non-induced clones.

Drug susceptibility assays with induced clones were performed as described above for NY-SM cells, with an initial induction period of 68 h with 1 µg mL⁻¹ tetracycline prior to the start of the drug susceptibility assay.

In vitro adenosine transporter assay. Transport via P1 was measured using B48 cells, which lack the P2 transport system26, using a [3H]adenosine concentration of 0.1 µM, whereas the transport via P2 was assayed in B48 cells transfected with TbAT1(P2) gene (B48 + TbAT1)33 for a constant, high level of expression, in the presence of 100 µM of inosine to block the P1 transporter (0.033 µM [3H]adenosine). The transport of [3H]adenosine (40 Ci mmol⁻¹) was determined by microscopic evaluation of tail vein blood samples at 4, 7, 10, 14 and 21 dpi (pre-set endpoint). As a test of cure, blood samples (250 µL) were collected from treated mice at 21 dpi and were sub-inoculated i.p. in naive Swiss mice (n = 3), followed by monitoring of parasitaemia as follow-up.
References

1. Bürcher, P., Cecchi, G., Jamonneau, V. & Prisotto, G. Human African trypanosomiasis. Lancet 390, 2397–2409 (2017).
2. Field, M. C. et al. Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. Nat. Rev. Microbiol. 15, 217–231 (2017).
3. Kennedy, P. G. E. Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). Lancet Neurol. 12, 186–194 (2013).
4. Bolton, R. Don, R., Jacobs, R. T., Wang, M. Z. & Barrett, M. P. Development of novel drugs for human African trypanosomiasis. Fst. Microbiol. 6, 677–691 (2011).
5. Babkovich, P., Sanyalo, A. O., Oyibo, W. A., Fagbenro-Beyioku, A. F. & Iriemenam, N. C. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. Pathog. Glob. Health 107, 422–252 (2013).
6. Mesu, V. et al. Oral fexinidazole for late-stage African trypanosomiasis: a pivotal multicentre, randomised, non-inferiority trial. Lancet 391, 144–154 (2018).
7. Pépin, J. et al. Gambiense trypanosomiasis: frequency of, and risk factors for, failure of melarsoprol therapy. Trans. R. Soc. Trop. Med. Hyg. 88, 447–452 (1994).
8. Kennedy, P. G. The continuing problem of human African trypanosomiasis (sleeping sickness). Ann. Neurol. 64, 116–126 (2008).
9. Sokolova, A. Y. et al. Cross-resistance to nitro drugs and implications for the proposal of a structural model for the protein. Mol. Microbiol. 96, 887–900 (2015).
10. Collar, C. et al. Predictive computational models of substrate binding by a nucleoside transporter. J. Biol. Chem. 284, 34028–34035 (2009).
11. Berg, M. et al. Evaluation of nucleoside hydrolase inhibitors for treatment of African trypanosomiasis. Antimicrob. Agents Chemother. 54, 2893–2900 (2010).
12. Wylie, S. et al. Nitroheterocyclic drug resistance mechanisms in Trypanosoma brucei. J. Antimicrob. Chemother. 71, 625–634 (2016).
13. Berg, M. et al. Evaluation of nucleoside hydrase inhibitors for treatment of African trypanosomiasis. Antimicrob. Agents Chemother. 54, 1900–1908 (2010).
14. Miller, R. W. et al. Laminoribitol transition state analogue inhibitors of protozoan nucleoside hydrases. Biochemistry 38, 13147–13154 (1999).
15. Kennedy, K. J., Bresi, J. C. & Gell, M. H. A disubstituted NAD+-analoge is a nanomolar inhibitor of trypanosomal glyceraldehyde-3-phosphate dehydrogenase. Bioorg. Med. Chem. Lett. 11, 95–98 (2001).
16. Berg, M., de Vekens, V., Grommeme, P., Haemers, A. & Augustyns, A. K. Inhibitors of the purine salvage pathway: a valuable approach for antiprotozoal chemotherapy? Curr. Med. Chem. 17, 2456–2481 (2010).
17. Williamson, J. Cordycepin, an antitumour antibiotic with trypanocidal properties. Trans. R. Soc. Trop. Med. Hyg. 60, 8–8 (1966).
18. Pena, I. et al. New compound sets identified from high throughput phenotypic screening against three kinetoplastid parasites: an open resource. Sci. Rep. 5, 8771 (2015).
19. Vodnala, S. K. et al. Preliminary assessment of the treatment of second-stage African trypanosomiasis with cordycepin and deoxycoformycin. PLoS Negl. Trop. Dis. 3, e495 (2009).
20. Vodnala, S. K. et al. Structure–activity relationships of synthetic cordycepin analogues as experimental therapeutics for African trypanosomiasis. J. Med. Chem. 56, 9861–9873 (2013).
21. Williamson, J. Activity of drugs on Trypanosoma congolense in vitro at 37 degrees C. Trans. R. Soc. Trop. Med. Hyg. 63, 422–423 (1969).
22. Aoki, J. L. et al. Characterization of a novel endoplasmic reticulum protein involved in tuberculin resistance in Leishmania major. PLoS Negl. Trop. Dis. 10, e004927 (2016).
23. Ranbjanari, F. et al. 9-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl) adenine is a potent antitrypanosomal adenine analogue that circumvents transport-related drug resistance. Antimicrob. Agents Chemother. 61, e02719 (2017).
24. Ho, H. T. B. & Wang, J. in Drug Transporters: Molecular Characterization and Role in Drug Disposition 2nd edn, 107–126 (Wiley, 2014).
25. Rottenberg, M. E. et al. Treatment of African trypanosomiasis with cordycepin and adenosine deaminase inhibitors in a mouse model. J. Infect. Dis. 192, 1658–1665 (2005).
26. Drew, M. E. et al. The adenosine analog tubercin inhibits glycolysis in Trypanosoma brucei as revealed by an RNA interference library. J. Biol. Chem. 278, 46596–46600 (2003).
27. Hulupa, F. et al. Revisiting tubercin against kinetoplastid parasites: aromatic substitutions at position 7 improve activity and reduce toxicity. Eur. J. Med. Chem. 164, 689–705 (2019).
28. Bridges, D. J. et al. Loss of the high-affinity pentamidine transporter is responsible for high levels of cross-resistance between arsenical and diamidines in African trypanosomes. Mol. Pharmacol. 71, 1098–1108 (2007).
29. Eze, A. A. et al. Reduced mitochondrial membrane potential is a late adaptation of Trypanosoma brucei to isometidium preceded by mutations in the γ-subunit of the F1F0-ATPase. PLoS Negl. Trop. Dis. 10, e004791 (2016).
30. Gonzalez-Andrade, P. et al. Diagnosis of trypanosomatis infections: targeting the spiked leader RNA. J. Mol. Diagn. 16, 400–404 (2014).
Acknowledgements
F.H. is indebted to the FWO-Flanders for a Ph.D. scholarship. G.D.C. thanks Science Without Borders for his scholarship (206385/2014-5, CNPq, Brazil). G.C. is supported by a research fund of the University of Antwerp (TT-ZAPBOF 33049). The present work has been funded by the FWO (G.C., L.M., S.V.C.; project number G013118N). S.V.C. thanks Prof. emeritus Fred Oppendoes for the useful suggestion to collaborate with H.P.d.K. to study the involvement of adenosine transporters in the uptake of the nucleoside analogues. The authors express their gratitude to Dr. Jennifer Ann Black for assistance with the microscopy experiments. We thank Rik Hendrickx, Pim-Bart Feijens, An Mathieuussen, Natasa Van Pelt, Mandy Vermont, Margot Desmet and Izet Karalic for excellent technical assistance.

Author contributions
Design of research: F.H., A.H., H.P.d.K., G.C., S.V.C. Performed experiments: F.H., D.M., G.D.C., G.S., I.R., L.M., A.H., H.P.d.K., G.C., S.V.C. Contributed RNAi library: I.R., G.S. Wrote the manuscript: F.H., D.M., G.D.C., G.S., I.R., L.M., A.H., H.P.d.K., G.C., S.V.C. D.M. and G.D.C. contributed equally to the mode of action studies in this work.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-13522-6.

Correspondence and requests for materials should be addressed to S.V.C.

Peer review information Nature Communications thanks Vern Schramm and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019