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Nucleoside analogues for the treatment of animal trypanosomiasis

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ABSTRACT

Animal trypanosomiasis (AT) is a parasitic disease with high socio-economic impact. Given the limited therapeutic options and problems of toxicity and drug resistance, this study assessed redirecting our previously identified antitrypanosomal nucleosides for the treatment of AT. Promising hits were identified with excellent *in vitro* activity across all important animal trypanosome species. Compound **7**, an inosine analogue, and our previously described lead compound, 3′-deoxytubercidin (**8**), showed broad spectrum anti-AT activity, metabolic stability in the target host species and absence of toxicity, but with variable efficacy ranging from limited activity to full cure in mouse models of *Trypanosoma congolense* and *T. vivax* infection. Several compounds show promise against *T. evansi* (surra) and *T. equiperdum* (dourine). Given the preferred target product profile for a broad-spectrum compound against AT, this study emphasizes the need to include *T. vivax* in the screening cascade given its divergent susceptibility profile and provides a basis for lead optimization towards such broad spectrum anti-AT compound.

1. Introduction

Trypanosomiasis is a neglected parasitic disease responsible for human and animal infections in Africa, Asia, South America and parts of Europe(Kennedy, 2013; Radwanska et al., 2018). Human African trypanosomiasis (HAT), mainly caused by *T. b. gambiense* and *T. b. rhodesiense*, is confined to a tsetse fly endemic region on the African continent due to its dependency on transmission by tsetse flies(Tirados et al., 2015). Animal trypanosomiasis (AT) is caused by a variety of species. *T. b. brucei* and *T. congolense* are transmitted by tsetse flies and cause infection of wild and domestic animals on the African continent (Truc et al., 2013). The transmission of *T. vivax* to the animal host includes a short developmental cycle in the anterior parts of the tsetse fly (Ooi et al., 2016), or mechanical transmission by tsetse flies and other blood sucking insects (horse flies: *Tabanus* spp.(Desquesnes and Dia, 2003) and stable flies: *Stomoxys* spp.)(Odeniran et al., 2019). The latter has contributed to the spread of *T. vivax* far outside the tsetse fly belt (Radwanska et al., 2018). A mechanical mode of transmission was also acquired by *T. evansi* explaining its spread outside the African continent; it is now endemic in Africa, Asia and South America, causing infection in many species including equines, bovines and camelids(Desquesnes et al., 2013). *T. equiperdum* is transmitted in equines during mating, without any vector involvement, and is prevalent in Africa, Asia, South America and parts of Europe(Gizaw et al., 2017).

Despite recent achievements in vaccine development for *T. vivax* (Autheman et al., 2021), global disease prevention through vaccination is not yet possible and AT control solely depends on vector control and chemotherapy. The drugs that are currently available to treat HAT have major limitations, including drug toxicity, parenteral administration, and the emergence of drug resistance(Babokhov et al., 2013; Baker et al., 2013; Sokolova et al., 2010; Wyllie et al., 2016). A new era in HAT treatment was ushered in with the global approval of fexinidazole, the first all-oral drug available (Mesu et al., 2018) and the positive clinical trial results for the one-day oral drug treatment with acoziborole(Dickie et al., 2020). Nonetheless, nifurtimox-eflornithine combination therapy remains the treatment of choice in severely ill patients and continued

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research remains important to achieve the goal of sleeping sickness elimination as set by the WHO.

For the treatment of AT, seven compounds are currently in use (diminazene aceturate, homidium bromide/chloride, isometamidium, pyrithidium bromide, quinapyramine, suramin and melarsomine dihydrochloride)(Giordani et al., 2016), however, each of these drugs has limitations of toxicity and increasing emergence of drug resistance in the field(Delespaux and de Koning, 2007; Geerts et al., 2001). Diminazene aceturate (DA) and isometamidium chloride (ISM) are mostly used, followed by suramin. In areas of high drug resistance, drugs are used sequentially alternating using compounds of distinct chemical classes (*e.g.* DA and ISM, called a sanative pair) or used at a higher dosage(Giordani et al., 2016; Leach and Roberts, 1981). Although in comparison, HAT burdens remain relatively low and are confined to African countries, the high prevalence of animal *Trypanosoma* species in domestic animals across different continents has a large socio-economic impact on communities via agricultural production and animal husbandry(Kasozi et al., 2021). It has been decades since the introduction of any new treatments for AT and the need for novel drug and/or vaccine candidates is now urgent.

African trypanosomes lack machinery for *de novo* purine synthesis and thus solely depend on the salvage of purines from the host environment(Berg et al., 2010). As purine uptake is essential for parasite replication, interfering with the involved pathways constitutes a logical strategy to find novel antitrypanosomal treatments. Our previous research contributed to the discovery of highly potent nucleoside analogues(Hulpia et al., 2019a, 2019b, 2020a), including 7-deaza adenosine analogues, 7-deaza inosine analogues, and 3′-deoxy-7-deaza adenosine analogues that represent interesting candidates for the treatment of late-stage HAT. Given the large socio-economic impact of AT, the present study evaluated the potential use of nucleoside analogues for the control of animal trypanosomiasis using *in vitro* drug sensitivity assays as well as animal (mouse) models.

2. Materials and methods

2.1. Ethics statement

The use of laboratory rodents was carried out in strict accordance with 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 01/01/2013, and the declaration of Helsinki in its latest version) and was approved by the Ethical Committee of the University of Antwerp, Belgium [UA-ECD 2019–26].

2.2. Animals and parasites

Female Swiss mice (8 weeks, ~25 g) were purchased from Janvier (France). Food for laboratory rodents (Carfil, Arendonk, Belgium) and drinking water were available *ad libitum*. The animals were kept in quarantine for at least 5 days before infection and were randomly allocated to the experimental units.

In vitro drug assays were performed with bloodstream forms of several trypanosome species. *T. congolense* IL3000 and the diminazene-resistant clone 6C3 derived from it by *in vitro* exposure to increasing concentrations of the drug(Carruthers et al., 2021) were cultured in Dulbecco's minimum essential medium (MEM) supplemented with 25 mM HEPES, 26 mM $NaHCO_3$, 5.6 mM D-glucose, 1 mM sodium pyruvate, 40 μM adenosine, 100 μM hypoxanthine, 16.5 μM thymidine, and 25 μM bathocuproinedisulfonic acid disodium salt. To this basal medium were added β-mercaptoethanol (0.0014% v/v), 1.6 mM glutamine, 10 units/mL penicillin, 0.1 mg/mL streptomycin, 20% goat serum (Gibco), and 5% Serum Plus (SAFC Biosciences)(Coustou et al., 2010).

T. evansi strain AnTat 3.3(Kageruka and Mortelmans, 1971) and *T. equiperdum* BoTat 1.1(Capbern et al., 1977) were cultured in Hirumi's Modified Iscove's (HMI-9) medium (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS (PAA Laboratories Linz, Austria)), 14 μL/L β-mercaptoethanol (BDH, Dorset, United Kingdom), and 3.0 g/L $NaHCO_3$ (Sigma) adjusted to pH 7.4. These parasites were kept at 37 °C in a humidified, 5% CO_2 environment.

T. vivax (ILRAD700, kindly provided by dr. Nick Van Reet and Prof. Philippe Büscher of the Institute of Tropical Medicine, Antwerp) and *T. congolense* (TC13, kindly provided by Prof. Benoît Stijlemans of the Vrije Universiteit Brussel) were used for *ex vivo* and *in vivo* experiments.

2.3. Compounds

A range of tubercidin analogues (Fig. 1) was tested for *in vitro* and *in vivo* activity against the most pathogenic animal trypanosomes. Experimental details regarding the chemical synthesis have been described elsewhere(Hulpia et al., 2019a, 2019b, 2020a, 2020b; Mabille et al., 2021). Purity of all nucleoside analogues was >95%, as assayed via analytical LC/MS (UV-integration), of which the methods are as described before(Hulpia et al., 2020a). Analogues **5** and **6** were prepared as described in literature(Seela and Ming, 2007).

2.4. Cytotoxicity assay on MRC-5 fibroblasts

MRC-5_{SV2} cells were cultured in MEM (Life Technologies) supplemented with L-glutamine, $NaHCO_3$ and 5% heat-inactivated fetal bovine serum (hiFBS). To determine the *in vitro* cytotoxicity, cells were seeded at a concentration of 15,000 cells/well. Four-fold dilutions of the test compounds were added to the cells with a highest in-test concentration of 64 μM. After 72 h of drug exposure at 37 °C and 5% CO_2, cell viability was determined by fluorescence reading (Tecan®, GENios) after a 4-h incubation with resazurin (Sigma Aldrich). The 50% cytotoxic concentration (CC_{50}) was calculated for each of the compounds. The obtained cytotoxicity values were compared to those of the AT reference compounds: suramin (>64 μM, n = 3), diminazene aceturate (>64 μM, n = 1), isometamidium (22.74 μM, n = 1) and quinapyramine (>64 μM, n = 1).

2.5. In vitro drug susceptibility assays

T. b. brucei Squib 427(Kaiser et al., 2015) was cultured in HMI-9 culture medium supplemented with 10% hiFBS. To determine the *in vitro* susceptibility of *T. b. brucei* to the test compounds, cells were seeded at a concentration of 1.5×10^4 cells/well. Drug stock solutions were prepared in 100% DMSO. Four-fold dilutions of the test compounds were added to the cells with a highest in-test concentration of 64 μM. DMSO concentration in the wells never exceeded 1%. After 72 h of drug exposure at 37 °C and 5% CO_2, cell viability was determined by fluorescence reading (Tecan®, GENios) after a 24-h incubation with resazurin (Sigma Aldrich) at a final concentration of 10 μg/mL. The 50% inhibitory concentration (IC_{50}) was calculated for each of the compounds.

For *T. evansi*, *T. equiperdum* and *T. congolense*, the assays were performed in their respective media and temperatures as described above, in white 96-well plates (Greiner Bio-one, Frickenhausen, Germany), using either 11 (1 row) or 23-doubling dilutions (2 rows) as required to obtain a full sigmoid curve, starting at 100 μM drug concentration and leaving one well as drug-free control. To each well was added 100 μL of trypanosome culture adjusted to 2×10^4 cells/mL (*T. equiperdum*), 4×10^4 cells/mL (*T. evansi*) or 5×10^5 cells/mL (*T. congolense*). The cells were incubated with the drug dilutions for 48 h, followed by the addition of 20 μL of resazurin solution and a further incubation of 24 h. Fluorescence was determined using a FLUOstar Optima (BMG Labtech, Durham, NC, USA) at wavelengths of 544 nm for excitation and 590 nm for emission. The EC_{50} values for the drugs/test compounds were calculated by non-linear regression fitted to a sigmoidal dose-response curve with variable slope (Prism 8.0, GraphPad Software Inc., San

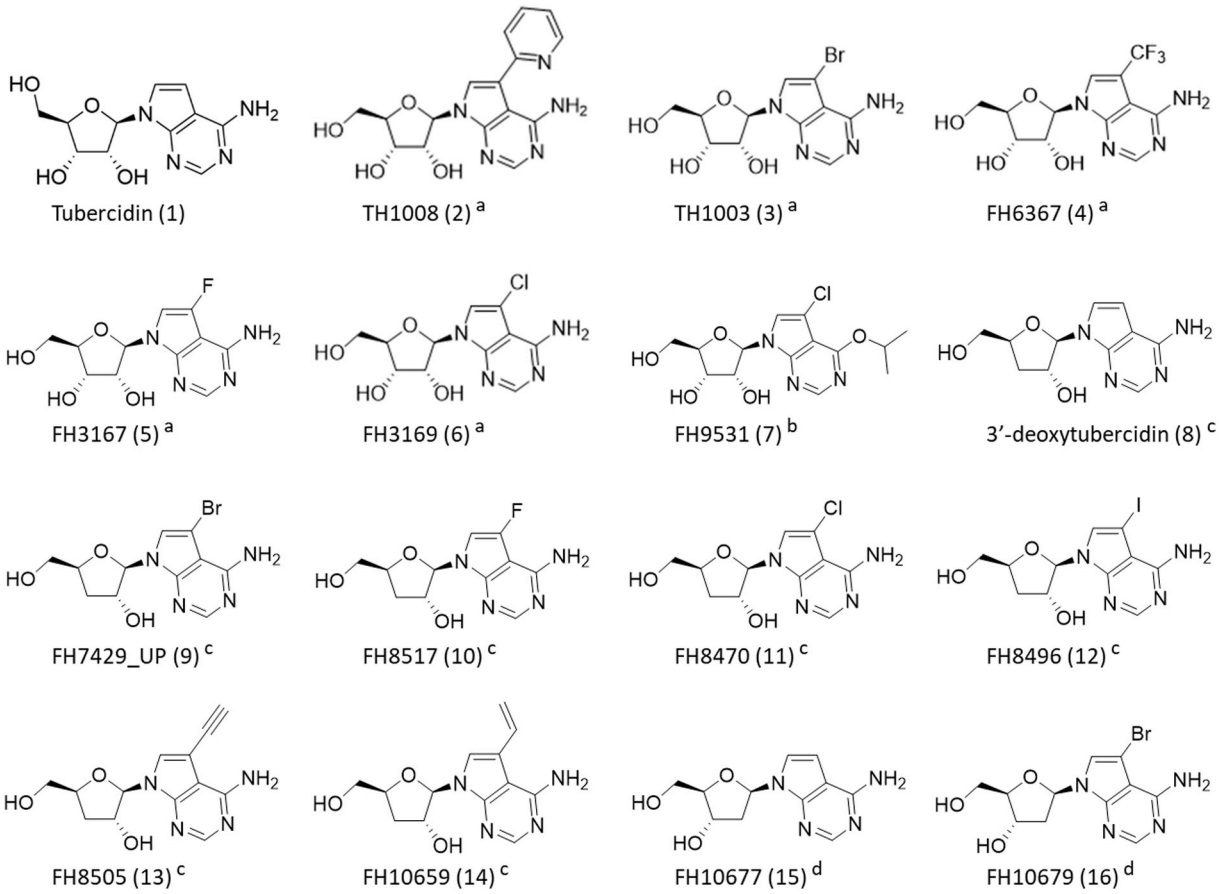


Fig. 1. Overview of the nucleoside analogues used in this study. Compound name (compound code). [a] 7-deaza adenosine analogues. [b] Inosine analogues. [c] 3′-deoxy-7-deaza adenosine analogues. [d] 2′-deoxy-7-deaza adenosine analogues. Substitution of C-7 of tubercidin (**1**) with a pyridin-2-yl (TH1008), bromide (TH1003), trifluoromethyl (FH6367), fluoride (FH3167), or chloride (FH3169) group led to compounds **2**–**6**. Compound **7** is a C6–*O*-alkylated 7-substituted 7-deazainosine analogue. Compound **8** is 3′-deoxytubercidin. Substitution of C-7 of 3′-deoxytubercidin with a bromide (FH7429_UP), fluoride (FH8517), chloride (FH8470), iodide (FH8496), propyn (FH8505), propene (FH10659) group led to compounds **9-14**. Compounds **15** (FH10677) and **16** (FH10679) are 2′-deoxytubercidin analogues with a bromide substitution at C-7 for compound **16**. Compound codes in original publication: (Tubercidin (1), TH1008 (13), TH1003 (31)) [(Hulpia et al., 2019a)], FH6367 (4) [(Mabille et al., 2021)], FH9531 (36) [(Hulpia et al., 2020a)], (3′-deoxytubercidin (9), FH7429_UP (10), FH10677 (11), FH10679 (12)) [(Hulpia et al., 2019b)], (FH8517 (7), FH8470 (8), FH8496 (9), FH8505 (12), FH10659 (16)) [(Hulpia et al., 2020b)].

Diego).

2.6. Ex vivo drug susceptibility assay for T. vivax

Donor mice were infected intraperitoneally with 10^4 *T. vivax* parasites derived from cryostabilates. At 5 days post-infection (dpi), blood was collected via cardiac puncture and parasites were isolated from the heparinized blood using the mini anion exchange centrifugation technique (mAECT) as used in the field for diagnosis of African trypanosomiasis(Buscher et al., 2009). Isolated parasites were seeded in HMI-9 culture medium supplemented with 10% hiFBS at a concentration of 10^5 parasites/well. Four-fold dilutions of the test compounds were added to the cells with a highest in-test concentration of 64 μM. After 24 h of drug exposure at 37 °C and 5% CO_2, the viability was determined by fluorescence reading (Tecan®, GENios) after a 6- and 24-h incubation with resazurin (Sigma Aldrich) at a final concentration of 10 μg/mL. The 50% inhibitory concentration (IC_{50}) was calculated for each compound by comparing cell viability of drug-treated wells to untreated control wells.

2.7. In vitro metabolic stability

The metabolic stability of selected hits was tested using human, bovine, horse and mouse liver microsomes (Corning) of phase-I (CYP_{450} and NADPH dependent enzymes) and phase-II (UGT enzymes) metabolism as described before (Hulpia et al., 2019a). In short, samples were collected after 0, 15, 30 and 60 min and the corresponding loss of parent compound was determined using ultra-performance liquid chromatography (UPLC) (Waters AquityTM) coupled with tandem quadrupole mass spectrometry (MS^2) (Waters XevoTM), equipped with an electrospray ionization (ESI) interface and operated in multiple reaction monitoring (MRM) mode.

2.8. Exploratory in vivo acute toxicity evaluation

The test compounds were formulated at 6.25 mg/mL in 10% PEG_{400}. Per compound, one uninfected mouse was treated twice daily for 5 consecutive days intraperitoneally at 50 mg/kg (3′-deoxytubercidin; **8**) or 25 mg/kg (**7**, **9**, **11** and **14**). Body weight and general clinical appearance were monitored daily during the next 4 days for signs of toxicity.

2.9. Mouse model of AT

Female Swiss mice were randomly divided into groups of 3 animals and intraperitoneally infected with 10^4 *T. congolense* (TC13) or *T. vivax* (ILRAD700) derived from a heavily infected donor mouse. Analogues **7** and **8** were formulated in 10% PEG_{400} in water at 2 mg/mL, freshly

prepared before each administration and administered by intraperitoneal injection for 5 days at 50 mg/kg (**7**) or 6.25 mg/kg (**8**) once a day. The reference drug diminazene aceturate was formulated in phosphate buffered saline at 2.5 mg/mL and administered intraperitoneally for 5 days at 10 mg/kg once a day. Treatment was initiated at day 3 post-infection when the parasitaemia reached 10^6/mL.

Parasitaemia was determined microscopically using an improved Neubauer haemocytometer, daily for the first 2 weeks, twice a week up to 30 dpi and once a week up to 60 dpi. At 60 dpi, mice were euthanized and 100 μL of blood was collected for RNA extraction using the QIAamp RNA blood mini kit (Qiagen) following the manufacturer's instructions prior to qPCR analysis to determine the presence of residual parasite burdens. Parasite levels were determined using quantitative real-time PCR targeting SL-RNA(Gonzalez-Andrade et al., 2014) with optimized assay conditions for *T. congolense* and *T. vivax* using the SensiFAST™ SYBR® Hi-ROX One-Step Kit (Table S1-2). RT-qPCR targeting the *eukaryotic translation elongation factor 2* (*Eef2*), a mouse reference gene, was performed in parallel to confirm appropriate RNA extraction in all tested samples(Eissa et al., 2016).

3. Results

3.1. In vitro activity across a broad panel of animal trypanosome species

All nucleoside analogues were tested *in vitro* on MRC-5_{SV2} cells to evaluate the level of cytotoxicity/selectivity, and on *T. b. brucei*, *T. evansi*, *T. equiperdum* and *T. congolense* (IL3000 and the derived diminazene-resistant 6C3 strain). Activity against *T. vivax* was evaluated in an *ex vivo* assay (Table 1).

Of the C-7 substituted 7-deazaadenosine analogues (compounds **2-6**), **2** was previously reported to have submicromolar activity against *T. b. brucei in vitro* (Hulpia et al., 2019a). The data in Table 1 shows that compounds **2-6** all exhibited (sub)micromolar activity against the entire panel of AT strains, except for compound **2**, which was not active against *T. vivax*. These analogues showed various degrees of cytotoxicity on MRC-5_{SV2} cells, leading to variable SI values for the different species. A 7-trifluoromethyl (**4**) or fluoride (**5**) group resulted in particularly high host cytotoxicity. Compound **7**, a C6–*O*-alkylated 7-substituted 7-deazainosine analogue, showed sub-micromolar activity against *T. b. brucei* (IC_{50} = 0.09 ± 0.04 μM), *T. vivax* (IC_{50} = 0.12 ± 0.01 μM), *T. evansi* (IC_{50} = 0.20 ± 0.02 μM) and *T. equiperdum* (IC_{50} = 0.11 ± 0.00 μM) and micromolar activity against both *T. congolense* strains (IC_{50} = 3.23–4.04 ± 0.36 μM) and very low toxicity against human cell lines.

3′-Deoxytubercidin (**8**)(Hulpia et al., 2019b) displayed sub-micromolar activity (IC_{50} = 0.003–0.048 ± 0.009 μM) against all animal trypanosomes examined, except *T. vivax* (IC_{50} of 3.48 ± 0.09 μM). The 3′-deoxytubercidin analogues **9-14** displayed potent activity against all AT strains, with selectivity indices up to 29800. 2′-Deoxytubercidin analogue **15**, an isomer of **8**, was inactive against all tested strains. Compound **16** exhibited (sub)micromolar activity, however accompanied by low micromolar host cell cytotoxicity. Based on the overall broad spectrum anti-AT activity and high selectivity, compounds **7**, **8**, **9**, **11** and **14** were selected for further *in vitro* metabolic stability and *in vivo* analysis.

3.2. Explorative in vivo acute toxicity of selected anti-AT compounds

Given the observed *in vitro* cytotoxicity on MRC-5_{SV2} cells for some of the compounds, single mice were exposed to a high dose of either compound **7**, **8**, **9**, **11**, or **14**, to assess toxicity prior to evaluating the *in vivo* efficacy of the compounds in various AT mouse models. Compound **8** showed no obvious signs of toxicity, as described previously(Hulpia et al., 2019b), at 5 daily doses of 50 mg/kg intraperitoneally (Fig. 2). For compounds **9**(Hulpia et al., 2019b), **11** and **14** (5 × 25 mg/kg intraperitoneally), a drastic decrease in body weight was observed. This resulted in death for compounds **9** and **11** and in severe clinical

Table 1
Evaluation of *in vitro* drug sensitivity of nucleoside analogues against animal trypanosomes. Cytotoxicity was evaluated against human lung fibroblasts (MRC-5_{SV2} cells). IC_{50} values are expressed in μM and represent the mean and SEM of at least two independent experiments each with 2 replicates.

Compound	EC_{50} (μM)							
	MRC-5	*T. b. brucei* Squib 427	*T. vivax* ILRAD700	*T. evansi* AnTat 3.3	*T. equiperdum* BoTat 1.1	*T. congolense* IL3000	*T. congolense* 6C3	[b]SI
2 (TH1008)	15.1 ± 4.1	0.31 ± 0.06	12.9 ± 4.4	0.04 ± 0.01	0.061 ± 0.005	0.17 ± 0.05	0.056 ± 0.013	1 – 378
3 (TH1003)	12 ± 2	1.2 ± 0.3	0.74 ± 0.04	0.48 ± 0.06	0.085 ± 0.026	0.79 ± 0.14	0.52 ± 0.03	10 – 141
4 (FH6367)	4.32	0.69 ± 0.11	0.82 ± 0.09	0.67 ± 0.11	0.19 ± 0.03	1.8 ± 0.4	0.89 ± 0.17	2 – 23
5 (FH3167)	0.16 ± 0.03	0.035 ± 0.001	0.038 ± 0.006	0.18 ± 0.02	0.033 ± 0.007	0.45 ± 0.06	0.78 ± 0.10	0 – 5
6 (FH3169)	13 ± 6	1.33 ± 0.34	0.53 ± 0.07	0.62 ± 0.06	0.12 ± 0.02	0.86 ± 0.12	0.75 ± 0.14	10 – 108
7 (FH9531)[a]	>64	0.09 ± 0.04	0.12 ± 0.01	0.20 ± 0.02	0.11 ± 0.00	4.04 ± 0.36	3.23 ± 0.36	16 – 711
8 (FH7429_D)[a]	>64	0.048 ± 0.009	3.48 ± 0.09	0.017 ± 0.002	0.003 ± 0.001	0.021 ± 0.001	0.019 ± 0.002	18 – 21333
9 (FH7429_UP)	14.9 ± 3.4	0.0013 ± 0.0003	0.018 ± 0.003	0.0009 ± 0.0002	0.0005 ± 0.0001	0.0007 ± 0.0001	0.0005 ± 0.0001	828 – 29800
10 (FH8517)	3.56 ± 0.8	0.002 ± 0.001	0.50 ± 0.01	0.004 ± 0.001	0.0005 ± 0.0001	0.0052 ± 0.0003	0.0031 ± 0.0003	7 – 7120
11 (FH8470)[a]	9.9 ± 1.5	0.00210 ± 0.00004	0.096 ± 0.082	0.0018 ± 0.0002	0.0006 ± 0.0001	0.0012 ± 0.0002	0.00066 ± 0.00003	103 – 16500
12 (FH8496)	3.4 ± 1.3	0.0085 ± 0.0004	0.056 ± 0.001	0.004 ± 0.001	0.002 ± 0.001	0.005 ± 0.001	0.0030 ± 0.0002	61 – 1700
13 (FH8505)	1.29 ± 0.56	0.005 ± 0.002	0.159 ± 0.009	0.008 ± 0.002	0.003 ± 0.001	0.017 ± 0.002	0.008 ± 0.002	8 – 430
14 (FH10659)[a]	25.6 ± 6.5	0.036 ± 0.005	1.57 ± 0.43	0.038 ± 0.002	0.011 ± 0.001	0.056 ± 0.004	0.040 ± 0.003	16 – 2327
15 (FH10677)	>64	48 ± 1	>64	>64	17.0 ± 2.3	43 ± 14	11.0 ± 2.3	1 – 6
16 (FH10679)	6.1 ± 0.7	0.46 ± 0.08	4.3 ± 0.9	1.7 ± 0.5	0.067 ± 0.013	0.51 ± 0.09	0.31 ± 0.07	1 – 91

[a] Compounds selected for *in vivo* analysis.
[b] The selectivity index (SI) represents the CC50/IC50.

a

Compound	*In vitro* cytotoxicity	Dose	Observed toxic effects during treatment
3'-deoxytubercidin (8)	> 64 μM	50 mg/kg BID IP	-no weight loss -no signs of organ toxicity
7	> 64 μM	25 mg/kg BID IP	-slight weight loss -no signs of organ toxicity
9	14.9 μM	25 mg/kg BID IP	-weight loss -mortality after 2 days of treatment -autopsy could not be performed
11	9.9 μM	25 mg/kg BID IP	-weight loss -mortality after 2 days of treatment -autopsy could not be performed
14	25.6 μM	25 mg/kg BID IP	-weight loss -pale and spotted liver -enlarged gallbladder

b

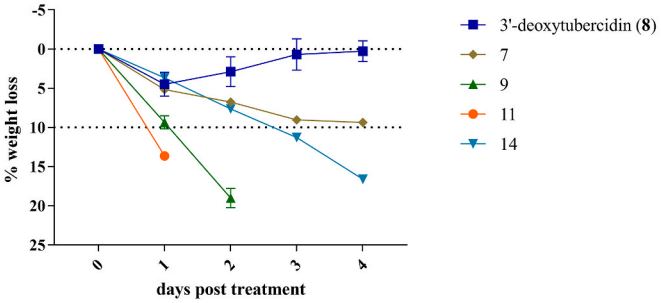


Fig. 2. Pilot acute toxicity of selected nucleoside analogues in mice. (a) Overview of the toxic effects and (b) percentage weight loss observed after exposure to 3′-deoxytubercidin (**8**) (n = 3), compound **7** (n = 1), **9** (n = 3), **11** (n = 1) and **14** (n = 1).

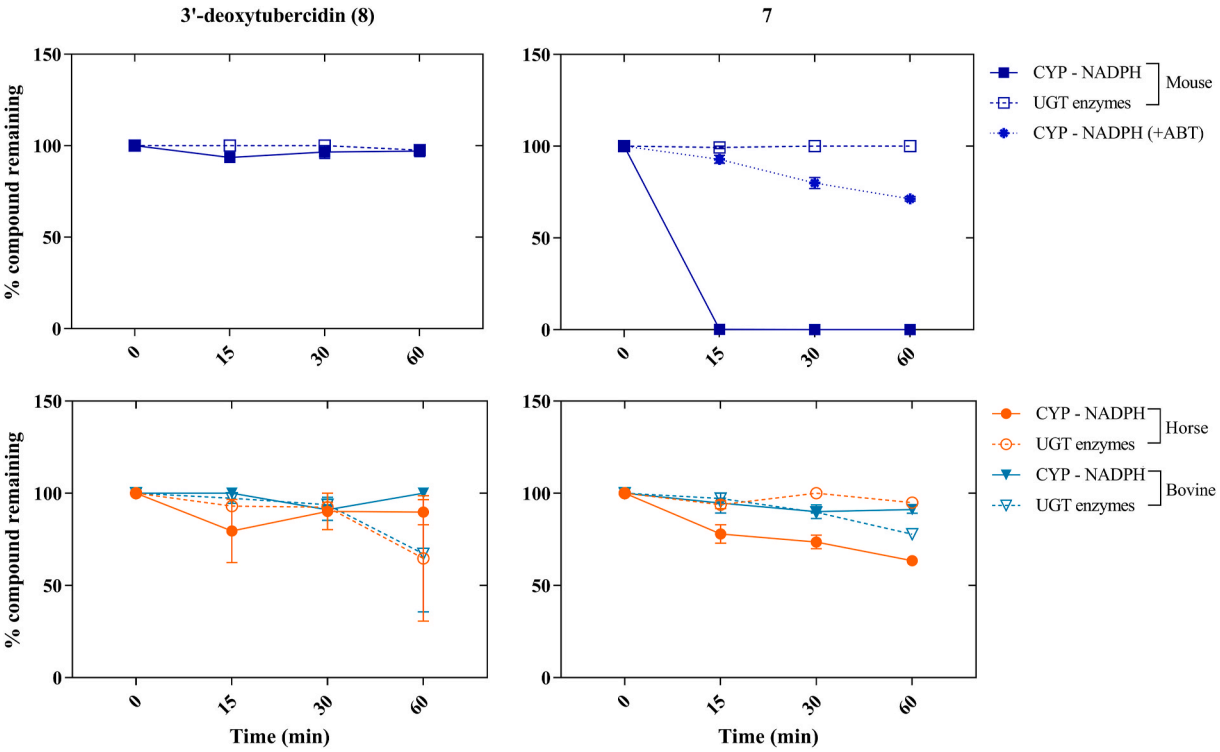
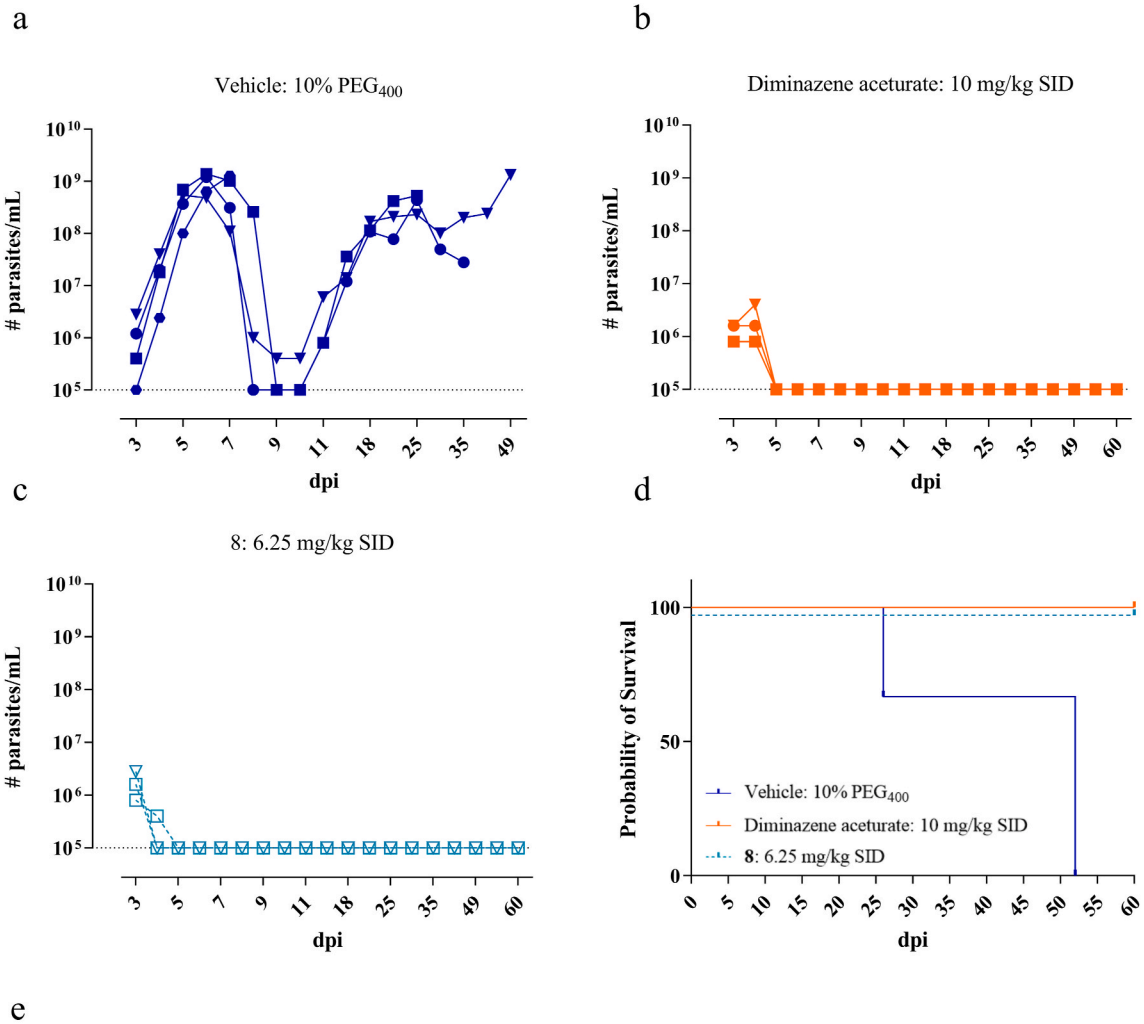


Fig. 3. *In vitro* metabolic stability (Phase-I and Phase-II) of nucleoside analogues (**7** and 3′-deoxytubercidin (**8**)) using mouse, horse and bovine S9 microsomal fractions. The figures represent the percentage of remaining parent compound assayed at various time points of incubation (0-15-30-60 min). 1-ABT = 1-aminobenzotriazole.

pathology for compound **14**. Treatment with compound **7** resulted in a minor weight loss within the acceptable range without observed organ toxicity (Fig. 2).

3.3. *In vitro* metabolic stability of selected anti-AT compounds

Compounds **7** and **8** were selected for further analysis. To evaluate their metabolic stability, both compounds were exposed to mouse, horse and bovine S9 liver microsomal fractions, followed by evaluating the percentage of parent compound remaining after incubation (Fig. 3). The results indicate that compound **7** is susceptible to Phase-I metabolism in mouse liver microsomes with a complete degradation of the parent compound within 15 min of incubation. However, acceptable levels of compound degradation (≥50% of parent compound remaining after 30 min) were observed in the target species, *i.e.* with horse and bovine microsomes. The Phase-I degradation of compound **7** in mice could partially be rescued by the addition of the non-selective CYP_{450} inhibitor 1-aminobenzotriazole (1-ABT) at 100 mg/kg, SID (*semel in die*, once a



e

Target	Group	Sample	Tissue	Ct		Tm	
SL-RNA	Untreated	1	Blood	14.24	14.27	66.00	66.00
	Diminazene	1		34.53	34.25	64.65	64.65
		2		37.11	34.99	64.95	64.65
		3		36.08	35.64	64.80	64.80
	8 - 6.25 mg/kg once a day	1		36.87	36.27	64.95	64.65
		2		35.85	35.60	64.80	64.65
		3		32.31	32.44	65.10	65.10
	(-) control			37.81	34.67	65.10	64.65

Fig. 4. *In vivo* activity of nucleoside analogue **8** (3′-deoxytubercidin) in a mouse model of *T. congolense*. (a) Vehicle group treated with 10% PEG_{400}. (b) Reference drug diminazene aceturate administered at 10 mg/kg SID for 5 consecutive days. (c) Nucleoside analogue 3′-deoxytubercidin administered at 6.25 mg/kg SID for 5 consecutive days. (a–c): Blood parasitaemia in tail vein blood. Squares, triangles and circles represent the individual mice. The dotted line represents the detection limit of the counting chamber. (d) Survival analysis. The colours correspond to the different test groups depicted in a-c. (e) qPCR analysis of blood samples from surviving animals to probe for potential residual parasite levels. Cells in the table that are coloured in green are positive for the specific amplification product (SL-RNA). Cells coloured in red are negative for the specific amplification product. All samples tested positive for the presence of the mouse reference gene *Eef2*, demonstrating appropriate RNA extraction efficiency. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

day) (Fig. 3), enabling the use of mice as a valid model for compound evaluation. Compound **8** was metabolically stable in all tested species warranting further *in vivo* evaluation. Based on the *in vitro* and *in vivo* results, compounds **7** and **8** were selected for further *in vivo* analysis against a range of animal trypanosome species.

3.4. *In vivo efficacy of selected anti-AT compounds against T. vivax and T. congolense*

First, **8** was evaluated in an animal model of *T. congolense*. Intraperitoneal administration of 6.25 mg/kg once a day for 5 consecutive days resulted in a negative blood parasitaemia and survival of all treated animals up to the pre-set endpoint of 60 dpi (Fig. 4A–D). In two out of three mice, the parasitaemia dropped below the detection limit already after 1 treatment dose. All treated animals showed complete absence of residual parasite burdens in the blood, as evaluated by a highly sensitive spliced-leader RNA (SL-RNA) quantitative PCR (qPCR) detection method (Fig. 4E). However, similar treatment against *T. vivax*, resulted in parasite burdens and animal mortality equal to the vehicle-treated control group (Fig. 5).

Treatment of *T. congolense* infected mice with compound **7** at 50 mg/kg, once a day, in combination with 1-ABT to overcome Phase-I metabolic degradation, resulted in parasitaemia levels below the detection limit. However, relapse occurred in all animals around 13 dpi resulting in animal death in part of the treated group (Fig. 6). Administration of compound **7** to *T. vivax* infected mice had variable results. One mouse died during the initial peak of infection while in two mice the parasitaemia dropped below the detection limit. One of them relapsed and died from infection around 37 dpi and the other survived the pre-set endpoint of 60 dpi without relapse (SL-RNA qPCR-negative in blood and spleen) (Fig. 7).

4. Discussion

While drug discovery initiatives for HAT have stagnated with the recent implementation of fexinidazole(Lindner et al., 2020) and the Phase-II/III clinical progression of acoziborole, the treatment of animal trypanosomiasis still relies on drugs causing severe local reactions as well as systemic side-effects and the further spread of drug resistance (Chitanga et al., 2011; Richards et al., 2021). Based on the exclusive dependency of trypanosomes on purine salvage, we recently explored several series of nucleoside analogues, some of which are highly effective for the treatment of second-stage sleeping sickness(Hulpia et al., 2019b; Mabille et al., 2021). Given the current more pressing need for novel compounds for the treatment of AT, this study aimed at redirecting several promising nucleoside analogues for HAT towards animal trypanosomes.

The preferred target product profile (TPP) for trypanocidal drugs in animals describes a single treatment, active against the entire range of AT species,(GALVmed) and including parasite strains resistant against

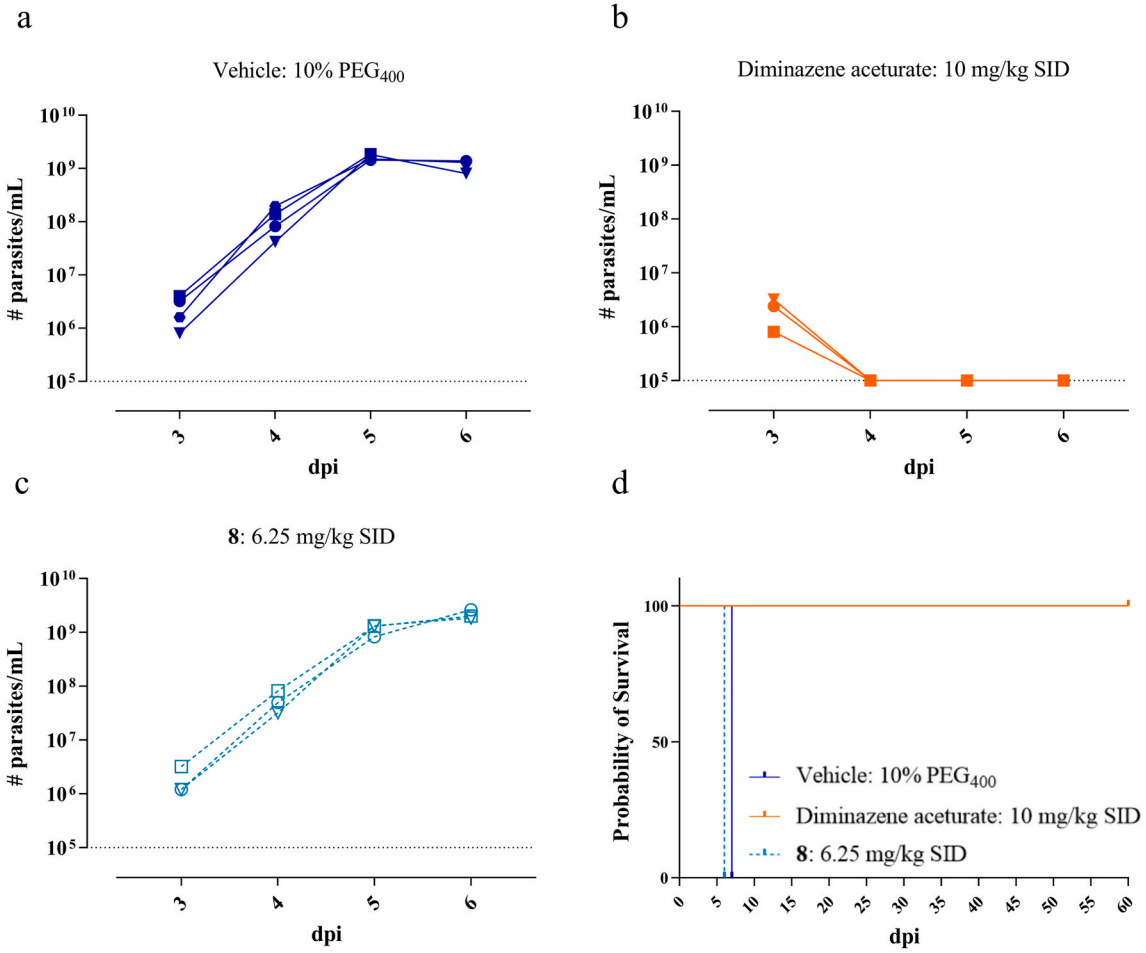


Fig. 5. *In vivo* activity of nucleoside analogue **8** (3′-deoxytubercidin) in a mouse model of *T. vivax*. (a) Vehicle group treated with 10% PEG_{400}. (b) Reference drug diminazene aceturate administered at 10 mg/kg SID for 5 consecutive days. (c) Nucleoside analogue 3′-deoxytubercidin administered at 6.25 mg/kg SID for 5 consecutive days. (a–c): Blood parasitaemia in tail vein blood. Squares, triangles and circles represent the individual mice. The dotted line represents the detection limit of the counting chamber. (d) Survival analysis. The colours correspond to the different test groups depicted in a-c. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

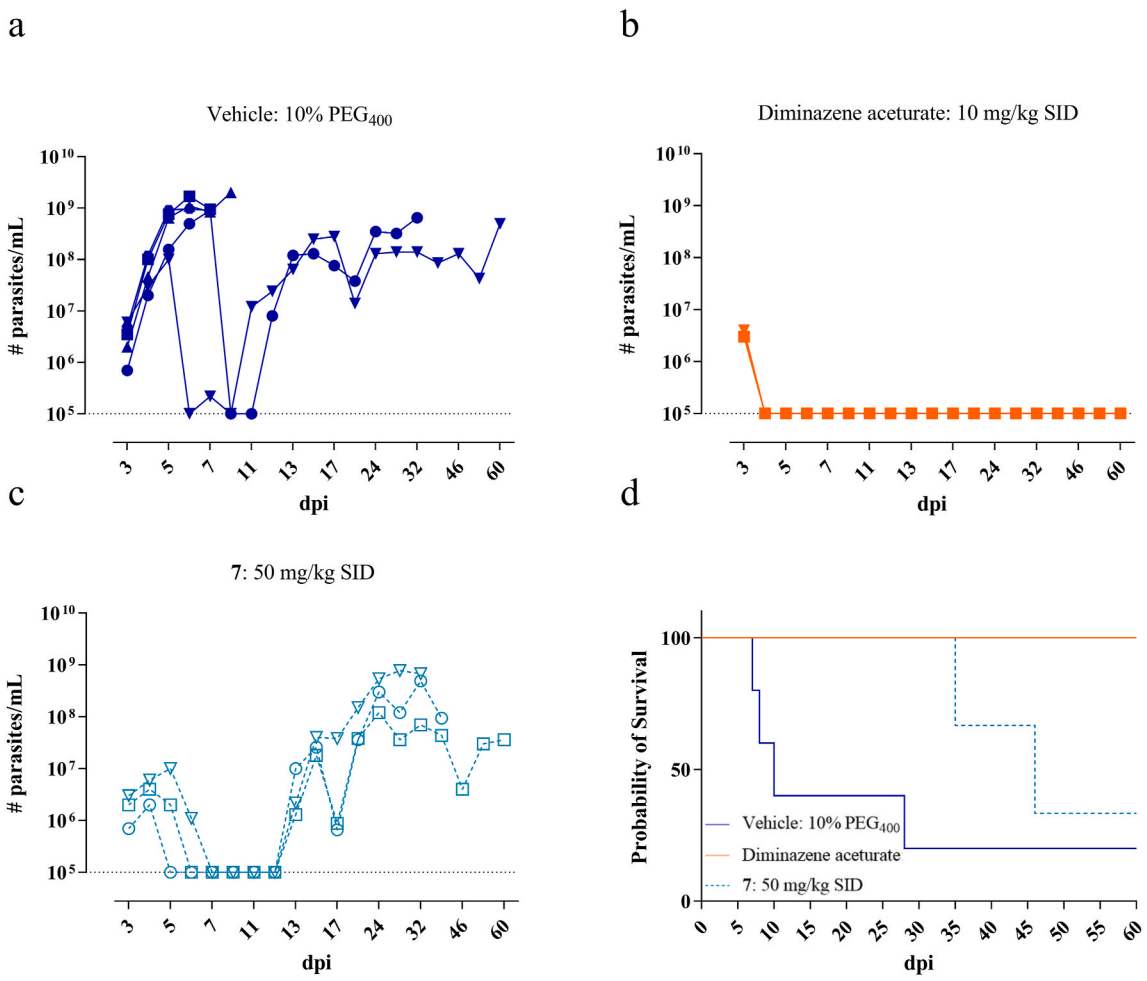


Fig. 6. *In vivo* activity of nucleoside analogue **7** in a mouse model of *T. congolense*. (a) Vehicle group treated with 10% PEG_{400}. (b) Reference drug diminazene aceturate administered at 10 mg/kg SID for 5 consecutive days. (c) Nucleoside analogue **7** administered at 50 mg/kg SID for 5 consecutive days. (a–c): Blood parasitaemia in tail vein blood. Squares, triangles and circles represent the individual mice. The dotted line represents the detection limit of the counting chamber. (d) Survival analysis. The colours correspond to the different test groups depicted in a-c. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the existing drugs(Carruthers et al., 2021; Degneh et al., 2019). The *in vitro* susceptibility assays demonstrated a clear difference in drug-susceptibility of individual trypanosome species, in particular *T. vivax* which generally showed a lower susceptibility, indicating that *T. vivax* requires additional focus. This divergent susceptibility of *T. vivax* can be explained by the phylogeny of the *Trypanosoma* species, positioning *T. vivax* the most distant from the *Trypanozoon* cluster (including *T. b. brucei spp., T. evansi* and *T. equiperdum*)(Fraga et al., 2016). Due to a collaboration between laboratories, different assay conditions were used for the different AT strains which might have an impact on the obtained IC_{50} values. However, the main aim was not to compare the IC_{50} values between the different species but within one species to select the compounds with the most promising activity. Compounds with the overall highest potency across the different species were then selected for further *in vivo* evaluation.

Our lead compound **8** to treat late-stage sleeping sickness(Hulpia et al., 2019b), also showed promising activity against *T. congolense in vivo*, with total clearance of blood parasitaemia confirmed by RT-qPCR. However, compound **8** was less active against *T. vivax* as shown by the *ex vivo* results (IC_{50} = 3.48 ± 0.09 μM) and an *in vivo* mouse experiment, making compound **8** not compliant with the TPP for AT treatment. This emphasizes the importance of including *T. vivax* in the screening panel for the selection of broad spectrum anti-AT agents. While it is difficult to put an absolute value for an *in vitro* EC_{50} for progression to *in vivo* studies, the extent of the difference with the activity against the other trypanosome species (72 to >1000-fold) would seem to be incompatible with a broad anti-AT treatment. However, the requirement for broad spectrum anti-AT activity derives from the situation in sub-Saharan Africa, where it is almost always unknown which trypanosome species (*T. b. brucei*, *T. b. rhodesiense*, *T. vivax*, *T. congolense*) has infected a particular animal, and mixed infections are common(Giordani et al., 2016). With the non-tsetse transmitted animal trypanosomiases, *e.g.* surra and dourine, the trypanosome species is usually known by geographical region, host species and clinical symptoms. As *T. vivax* is limited to the tsetse belt and South America(Desquesnes, 2004; Garcia et al., 2014), compound **8** might still have very important use as treatment of *T. evansi* surra in Northern Africa, the Middle East and Asia, as well as the fatal equine disease dourine (*T. equiperdum*), for which there currently is no treatment.(OIE, 15/12/2020)

Based on the *in vitro* and *ex vivo* results, four compounds (**7**, **9**, **11** and **14**) showed promise and were selected for further analysis and confirmation of potency. The halogenated compounds (incl. compound **11**) were found to display some cytotoxicity against MRC-5_{SV2} cells but maintained, nonetheless, an excellent selectivity index. Similar observations were made for the unsaturated carbon-based 7-substituents (incl. compound **14**)(Hulpia et al., 2020b). Despite their excellent selectivity indices, however, compounds **9**, **11** and **14** caused severe toxicity at 25 mg/kg twice a day intraperitoneally in a mouse,

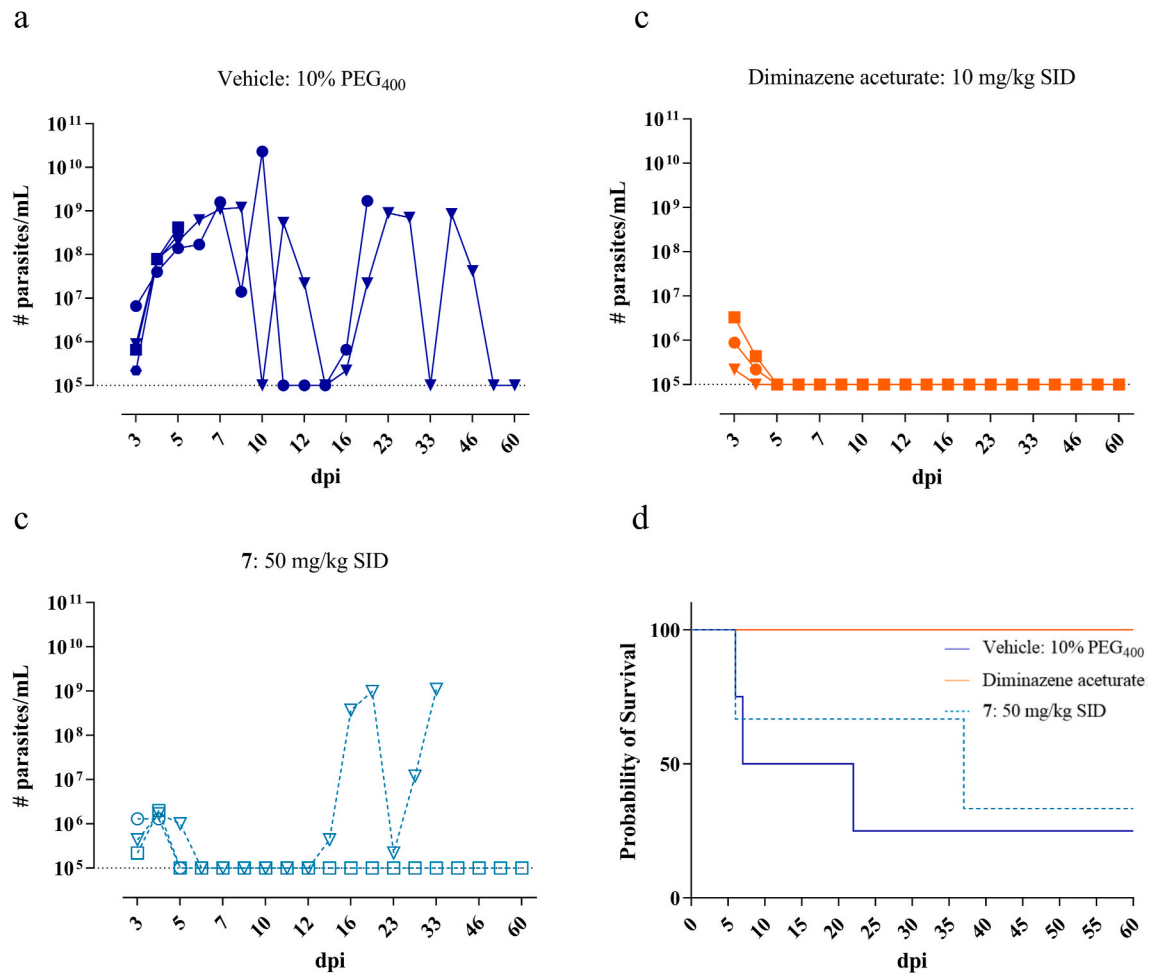


Fig. 7. *In vivo* activity of nucleoside analogue **7** in a mouse model of *T. vivax*. (a) Vehicle group treated with 10% PEG_{400}. (b) Reference drug diminazene aceturate administered at 10 mg/kg SID for 5 consecutive days. (c) Nucleoside analogue **7** administered at 50 mg/kg SID for 5 consecutive days. (a–c): Blood parasitaemia in tail vein blood. Squares, triangles and circles represent the individual mice. The dotted line represents the detection limit of the counting chamber. (d) Survival analysis. The colours correspond to the different test groups depicted in a-c. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

precluding further *in vivo* analysis. Compounds **8** and **9** were tested at a different time-point using 3 mice per group. Based on these results and the observed toxic effect of compound **9** in all three mice we decided that explorative evaluation in single mice (n = 1) is sufficiently indicative of toxicity within this chemical series. Treated mice showed hepatic injury as has been described for tubercidin(Kolassa et al., 1982). Previous studies on the co-administration of the nucleoside transport inhibitor NBMPR-P showed protection of mice from the hepatic and renal injury caused by tubercidin by limiting compound uptake in these organs(el Kouni et al., 1989). However, the basis of the *in vivo* toxicity remains to be clarified and progression of compounds **11** and **14** was not pursued further. Compound **7** did not cause toxicity but was unable to completely clear parasite burdens in all *T. congolense* and *T. vivax* infected mice, leading to post-treatment relapse. Based on observations in the *in vitro* assay system, this might be due to remaining Phase-I metabolism despite the co-administration of 1-ABT.

In summary, we conclude that nucleoside analogues hold promise for a broadly applicable treatment of animal trypanosomiasis provided further improvement on metabolic stability and absence of toxicity. Several compounds with promising profiles against surra and dourine were identified and could be developed further. Regardless, further drug discovery efforts will focus on activity against an extended panel of animal trypanosomes, with special attention for *T. vivax* given its divergent susceptibility profile.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2022.05.001.

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