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Structure-activity relationship exploration of 3'-deoxy-7-deazapurine nucleoside analogues as anti-*Trypanosoma brucei* agents

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Human African trypanosomiasis is a neglected tropical disease caused by *Trypanosoma brucei* parasites. These protists are unable to produce the purine ring, making them vulnerable to the effects of purine nucleoside analogues. Starting from 3'-deoxytubercidin **5**, a lead compound with activity against central nervous stage human African trypanosomiasis, we investigate the structure-activity relationship of the purine and ribofuranose ring. The purine ring tolerated only modifications at C7, while from the many alterations of the 3'-deoxyribofuranosyl moiety only the *arabino* analogue **48** showed pronounced antitrypanosomal activity. Profiling of the most potent analogues against resistant *T. brucei* strains (resistant to pentamidine, diminazene and isometamidium) showed reduced dependence on uptake mediated by the P2 aminopurine transporter compared to **5**. The introduction of a 7-substituent confers increased affinity for the P1 nucleoside transporter up to 10-fold, while generally retaining high affinity for P2. Four of the most promising analogues were found to be metabolically stable, earmarking them suitable back-up analogues for lead **5**.

Keywords:
Trypanosoma brucei, purine nucleoside, nucleoside transporter

Trypanosoma brucei spp. parasites are the causative agents of the deadly infectious disease Human African Trypanosomiasis (HAT). This Neglected Tropical Disease (NTD) is spread via the bite of an infected tsetse fly (*Glossina* spp.), and incidence of HAT coincides with the natural habitat of this vector in sub-Saharan Africa. Infected individuals initially represent rather non-specific, flu-like symptoms (*i.e.* fever, general malaise) and are elicited by *T. brucei* parasites replicating in the haemolymphatic system (stage 1 disease). Therefore, misdiagnosis and underdiagnosis are common, leaving patients untreated and allowing the parasites to eventually invade the central nervous system (stage 2 disease) and cause alterations in behaviour, and in circadian rhythm. This explains the more commonly known name for HAT, 'sleeping sickness'.[1-2] The neurological phase progresses to coma and death unless effectively treated with chemotherapy.

Current estimates put about 57 million people, spread over 36 countries, at risk of contracting HAT. Nevertheless, strenuous efforts of the WHO and Médecins sans Frontières regarding active case finding, treatment, and vector control have been successful to bring down the number of newly diagnosed cases per annum[3] (2164 reported new cases in 2016[3], 1442 in 2017 and 977 in 2018 according to the WHO[4]). Additionally, the recent approval of oral fexinidazole for advanced sleeping sickness represents a beacon of hope for people living in afflicted areas and is a leap forward to reach the goal of the WHO to eliminate HAT by 2020.[5] However, caution is warranted, as elimination was equally close in the 1960s. So-called cryptic reservoirs of parasites that might re-initiate infections in apparently HAT-free regions, have been suggested to be the underlying cause of this re-emergence.[5-7] Furthermore, fexinidazole is only recommended for use in infections caused by *T. b. gambiense*, while treatment of infections with *T. b. rhodesiense* still requires the use of melarsoprol,[8] an organo-arsenical, causing treatment-related mortality in 2.5 to 5 % of cases.[9] Additionally, drug resistance can be readily induced against fexinidazole *in vitro*,[10-11] and this compound shows cross-resistance with nifurtimox, another nitro-imidazole employed in the nifurtimox-eflornithine combination therapy (NECT).[11] Moreover, fexinidazole treatment is characterized by a high pill burden.[12] Finally, the need for stage-determination has not been eliminated as fexinidazole treatment was found inferior to NECT in patient with severe meningoencephalitic compromise.[12-13] Therefore, the need for continued research efforts into new

chemical entities with antitrypanosomal activity remains, particularly as no vaccine is available or in development.[1-2]

African trypanosomes, just like all other protozoan parasites, are known to be unable to biosynthesize the purine ring from amino acid precursors.[14-15] Hence, they acquire host-assembled purines (either as purine nucleobases or purine nucleosides) to fulfil the demands of their replication and metabolism. To this end, they make use of multiple purine (nucleoside and/or nucleobase) transporters to actively salvage these metabolites,[14] which are then employed by the enzymes of the purine salvage pathway. Purine salvage dependency represents a potentially fertile ground for the discovery of antitrypanosomal agents with a purine nucleoside scaffold. The potential utility of exploiting purine auxotrophy was realized as early as the 1960s, when Jim Williamson found that naturally occurring 'nucleoside antibiotics' such as tubercidin and cordycepin (Figure 1) displayed considerable trypanocidal efficacy.[16-17]

Recently, we have reported on the discovery of 7-substituted tubercidin analogues (*e.g.* **4**, Figure 1) as potent and selective anti-*T. brucei* agents.[18] Additionally, we discovered that the combination of the 3'-deoxyribuforanose sugar of cordycepin with the 7-deazapurine base of tubercidin resulted in highly potent 'hybrid' compounds, of which **5** was able to cure CNS-stage *T. b. brucei* infections in mice by oral dosing, marking it a promising lead compound for further development.[19] In the present study, we describe our efforts to systematically investigate the structure-activity-relationship (SAR) of these 3'-deoxy-7-deazapurine nucleoside analogues and identify several potent and selective back-up derivatives that are ready for further evaluation in animal models of HAT. Furthermore, we provide *in vitro* data on resistant parasites cell lines. Finally, we generate insight into the transporter-mediated uptake of selected analogues into the trypanosome, resulting in a refinement of the recognition motif for the *T. brucei* P1 adenosine transporter.

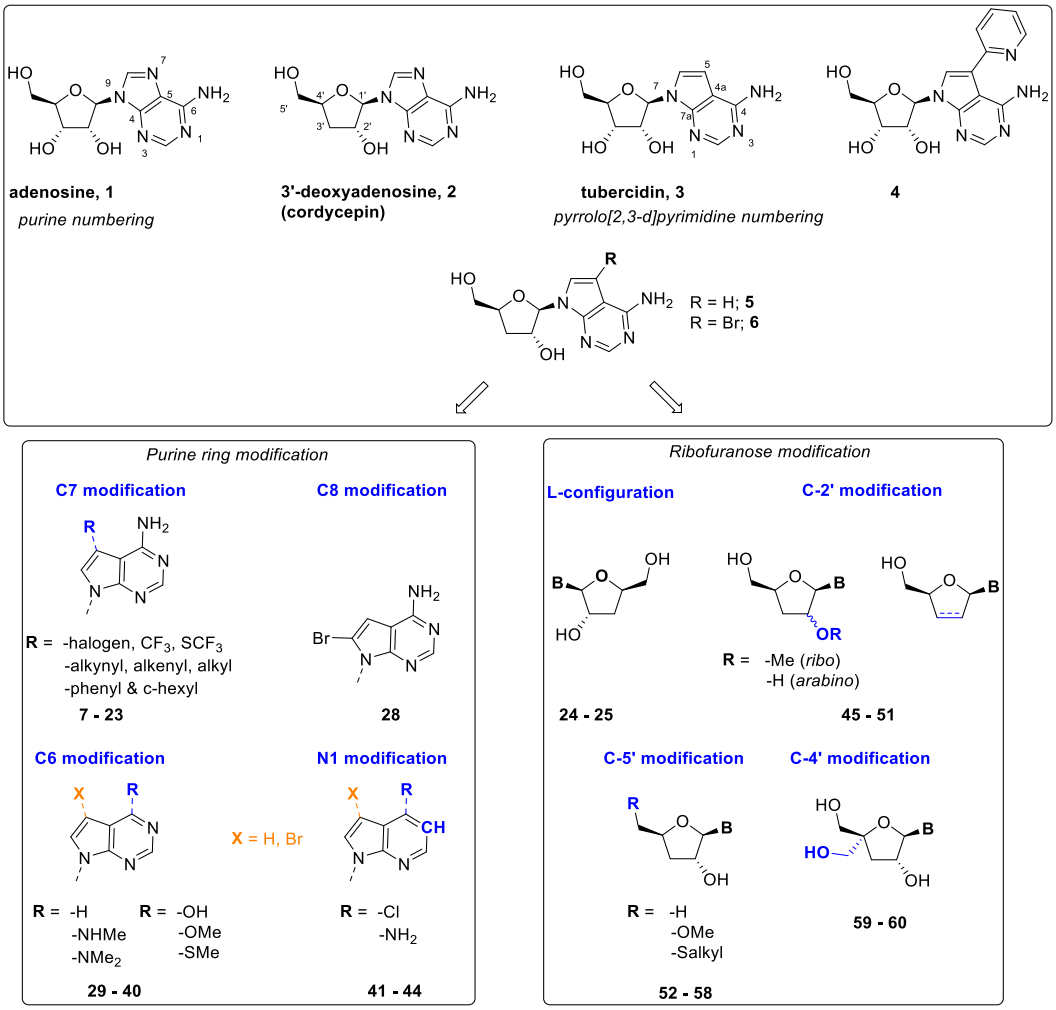
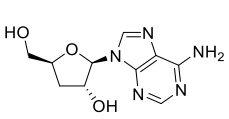


Figure 1: Structures of purine nucleoside analogues and antitrypanosomal agents. Structures in the lower two boxes represent analogues prepared in this study; B, nucleobase.

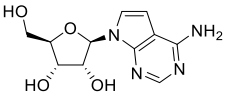
Results and discussion

The preparation of nucleoside analogues is presented and discussed in the Supporting Information. The prepared nucleoside analogues were evaluated *in vitro* by drug sensitivity assays (Alamar Blue) against *T. b. brucei* and *T. b. rhodesiense*. Simultaneously, cytotoxicity was assayed by employing MRC-5_{SV2} human fibroblast cells. The results of the *in vitro* evaluation are presented in Table 1 – 3.

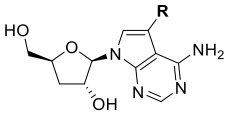
Table 1: *In vitro* antitrypanosomal activity of prepared nucleosides analogues. pEC_{50} values are depicted as average and SEM of 2–5 independent determinations. Values from different experiments were reported between brackets when an average could not be calculated.



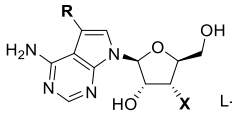
2- Cordycepin



3 - Tubercidin



5 - 23



L-nucleoside

R = H; X = H **24**
R = Br; X = H **25**
R = H; X = OH; **26**

Cpd.	R =	*T. b. brucei* pEC_{50}	*T. b. rhodesiense* pEC_{50}	MRC-5 pEC_{50}	SI (MRC-5 / *T. b. brucei*)	SI (MRC-5 / *T. b. rhod.*)
2 cordycepin	--	6.77 ± 0.08	6.95 ± 0.16	<4.20	>370	>560
3 tubercidin	--	6.32 ± 0.10	7.45 ± 0.01	5.65 ± 0.14	4.6	61
5[19]	**H**	7.32 ± 0.07	9.28 ± 0.03	<4.20	>1300	>120000
6[19]	**Br**	8.89 ± 0.10	9.40 ± 0.09	4.83 ± 0.09	>11000	>37000
7	**F**	8.70 ± 0.17	[>9.70 ; 9.40]	5.45 ± 0.09	>1700	8900
8	**Cl**	8.69 ± 0.01	9.43 ± 0.13	5.00 ± 0.06	>4800	>26000
9	**I**	8.07 ± 0.02	8.87 ± 0.17	5.47 ± 0.14	>400	2500
10	**CF_3**	6.03 ± 0.18	5.73 ± 0.04	6.18 ± 0.06	0.7	0.4
11	**SCF_3**	4.57 ± 0.01	4.68 ± 0.14	4.73 ± 0.09	0.7	0.9
12	**≡-H**	8.28 ± 0.17	9.27 ± 0.03	5.89 ± 0.16	240	>2300
13	**≡-propyl**	6.49 ± 0.21	7.45 ± 0.04	<4.20	>190	>1800
14	**≡-butyl**	6.49 ± 0.21	7.22 ± 0.16	<4.20	>190	>1050
15	**≡-Ph**	6.52 ± 0.18	6.92 ± 0.02	5.02 ± 0.06	31	79

16	**-vinyl**	7.45 ± 0.05	8.26 ± 0.19	4.59 ± 0.10	>710	4700
17	**-ethyl**	5.75 ± 0.07	6.19 ± 0.07	<4.20	>36	>98
18	***E*-styryl**	5.75 ± 0.02	6.78 ± 0.27	4.31 ± 0.11	28	290
19	**-$(CH_2)_2$Ph**	5.08 ± 0.01	6.18 ± 0.07	<4.20	>7	>96
20[20]	**c-hexen-2-yl**	4.40 ± 0.05	4.44 ± 0.02	<4.20	>1.6	>1.8
21[20]	**c-hexyl**	<4.20	<4.20	<4.20	--	--
22[20]	**Ph**	5.10 ± 0.02	5.91 ± 0.14	4.21 ± 0.02	7.8	49
23	**2-pyridinyl**	5.12 ± 0.00	[<4.20 ; 5.00]	4.47 ± 0.10	4.4	0.9
24 X = H	**H**	<4.20	<4.20	<4.20	--	--
25 X = H	**Br**	<4.20	<4.20	<4.20	--	--
26 X = OH	**H**	<4.20	<4.20	<4.20	--	--
Suramin	**--**	7.50 ± 0.12	7.50 ± 0.06	--	--	--

Building on the discovery of the highly potent nucleoside analogues **5** and **6**,[19] the SAR in the sugar as well as the heterocyclic moiety was thoroughly investigated by modifying virtually every position. First, the importance of the 7-substituent was probed (Table 1) by changing the nature of the halogen (**7**, **8** & **9**). This revealed that all of these halogenated analogues displayed highly potent *in vitro* antitrypanosomal activity (*T. b. brucei* pEC_{50} > 8.00), with only the iodide-substituted derivative **9** being slightly less potent than the others. All halogenated analogues did display some cytotoxicity against MRC-5 cells; nonetheless, each had an excellent selectivity index. Introduction of two potential halogen bio-isosters, CF_3 (**10**) and SCF_3 (**11**), led to a large drop in activity, particularly for the SCF_3 substituted

analogue. Next, we evaluated different carbon-based substituents, and found these, in general, to be less active than the 7-halogenated analogues. However, among these, small and linear substituents conferred the highest *in vitro* activity (**12** and **16**), with the ethynyl analogue giving similar activity as the halogenated compounds. Elongation of the carbon chain resulted in reduced potency (compare **12** with **13**, **14** and **15**), albeit that the latter three still exhibited sub-micromolar EC_{50} values. Complete saturation (**17**) or elongation of the 7-vinyl substituent (**18**) equally resulted in the loss of *in vitro* activity, underscoring that a small unsaturated group is optimal, presumably for steric reasons. This is further evidenced by the cyclohexenyl analogue **20**,[20] which can be seen as a substituted vinyl analogue, displaying almost no antitrypanosomal activity at all. An aromatic ring immediately attached to the 7-position of the purine ring (**22**[20] and **23**) resulted in analogues with supramicromolar potency. Additionally, none of our previously reported substituted 7-phenyl analogues, which displayed potent anti-*Trypanosoma cruzi* activity,[20] surpassed the 1 μM anti-*T. brucei* activity mark (data not shown). Most remarkably, the 2-pyridine substituted derivative **23**, for which the corresponding ribofuranose (**4**) emerged as the most potent analogue from our previous study,[18] displayed substantially lower *in vitro* potency than **4**, in sharp contrast to the effect observed upon 3'-deoxygenation of tubercidin (compare **3** and **5**).

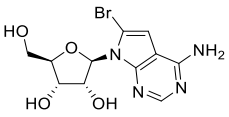
Lastly, L-nucleosides **24** and **25**, which are enantiomers of **5** and **6** respectively, failed to elicit any antitrypanosomal activity, indicating the absolute requirement of the D-configuration for activity. This was also observed for L-tubercidin **27**.

Having identified that high *in vitro* potency is conferred by 7-halogens and small, linear carbon-based substituents, we turned our attention to other positions of the purine ring (C8, N6 and N1; Table 2). Based on previously discovered differences in trypanosomal nucleoside transporter substrate preferences,[19] it was decided to create matched pairs to compare every modification for two 7-substituents: 7-H and 7-Br.

The introduction of a substituent (Br) in the 8-position of the purine ring (**28**) led to two orders of magnitude lower pEC_{50} (compare with **5**), which indicates that a fixed *syn*-conformation of the nucleobase is detrimental for antitrypanosomal activity. Generally, methylation of the 6-amino group or

substituting it for an OH (keto tautomer), OMe[21], or SMe is not tolerated. Interestingly, removal of any functionality (analogue **40**) gave rise to the most potent 6-amino modified analogue of **6**. This is noteworthy as in the corresponding N7 purine analogues, 3'-deoxygenation resulted in weaker *in vitro* antitrypanosomal activity.[22] Indeed, 7-bromide substituted nucleosides systematically displayed better *in vitro* activity than their 7-unsubstituted matched pairs. Lastly, the importance of the N1 nitrogen was investigated (compounds **41** and **42**), from which it was learned that it is absolutely required for activity, which was also observed for the corresponding 6-chloride substituted derivatives (**43** and **44**).

Table 2: *In vitro* antitrypanosomal activity of prepared nucleosides analogues. pEC_{50} values are depicted as average and SEM of 2–5 independent determinations. Suramin was used as a reference drug, of which the data are presented in Table 1.



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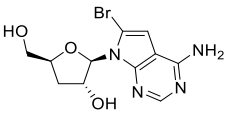

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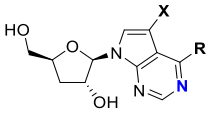
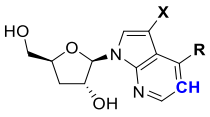


29 - 40



41 - 44

Cpd.	X =	R =	*T. b. brucei* pEC_{50}	*T. b. rhodesiense* pEC_{50}	MRC-5 pEC_{50}	SI (MRC-5 / *T. b. brucei*)	SI (MRC-5 / *T. b. rhod.*)
5[19]	**H**	**NH_2**	7.32 ± 0.07	9.28 ± 0.03	<4.20	>1300	>120000
6[19]	**Br**	**NH_2**	8.89 ± 0.10	9.40 ± 0.09	4.83 ± 0.09	>11000	>37000
27	**--**	**--**	<4.20	<4.20	<4.20	--	--
28	**--**	**--**	5.30 ± 0.19	5.99 ± 0.18	<4.20	>12	>62
29	**H**	**NHMe**	4.57 ± 0.02	4.82 ± 0.08	<4.20	>2	>4
30	**Br**	**NHMe**	5.21 ± 0.04	5.08 ± 0.01	<4.20	>10	>7
31	**H**	**NMe_2**	<4.20	<4.20	<4.20	--	--
32	**Br**	**NMe_2**	5.73 ± 0.03	6.10 ± 0.15	<4.20	>34	>80
33	**H**	**OH**	4.53 ± 0.00	5.54 ± 0.10	<4.20	>2	>22
34	**Br**	**OH**	5.09 ± 0.00	5.65 ± 0.02	<4.20	>7	>28
35	**H**	**-OMe**	<4.20	<4.20	<4.20	--	--
36	**Br**	**OMe**	5.58 ± 0.08	5.43 ± 0.16	5.57 ± 0.14	1	0.7
37	**H**	**SMe**	<4.20	4.24 ± 0.04	<4.20	--	--
38	**Br**	**SMe**	5.77 ± 0.01	5.36 ± 0.07	6.53 ± 0.12	0.2	0.07
39	**H**	**H**	4.55 ± 0.02	4.64 ± 0.04	<4.20	>2	>2

40	**Br**	**H**	6.30 ± 0.00	6.04 ± 0.20	4.68 ± 0.02	41	23
41	**H**	**NH_2**	<4.20	4.51 ± 0.02	<4.20	--	>2
42	**Br**	**NH_2**	4.45 ± 0.02	<4.20	<4.20	1.8	--
43	**H**	**Cl**	4.51 ± 0.02	5.10 ± 0.01	<4.20	>1.8	--
44	**Br**	**Cl**	4.83 ± 0.17	4.54 ± 0.05	4.57 ± 0.15	1.8	0.9

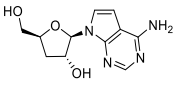
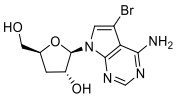
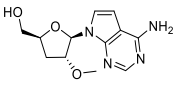
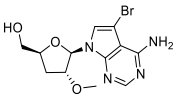
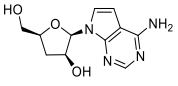
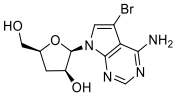
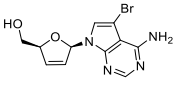
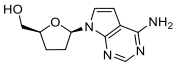
Next, we turned our focus towards modifying the sugar ring (Table 3). First, we explored the influence of the 2'-hydroxyl group by alkylating it (**45** and **46**), changing its relative orientation (from 3'-deoxy*ribo* to 3'-deoxy*arabino*, **47** and **48**), and removing it (**49**, **50** and **51**). Alkylation or removal led to an almost complete loss in activity. This is in line with the reported low antitrypanosomal potency of 2,3-dideoxyadenosine,[23] which we hypothesize, results from a lack of downstream activation rather than insufficient transporter-mediated uptake, as both the P1 and P2 transporters have at least equally good affinity for 2'-deoxyadenosine as for adenosine.[24] The 3'-deoxy*arabino* epimer **48** did display submicromolar activity, although it remains roughly two orders of magnitude less potent than its 2'-epimer **6**. The loss of potency is in agreement with 2-fluorocordycepin, which lost its activity even more dramatically upon 2'-epimerization.[22]

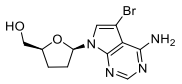
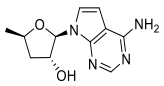
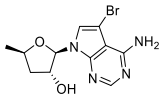
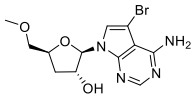
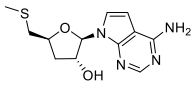
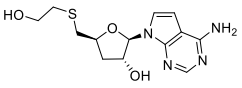
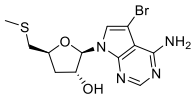
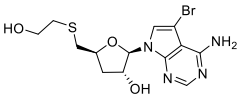
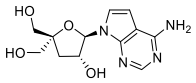
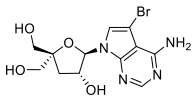
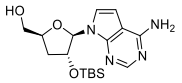
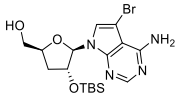
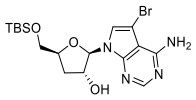
Then, we investigated the C5' position of the ribofuranose. Removal (**52** and **53**) or methylation (**54**) of the 5'-OH strongly impacted the antitrypanosomal activity, and would be consistent with a requirement for adenosine kinase-mediated phosphorylation (as demonstrated for **5**[19]) at this position (*i.e.* the subversive substrate hypothesis[15]). The introduction of C5'-*S*-alkyl groups[25] was also strongly detrimental to activity and resulted in only one analogue with low micromolar activity, **58**, suggesting significant differences with the C5'-thioalkyl nucleoside series investigated by Cyrus Bacchi and co-workers (*e.g.* HETA).[25-27]

Finally, we also evaluated intermediates that feature a silyl group at the 2'-OH (**61**, **62**) or 5'-OH (**63**). Analogue **62** in particular, was endowed with submicromolar antitrypanosomal activity (pEC_{50} = 6.20

± 0.09 against *T. b. brucei*) and no cytotoxicity. The antitrypanosomal activity cannot be readily explained based on the SAR trends described above. This remaining protecting group as a specific nucleoside modification is reminiscent of certain antiviral nucleosides *e.g.* TSAO-T[28-30] and reported *Plasmodium falciparum* dUTPAse inhibitors.[31-32]

Table 3: *In vitro* antitrypanosomal activity of ribofuranose-modified analogues of **5** and **6**. pEC_{50} values are depicted as average and SEM of 2–5 independent determinations. Values from different experiments are reported between brackets when an average could not be calculated. Suramin was used as a reference drug, of which the data are presented in Table 1.

Cpd.	Structure	*T. b. brucei* pEC_{50}	*T. b. rhodesiense* pEC_{50}	MRC-5 pEC_{50}	SI (MRC-5 / *T. b. brucei*)	SI (MRC-5 / *T. b. rhod.*)
5[19]		7.32 ± 0.07	9.28 ± 0.03	<4.20	>1300	>120000
6[19]		8.89 ± 0.10	9.40 ± 0.09	4.83 ± 0.09	>11000	>37000
45		[<4.20 ; 4.40]	4.51 ± 0.05	4.19 ± 0.00	--	>2
46		4.49 ± 0.00	4.60 ± 0.07	4.19 ± 0.00	>2	>2
47		5.18 ± 0.00	6.22 ± 0.01	<4.20	>9	>106
48		6.90 ± 0.01	7.56 ± 0.01	4.34 ± 0.03	360	>1600
49		<4.20	4.22 ± 0.01	4.34 ± 0.15	--	0.7
50		<4.20	<4.20	<4.20	--	--

51		[<4.20 ; 4.30]	4.61 ± 0.07	4.78 ± 0.14	--	0.6
52		<4.20	<4.20	<4.20	--	--
53		4.48 ± 0.01	4.70 ± 0.14	4.99 ± 0.05	0.3	0.5
54		4.49 ± 0.01	4.73 ± 0.22	4.64 ± 0.13	0.7	1.2
55		4.36 ± 0.13	4.50 ± 0.01	<4.20	>1.5	>2.0
56		[<4.20; 4.40]	4.58 ± 0.13	<4.20	--	>2.4
57		4.49 ± 0.01	4.65 ± 0.11	4.82 ± 0.04	0.5	0.7
58		5.30 ± 0.21	6.15 ± 0.10	4.58 ± 0.02	5	37
59		<4.20	<4.20	<4.20	--	--
60		4.57 ± 0.00	5.25 ± 0.13	<4.20	>2	>11
61		<4.20	<4.20	<4.20	--	--
62		6.20 ± 0.09	6.56 ± 0.20	<4.20	>102	>230
63		5.90 ± 0.21	7.10 ± 0.18	5.30 ± 0.02	4	67

In summary, the thorough SAR investigation of lead molecule **5** presented above, shows narrow but also relatively unpredictable trends. Only position 7 of the purine ring tolerates other halogens or small and linear carbon-based substituents, with either no (halogens) or modest (*e.g.* substituted alkynyl) loss of *in vitro* antitrypanosomal activity. The sugar ring does not tolerate additional modification. Matched

molecular pair analysis showed that, in general, modifications were more tolerated for the 7-Br substituted 7-deazapurines than for the 7-H analogues.

Since nucleoside analogues inherently are polar molecules, uptake via passive diffusion is unlikely, and not consistent with documented resistance to nucleoside analogues in transporter mutants of various protozoa.[14] As purines are essential nutrients for all protozoan parasites including *T. brucei*, they have evolved an array of efficient purine nucleoside and nucleobase transporters. Of particular relevance for purine nucleoside analogues are the P1 and P2 adenosine transporter systems in *T. brucei*.[14, 24, 33] The P2 transporter has a clear link to the development of (uptake-related) drug resistance, considering that its encoding gene (*AT1*)[34] is single-copy and non-essential for parasite survival.[35] Extensive investigation of this transporter showed that it is in fact a nucleobase transporter, with adenine being its preferred substrate, whilst tolerating the sugar moiety of adenosine. On a molecular level, this originates from the amidine recognition motif present as the N1-C6-NH_2 aminopyrimidine element in adenine and adenosine,[14, 24, 36-37] but also in the melamine part of melarsoprol and the benzamidine portion of pentamidine[38], furamidine[39], and diminazene[40].

Several nucleoside analogues have previously been shown to display cross-resistance with known trypanocides that also (partially) depend on the P2 transporter for internalization. This resistance profile has been reported for tubercidin and cordycepin,[23] as well as for the recently reported analogue **5**,[19] albeit that the latter still displays submicromolar *in vitro* activity against P2 transporter knock-out parasites.[19] On the other hand, the P1 transporter is encoded by several closely related genes, and P1-based resistance has never been reported.[14, 41-42] In view of this, nucleoside analogues that are (also) substrates of P1 transporters can be expected to withstand resistance, at least at the level of compound accumulation, and not to show cross-resistance with any currently used trypanocides. We have thus evaluated several of the nucleoside analogues prepared in this study against a panel of drug resistant *T. brucei* cell lines (Table 4). Resistance of two of these is due wholly or in part to the removal of the P2 transporter (*TbAT1*-KO and B48), both are to different degrees resistant to existing amidine-bearing drugs such as melarsoprol, pentamidine and diminazene. The B48 clone, which was developed from the

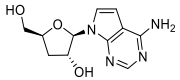
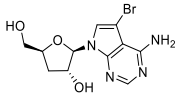
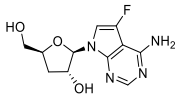
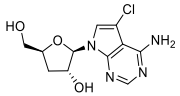
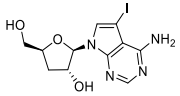
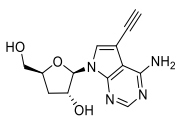
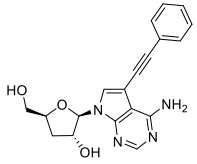
TbAT1-KO cell line,[43] has acquired additional resistance to pentamidine through the loss of the *AQP2* gene, which is the primary uptake mechanism of pentamidine.[44] Drug sensitivity against ISRM1, an isometamidium-resistant cell line with considerable diamidine cross-resistance due to reduced mitochondrial membrane potential,[45] was also determined. In all instances, a comparison to the parental cell line (Lister-427) was made, and a resistance factor (RF) was calculated.

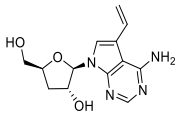
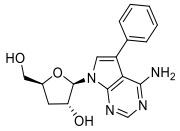
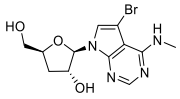
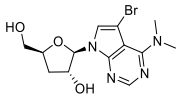
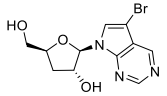
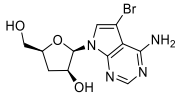
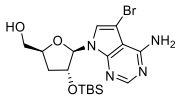
The *TbAT1*-KO cell line demonstrated an approximately 10-fold reduced drug sensitivity to **5**.[19] This concurs with the current substrate recognition model for the P1 transporter, which considers the 3'-OH, 5'-OH, N7 and N3 of purine nucleosides as the key interaction elements.[46] In line with this model, removal of two elements (*in casu* 3'-OH and N7) results in impaired binding to the P1 transporter, whilst maintaining excellent affinity for the P2 transporter, and thus explains the observed resistance profile for *TbAT1*-KO parasites.[19] Interestingly, the addition of a halogen (Br in the case of **6**) was able to mitigate this resistance profile by restoring affinity for P1. Therefore, we first investigated all other 7-halogenated 3'-deoxy nucleoside analogues **7** (F), **8** (Cl) and **9** (I) in the *AT1*-KO cell lines. This revealed that, although a small shift in drug sensitivity was observed, it was much attenuated with regard to the parent 7-unsubstituted analogue **5**, with a mere 3-fold shift rather than a RF >11. The same trend was observed for the B48 clone, indicating that these analogues, as expected, are not substrates for AQP2.

Interestingly, this trend was even stronger for other 7-substituted (non-halogen) analogues, such as ethynyl (**12**), 2-phenylethynyl (**15**), vinyl (**16**) and phenyl (**22**[20]), with *TbAT1*-KO RF values ≤ 2.2. Although a limited decrease in activity could be noted against *TbAT1*-KO and B48 strains, this was much smaller than observed for **5**, which has no 7-substituent. This is also in agreement with our previous findings for the 7-(pyridin-2-yl)-substituted ribofuranose analogue **4**[18], which equally demonstrated reduced activity against P2-compromised strains (also *vide infra*). We also assessed the effects of N6-methylation (**30** and **32**) as well as N6 removal (**40**), and observed that these analogues are not subject to P2-conferred resistance, but if anything, tended to be somewhat more active against *AT1*-KO strains (P>0.05, unpaired Student t-test). This may be explained by compensatory upregulation of P1 transporter activity in the *TbAT1*-null trypanosomes,[23, 47] an effect that has previously been observed for 2,6-*N*-disubstituted adenosine analogues.[36, 48] Also, the antitrypanosomal activity of the 3'-

deoxy*arabino* analogue **48** proved to be independent of the P2 transporter and thus represents a suitable back-up compound for further investigation.

Table 4: Evaluation of *in vitro* drug sensitivity of selected nucleoside analogues against resistant *T. brucei* cell lines. Depicted in the table are the pEC_{50} values, which represents the mean and SEM of at least three independent determinations. The resistance factor (RF) was calculated as the ratio of EC_{50} values from the resistant cell lines and the Lister-427 line. *TbAT1*-KO: *T. brucei* cell line lacking the *TbAT1*/P2 transporter gene. B48: *TbAT1*-KO derived clone, resistant to pentamidine, diminazene and melaminophenyl arsenical. ISMR1: isometamidium resistant cell line. Statistical difference between control (Lister-427) and resistant strain was evaluated using unpaired Student t-test: *, P<0.05; **, P<0.01; ***, P<0.001.

Cpd.	Structure	Lister-427 pEC_{50}	TbAT1-KO pEC_{50}	RF	B48 pEC_{50}	RF	ISMR1 pEC_{50}	RF
5[19]		7.48 ± 0.01	6.43 ± 0.01	**11**	6.43 ± 0.01	**11**	7.49 ± 0.05	0.97
6[19]		8.75 ± 0.07	8.67 ± 0.05	1.2	8.88 ± 0.08	0.75	8.96 ± 0.06	0.63
7		8.57 ± 0.05	8.03 ± 0.02	3.5***	7.85 ± 0.04	5.3**	8.33 ± 0.03	1.7*
8		9.11 ± 0.09	8.59 ± 0.07	3.3*	8.58 ± 0.05	3.4**	8.53 ± 0.07	3.8**
9		8.46 ± 0.14	7.88 ± 0.07	3.8**	8.02 ± 0.10	2.8	7.64 ± 0.03	6.7***
12		7.94 ± 0.03	7.60 ± 0.03	2.2**	7.43 ± 0.03	3.2***	7.64 ± 0.03	2.0**
15		6.16 ± 0.04	6.17 ± 0.00	0.97	6.02 ± 0.03	1.4*	6.05 ± 0.10	1.8*

16		7.13 ± 0.02	7.09 ± 0.02	1.2	6.93 ± 0.02	1.5**	6.98 ± 0.02	1.5**
22		5.66 ± 0.08	5.30 ± 0.07	2.2*	5.39 ± 0.11	1.8	5.33 ± 0.13	2.1
30		4.91 ± 0.07	5.19 ± 0.01	0.54	5.16 ± 0.02	0.57	4.90 ± 0.05	1.0
32		5.13 ± 0.07	5.31 ± 0.06	0.66	5.12 ± 0.01	1.0	5.49 ± 0.03	0.43*
40		6.39 ± 0.05	6.54 ± 0.02	0.7	6.68 ± 0.06	0.52*	6.45 ± 0.04	0.87
48		6.49 ± 0.03	6.52 ± 0.02	0.94	6.42 ± 0.03	1.3	6.53 ± 0.00	0.91
62		5.63 ± 0.01	5.66 ± 0.02	0.94	5.57 ± 0.01	1.2*	5.67 ± 0.01	0.92
Pentamidine	--	7.98 ± 0.05	7.73 ± 0.04	1.8***	6.00 ± 0.07	95***	6.84 ± 0.10	14**
Diminazene	--	6.37 ± 0.06	5.35 ± 0.08	11***	5.15 ± 0.09	17***	5.53 ± 0.06	6.9***
Isometamidium	--	6.19 ± 0.05	6.13 ± 0.07	1.2	6.25 ± 0.09	0.85	5.50 ± 0.06	4.8***

Based on the observation that the activity of several 7-substituted nucleoside analogues was only marginally affected by the removal of the P2 transporter (Table 4), we sought to gain further insight into the engagement of the purine transporters by performing competition experiments with [^{3}H]-adenosine for three representative 7-substituted analogues, *i.e.* **7**, **12** and **22** (Figure 2 and Table 5).

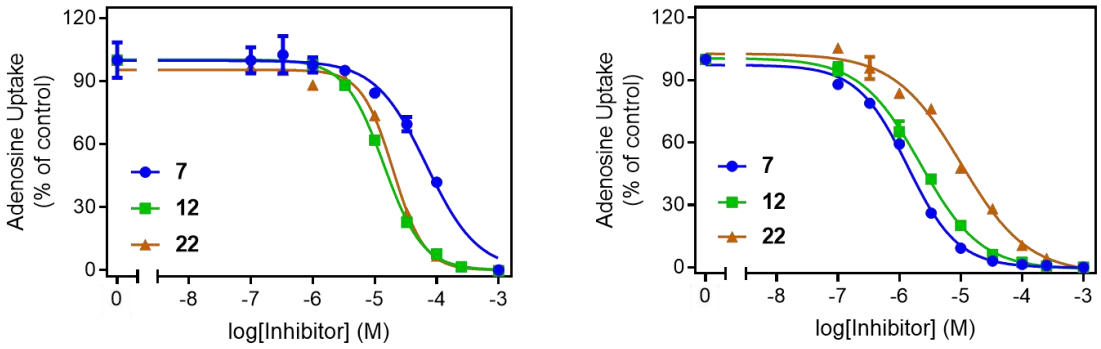
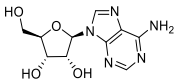
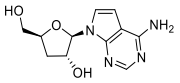
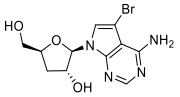
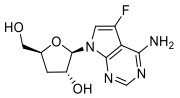
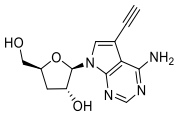
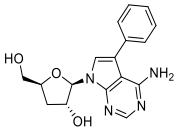
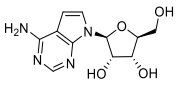


Figure 2: Uptake of [^{3}H]-adenosine via *T. brucei* P1 and P2 transporter systems in the presence of an increasing concentration of nucleoside analogues **7**, **12** and **22**. P1 transport is measured in B48 cells, which lack the P2 transporter. P2-mediated uptake is measured in B48 cells, stably overexpressing the P2 transporter protein. P2-transporter assays are performed in the presence of 100 µM inosine to block all P1-mediated uptake.[37] Each panel depicts one representative example of three independent experiments, each performed in triplicate. Error bars depict SEM, and when not shown, fall within the symbol. K_i values derived from these experiments are represented in Table 5.

Table 5: Kinetic parameters of selected nucleoside analogues for P1 and P2 transporters of bloodstream form *T. brucei*. K_m (indicated in **bold**) and K_i values are stated in µM (± SEM, $n \geq 3$). ΔG^0 values are stated in kJ/mol. $\delta(\Delta G^0)$ was calculated with respect to the ΔG^0 of the permeant adenosine, and is stated in kJ/mol. The values for **5** and **6** were taken from reference [19].

Cpd.	Structure	P1 transporter			P2 transporter		
		K_m or K_i	ΔG^0	$\delta(\Delta G^0)$	K_m or K_i	ΔG^0	$\delta(\Delta G^0)$
Adenosine, 1		**0.12 ± 0.02**	-39.4	--	**0.53 ± 0.02**	-35.8	--
5[19]		99.9 ± 21.7	-22.8	16.6	0.15 ± 0.03	-38.9	-3.1
6[19]		9.47 ± 1.4	-28.7	10.7	0.50 ± 0.14	-35.9	-0.1
7		75.6 ± 5.6	-23.5	15.9	1.10 ± 0.1	-33.9	1.9
12		9.71 ± 0.41	-28.6	10.8	2.35 ± 0.13	-32.1	3.7
22		26.0 ± 7.1	-26.2	13.2	10.9 ± 0.9	-28.3	7.5
26 (L-tubercidin)		N.E.	--	--	107 ± 3.4	-22.7	13.1

None of the three nucleoside analogues had a profile similar to that of **5**, which overwhelmingly depended on P2-mediated uptake with a P1/P2 K_i ratio of 670, explaining the RF of 11.2 for the *TbAT1*-KO strain (Table 4). The 7-fluoro nucleoside analogue **7** did display comparable P1 binding affinity to **6**, but showed reduced P2 binding energy (K_i ratio 68.7; RF = 3.5), indicating an attenuation of P2-dependent uptake, thus explaining the modest increase in EC_{50} for *TbAT1*-KO parasites (see Table 4). Remarkably, both 7-ethynyl and 7-phenyl analogues **12** and **22** displayed markedly better binding to the P1-transporter than tubercidin ($K_{i,\ tubercidin}$ = 78 ± 6.4 µM for P1[24]) and **5**. This concurs with the very modest resistance factor observed for *TbAT1*-KO parasites for **12** and **22** (Table 4). We have previously observed a similar effect for analogue **4**, featuring a 7-(pyridin-2-yl) group.[18] Al together, these results

allow the further refinement of the earlier model[24] of the P1-transporter molecular recognition motifs. We demonstrate here that the loss associated with substituting N7 (presumed to engage in an H-bonding interaction with the P1 adenosine binding site) for CH, can be compensated for by the introduction of a halogen (with the exception of fluorine), a linear carbon chain or an aryl ring at C7. We hypothesize that the reason for the exception of the fluorinated analogue is because of its small size.

Regarding transporter-dependent resistance, we found a strong correlation (Figure 3) between the RF values for *TbAT1*-KO and the ratio of the P1/P2 K_i values (r^2=0.978; P=0.0014, F-test). On the other hand, the RF did not significantly correlate with the K_i for either transporter alone, although the correlation with the P1 K_i was much better than for P2 (r^2=0.73/P=0.063 *vs* r^2=0.12/P=0.56).

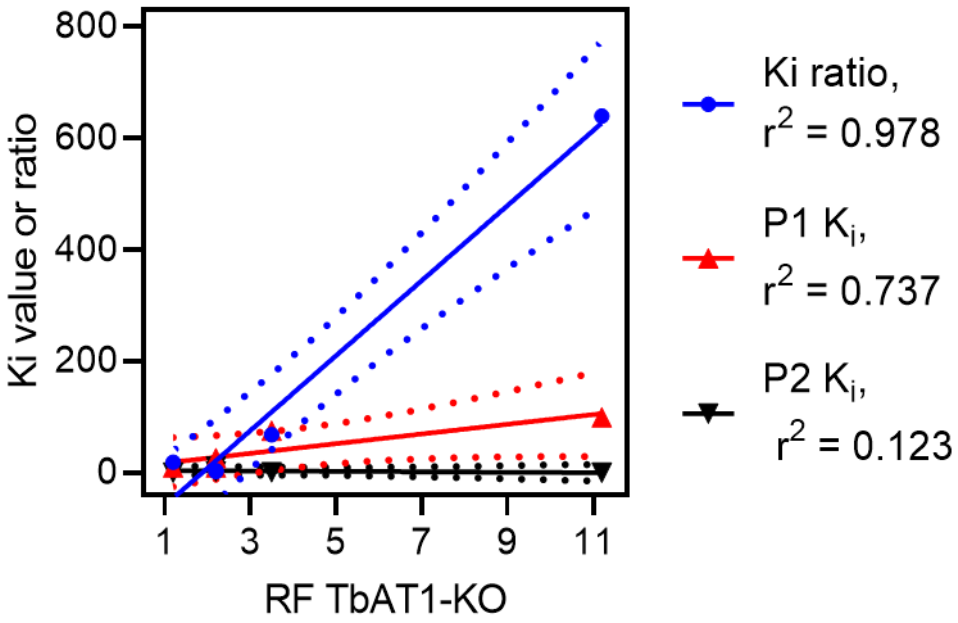


Figure 3: Correlation plot between the Resistance Factor (RF) of compounds **5**, **6**, **7**, **12**, and **22** and their K_i for the P1 transporter (red line and symbols), the K_i for the P2 transporter (black line and symbols), and their ratio (P1/P2; blue line and symbols). 95% confidence intervals are indicated by dotted lines and correlation coefficient r^2 is given for each line.

Finally, we also determined the ability of L-7-deazaadenosine (**26**) to inhibit [^{3}H]-adenosine uptake via the P1 and P2 transporter as the effect of enantiomeric nucleoside analogues for these *T. brucei* transporters has remained unexplored. The L-nucleoside **26** analogue was unable to affect the uptake of [^{3}H]-adenosine via P1, which is expected as recognition of D-7-deazaadenosine occurs via elements in both the sugar as well as the purine ring part. The P2 transporter affinity (K_i = 107 ± 3 μM) was also much lower than previously reported for D-7-deazaadenosine, (K_i = 3.8 ± 0.7 μM[24]), indicating an unfavourable binding of the L-nucleoside with the transporter. These data are likely to be (part of) the reason for the inactivity of L-nucleosides against *T. brucei*.

Selected nucleoside analogues (**8**, **9**, **16** and **48**) were then assayed for microsomal stability (Figure 4), to gain information on their potential for development as a therapeutic agent. Assays were run in the presence of either NADPH (mimicking phase I metabolism) or uridine glucuronyl transferase (UGT) enzymes (mimicking phase II metabolism). All four analogues were shown to be metabolically stable in both phase I and phase II metabolism mimicking experiments as virtually no reduction in percentage remaining parent compound could be observed.

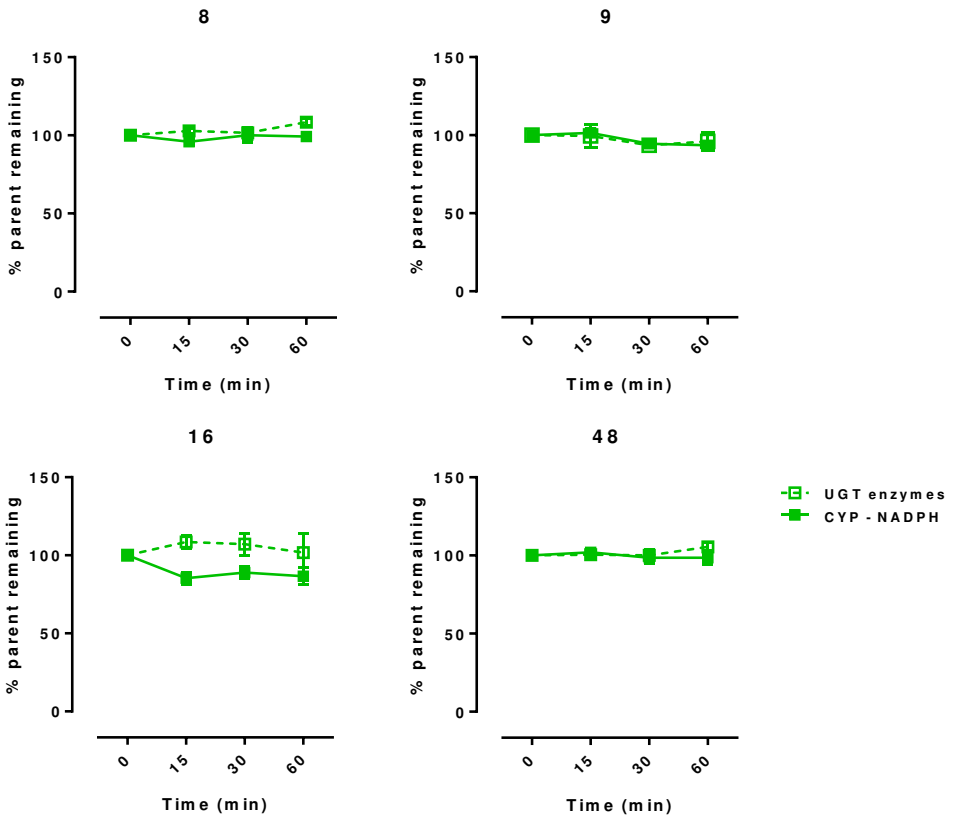


Figure 4: Analysis of metabolic stability in the presence of human liver microsomes for nucleoside analogues **8**, **9**, **16** and **48**. Indicated is the percentage of parent compound remaining (y-axis) as a function of time. Filled symbols represent data from NADPH-fortified experiments (phase I metabolism), while open symbols represent data from UGT-enzyme added experiments (phase II metabolism). Fluconazole (stable in phase I assays) and diclofenac (susceptible to degradation in both phase I and phase II assays) were added to assure proper functioning of the assay (data not shown). Data points are the mean from two independent experiments, and error bars are SD. When not shown, error bars fall withing the symbol.

Conclusion

In the present publication we have extensively studied the SAR of a series of 3'-deoxytubercidin nucleoside analogues. Starting from the previously described analogues **5** and **6**,[19] we have modified the

N1, C6, C7 and C8 position of the purine ring, the C2', C4' and C5' of the ribofuranose ring, and investigated the corresponding L-enantiomers of **5** and **6**. This revealed that the 7-position was most tolerant for modification and that halogens or small (non-aromatic) carbon-based substituents (such as ethynyl) represent the most favourable substituents. Other modifications in the purine ring were much less tolerated. Investigation of modifications of the ribofuranose ring revealed that only the 3'-deoxy*arabino* epimer **48** displayed submicromolar trypanocidal activity *in vitro*. An *anti*-conformation for nucleoside analogues and a D-ribofuranose stereochemistry appear to be an absolute requirement for adenosine transporter recognition and antitrypanosomal activity; although, as yet, this conclusion is based on a small sample size. Analysis of drug sensitivity against resistant *T. brucei* cell lines showed that the activity of several 7-substituted analogues was at most only modestly affected by the removal of the P2 transporter, representing an improvement over the previously discovered nucleoside analogue **5**. Detailed analysis of the binding affinity for the P1 transporter revealed that this transporter can accommodate a wide range of 7-substituted analogues, while N7 was previously reported to be a key recognition element for this transporter. Transporter-related resistance correlated best with the ratio of the K_i values for P1 and P2. Summarizing, this study has identified several 3'-deoxy nucleoside analogues that display potent antitrypanosomal activity and may overcome P2-related resistance in *T. brucei*. Several of these derivatives were found to be metabolically stable, providing confidence to translate the obtained data from this study into an *in vivo* setting.

Methods

Details on the chemical synthesis of discussed nucleoside analogues can be found in the Supporting Information.

Drug sensitivity assays

Standardised drug tests were performed on *T. b. brucei* Squib 427 (seeding density $1.5x10^4$ parasites/well) and *T. b. rhodesiense* strain STIB-900 (seeding density $4x10^3$ parasites/well) at the University of Antwerp, Belgium, and on a panel of strains derived from *T. b. brucei* Lister-427 (seeding density $2x10^4$ parasites/well) at the University of Glasgow, UK. The latter panel consisted of the multi-drug resistant strains *TbAT1*-KO,[35] B48,[43] and ISMR1[45] (all at a seeding density of 2×10^4 parasites/well). Culturing and drug testing were performed exactly as described previously.[18-19] All experiments were performed with bloodstream trypomastigotes, cultured in HMI-9 with 10% Fetal Bovine Serum (Gibco) at 37 ºC in a 5% CO_2 incubator. Drug tests were performed on doubling dilutions of test compounds in 96-well plates, incubated with 2×10^4 trypanosomes/well for 48 h before the addition of 'Alamar blue' solution (resazurin sodium salt; Sigma) and a further 24 h incubation. Fluorescence was read in a FLUOstar Optima (BMG Labtech, Aylesbury, Bucks, UK) and plotted to a sigmoid curve with variable slope (PRISM 7.0, GraphPad Software, San Diego, CA, USA) to obtain EC_{50} values (Effective Concentration 50%). Cytotoxicity against MRC-5_{SV2} fibroblasts was performed exactly as described previously.[18, 20, 49]

Transport studies

Transport of 50 nM 2,8-[^{3}H]-adenosine (American Radiolabelled Chemicals, 40 Ci/mmol) was performed exactly as described previously, using a rapid oil-stop technique,[50] where the incubation of 10^7 cells with radiolabel and test compound is terminated by the addition of 1 mL ice-cold 250 µM unlabelled adenosine followed by immediate centrifugation through an oil layer. The cell pellet is harvested after flash freezing in liquid nitrogen, by cutting of the tip of the microfuge tube into a scintillation vial. Radioactivity was determined in a Hidex 300SL scintillation counter (Lablogic, Sheffield, UK).

Metabolic stability

Metabolic stability assays were performed with pooled human liver microsomes (Corning), which were stored at -80 °C until use. Solutions containing NADPH-generating system A and B (Corning) and UGT-reaction mix A and B (Corning) were stored at -20 °C, until used in the assay. The test compounds (analogues **8**, **9**, **16** and **48**) were dissolved in DMSO at a concentration of 10 mM, as well as the two reference compounds diclofenac and fluconazole (metabolic stability data for the reference analogues not shown). The assay protocol follows the BD Biosciences Guidelines for use (TF000017 Rev1.0), with only minor changes. Metabolic stability was assayed for either phase I metabolism (NADPH fortification) or for phase II metabolism (uridine diphosphate glucuronic acid, UDPGA fortification). For phase I metabolism mimicking assays, the test compound was incubated at 1 µM with 0.5 mg/mL liver microsomes in potassium phosphate buffer solution. The reaction was started by the addition of 1 mM NADPH (t=0). At several times points (0, 15, 30 and 60 min), 20 µL samples were withdrawn, mixed with 80 µM ice-cold acetonitrile, containing tolbutamide as an internal standard. Then, the mixture was vortexed for 30 s, centrifuged at 4 °C for 5 min at 15 000 rpm. The supernatant was kept at -80 °C until the analysis by UPLC/MS-MS (see below). For phase II stability assays, compounds, at a concentration of 5 µM, were incubated with 0.5 mg/mL liver microsomes, and the reaction was started by adding 2 mM UDPGA cofactor to the mixture. Samples were taken in the same time schedule as described above for the phase I metabolic stability assays. The samples were then analysed by UPLC-MS², employing a Waters Aquity UPLC module coupled to a tandem quadrupole (Waters Xevo), equipped with an electrospray (ESI) interface, operated in the multiple-reaction-monitoring (MRM) mode. Data are expressed as the percentage loss of the parent compound. Fifty percent remaining parent compound at the 30 min time point was considered the cut-off for calling a compound metabolically stable.

Ancillary information

Supporting information

Schemes and discussion on the chemistry are presented in the Supporting Information.

Experimental data of the prepared nucleoside analogues in this study can be found in the Supporting Information.

Copies of ^{1}H, ^{13}C and ^{19}F spectra of compounds **8**-**19**, **23-26, 28-60**, **70**, **74**, **75**, **89** and **92** can be found in the Supporting Information. ^{1}H-^{13}C gHMBC of compounds **13**, **14**, **70**, **74**, **75**, **89** and **92** as well as 2D (^{1}H-^{1}H) NOESY spectra of compounds **70**, **74**, **75**, **89** and **92** can be found in the Supporting Information.

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Abbreviations used:

NTD, Neglected tropical disease.

References

1. Field, M. C.; Horn, D.; Fairlamb, A. H.; Ferguson, M. A.; Gray, D. W.; Read, K. D.; De Rycker, M.; Torrie, L. S.; Wyatt, P. G.; Wyllie, S.; Gilbert, I. H., Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. *Nat. Rev. Microbiol.* **2017,** *15* (4), 217-231.

2. De Rycker, M.; Baragaña, B.; Duce, S. L.; Gilbert, I. H., Challenges and recent progress in drug discovery for tropical diseases. *Nature* **2018,** *559* (7715), 498-506.

3. Franco, J. R.; Cecchi, G.; Priotto, G.; Paone, M.; Diarra, A.; Grout, L.; Simarro, P. P.; Zhao, W.; Argaw, D., Monitoring the elimination of human African trypanosomiasis: Update to 2016. *PLoS Neglected Trop. Dis.* **2018,** *12* (12), e0006890.

4. WHO Fact Sheet - Trypanosomiasis, human African (sleeping sickness) https://www.who.int/en/news-room/fact-sheets/detail/trypanosomiasis-human-african-(sleeping-sickness) (accessed 3/11/2019).

5. Reservoirs., I. E. G. o. G. H.; Buscher, P.; Bart, J. M.; Boelaert, M.; Bucheton, B.; Cecchi, G.; Chitnis, N.; Courtin, D.; Figueiredo, L. M.; Franco, J. R.; Grebaut, P.; Hasker, E.; Ilboudo, H.; Jamonneau, V.; Koffi, M.; Lejon, V.; MacLeod, A.; Masumu, J.; Matovu, E.; Mattioli, R.; Noyes, H.; Picado, A.; Rock, K. S.; Rotureau, B.; Simo, G.; Thevenon, S.; Trindade, S.; Truc, P.; Van Reet, N., Do cryptic reservoirs threaten gambiense-sleeping sickness elimination? *Trends Parasitol.* **2018,** *34* (3), 197-207.

6. Capewell, P.; Cren-Travaillé, C.; Marchesi, F.; Johnston, P.; Clucas, C.; Benson, R. A.; Gorman, T.-A.; Calvo-Alvarez, E.; Crouzols, A.; Jouvion, G.; Jamonneau, V.; Weir, W.; Stevenson, M. L.; O'Neill, K.; Cooper, A.; Swar, N.-r. K.; Bucheton, B.; Ngoyi, D. M.; Garside, P.; Rotureau, B.; MacLeod, A., The skin is a significant but overlooked anatomical reservoir for vector-borne African trypanosomes. *eLife* **2016,** *5*, e17716.

7. Capewell, P.; Atkins, K.; Weir, W.; Jamonneau, V.; Camara, M.; Clucas, C.; Swar, N. K.; Ngoyi, D. M.; Rotureau, B.; Garside, P.; Galvani, A. P.; Bucheton, B.; MacLeod, A., Resolving the apparent transmission paradox of African sleeping sickness. *PLoS Biol.* **2019,** *17* (1), e3000105.

8. Bottieau, E.; Clerinx, J., Human African Trypanosomiasis: progress and stagnation. *Infect. Dis. Clin. North. Am.* **2019,** *33* (1), 61-77.

9. Kennedy, P. G., The continuing problem of human African trypanosomiasis (sleeping sickness). *Ann. Neurol.* **2008,** *64* (2), 116-126.

10. Sokolova, A. Y.; Wyllie, S.; Patterson, S.; Oza, S. L.; Read, K. D.; Fairlamb, A. H., Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. *Antimicrob. Agents Chemother.* **2010,** *54* (7), 2893-2900.

11. Wyllie, S.; Foth, B. J.; Kelner, A.; Sokolova, A. Y.; Berriman, M.; Fairlamb, A. H., Nitroheterocyclic drug resistance mechanisms in *Trypanosoma brucei*. *J. Antimicrob. Chemother.* **2016,** *71* (3), 625-634.

12. Mesu, V.; Kalonji, W. M.; Bardonneau, C.; Mordt, O. V.; Blesson, S.; Simon, F.; Delhomme, S.; Bernhard, S.; Kuziena, W.; Lubaki, J. F.; Vuvu, S. L.; Ngima, P. N.; Mbembo, H. M.; Ilunga, M.; Bonama, A. K.; Heradi, J. A.; Solomo, J. L. L.; Mandula, G.; Badibabi, L. K.; Dama, F. R.; Lukula, P. K.; Tete, D. N.; Lumbala, C.; Scherrer, B.; Strub-Wourgaft, N.; Tarral, A., Oral fexinidazole for late-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a pivotal multicentre, randomised, non-inferiority trial. *Lancet* **2018,** *391* (10116), 144-154.

13. Pelfrene, E.; Harvey Allchurch, M.; Ntamabyaliro, N.; Nambasa, V.; Ventura, F. V.; Nagercoil, N.; Cavaleri, M., The European Medicines Agency's scientific opinion on oral fexinidazole for human African trypanosomiasis. *PLoS Neglected Trop. Dis.* **2019,** *13* (6), e0007381.

14. de Koning, H. P.; Bridges, D. J.; Burchmore, R. J. S., Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. *FEMS Microbiol. Rev.* **2005,** *29* (5), 987-1020.

15. Berg, M.; Van der Veken, P.; Goeminne, A.; Haemers, A.; Augustyns, K., Inhibitors of the purine salvage pathway: a valuable approach for antiprotozoal chemotherapy? *Curr. Med. Chem.* **2010,** *17* (23), 2456-2481.

16. Williamson, J., Cordycepin, an antitumour antibiotic with trypanocidal properties. *Trans. R. Soc. Trop. Med. Hyg.* **1966,** *60* (1), 8-8.

17. Williamson, J., Activity of drugs on *Trypanosoma congolense* in vitro at 37 degrees C. *Trans. R. Soc. Trop. Med. Hyg.* **1969,** *63* (4), 422-423.

18. Hulpia, F.; Campagnaro, G. D.; Scortichini, M.; Van Hecke, K.; Maes, L.; de Koning, H. P.; Caljon, G.; Van Calenbergh, S., Revisiting tubercidin against kinetoplastid parasites: aromatic substitutions at position 7 improve activity and reduce toxicity. *Eur. J. Med. Chem.* **2019,** *164*, 689-705.

19. Hulpia, F.; Mabille, D.; Campagnaro, G. D.; Schumann, G.; Maes, L.; Roditi, I.; Hofer, A.; de Koning, H. P.; Caljon, G.; Van Calenbergh, S., Combining tubercidin and cordycepin scaffolds results in highly active candidates to treat late-stage sleeping sickness. *Nat. Commun.* **2019,** *10* (1), 5564.

20. Hulpia, F.; Van Hecke, K.; Franca da Silva, C.; da Gama Jaen Batista, D.; Maes, L.; Caljon, G.; de Nazare, C. S. M.; Van Calenbergh, S., Discovery of novel 7-aryl 7-deazapurine 3'-deoxy-ribofuranosyl nucleosides with potent activity against *Trypanosoma cruzi*. *J. Med. Chem.* **2018,** *61* (20), 9287-9300.

21. Hulpia, F.; Bouton, J.; Campagnaro, G. D.; Alfayez, I. A.; Mabille, D.; Maes, L.; de Koning, H. P.; Caljon, G.; Van Calenbergh, S., C6-O-alkylated 7-deazainosine nucleoside analogues: Discovery of potent and selective anti-sleeping sickness agents. *Eur. J. Med. Chem.* **2020,** *188*, 112018.

22. Vodnala, S. K.; Lundbäck, T.; Yeheskieli, E.; Sjöberg, B.; Gustavsson, A.-L.; Svensson, R.; Olivera, G. C.; Eze, A. A.; de Koning, H. P.; Hammarström, L. G. J.; Rottenberg, M. E., Structure–activity relationships of synthetic cordycepin analogues as experimental therapeutics for African trypanosomiasis. *J. Med. Chem.* **2013,** *56* (24), 9861-9873.

23. Geiser, F.; Lüscher, A.; de Koning, H. P.; Seebeck, T.; Mäser, P., Molecular pharmacology of adenosine transport in *Trypanosoma brucei*: P1/P2 revisited. *Mol. Pharmacol.* **2005,** *68* (3), 589-595.

24. de Koning, H. P.; Jarvis, S. M., Adenosine transporters in bloodstream forms of *Trypanosoma brucei brucei*: substrate recognition motifs and affinity for trypanocidal drugs. *Mol. Pharmacol.* **1999,** *56* (6), 1162-1170.

25. Sufrin, J. R.; Spiess, A. J.; Marasco, C. J.; Rattendi, D.; Bacchi, C. J., Novel trypanocidal analogs of 5′-(methylthio)-adenosine. *Antimicrob. Agents Chemother.* **2008,** *52* (1), 211-219.

26. Bacchi, C. J.; Sufrin, J. R.; Nathan, H. C.; Spiess, A. J.; Hannan, T.; Garofalo, J.; Alecia, K.; Katz, L.; Yarlett, N., 5'-Alkyl-substituted analogs of 5'-methylthioadenosine as trypanocides. *Antimicrob. Agents Chemother.* **1991,** *35* (7), 1315-1320.

27. Bacchi, C. J.; Sanabria, K.; Spiess, A. J.; Vargas, M.; Marasco, C. J.; Jimenez, L. M.; Goldberg, B.; Sufrin, J. R., In vivo efficacies of 5'-methylthioadenosine analogs as trypanocides. *Antimicrob. Agents Chemother.* **1997,** *41* (10), 2108-2112.

28. Balzarini, J.; Pérez-Pérez, M. J.; San-Félix, A.; Schols, D.; Perno, C. F.; Vandamme, A. M.; Camarasa, M. J.; De Clercq, E., 2',5'-Bis-*O*-(tert-butyldimethylsilyl)-3'-spiro-5"-(4'-amino-1",2"-oxathiole-2",2"dioxide)pyrimidine (TSAO) nucleoside analogues: highlyselective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* **1992,** *89* (10), 4392-4396.

29. Camarasa, M. J.; Perez-Perez, M. J.; San-Felix, A.; Balzarini, J.; De Clercq, E., 3'-Spiro nucleosides, a new class of specific human immunodeficiency virus type 1 inhibitors: synthesis and antiviral activity of [2', 5'-bis-*O*-(tert-butyldimethylsilyl)-β-D-xylo- and -ribofuranose]-3'-spiro-5"-[4"-amino-1", 2"-oxathiole 2", 2"-dioxide] (TSAO) pyrimidine nucleosides. *J. Med. Chem.* **1992,** *35* (15), 2721-2727.

30. Perez-Perez, M. J.; San-Felix, A.; Balzarini, J.; De Clercq, E.; Camarasa, M. J., TSAO analogs. Stereospecific synthesis and anti-HIV-1 activity of 1-[2',5'-bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)pyrimidine and pyrimidine-modified nucleosides. *J. Med. Chem.* **1992,** *35* (16), 2988-2995.

31. Hampton, S. E.; Baragaña, B.; Schipani, A.; Bosch-Navarrete, C.; Musso-Buendía, J. A.; Recio, E.; Kaiser, M.; Whittingham, J. L.; Roberts, S. M.; Shevtsov, M.; Brannigan, J. A.; Kahnberg, P.; Brun, R.; Wilson, K. S.; González-Pacanowska, D.; Johansson, N. G.; Gilbert, I. H., Design, synthesis, and evaluation of 5′-diphenyl nucleoside analogues as inhibitors of the *Plasmodium falciparum* dUTPase. *ChemMedChem* **2011,** *6* (10), 1816-1831.

32. Ruda, G. F.; Nguyen, C.; Ziemkowski, P.; Felczak, K.; Kasinathan, G.; Musso-Buendia, A.; Sund, C.; Zhou, X. X.; Kaiser, M.; Ruiz-Perez, L. M.; Brun, R.; Kulikowski, T.; Johansson, N. G.; Gonzalez-Pacanowska, D.; Gilbert, I. H., Modified 5'-trityl nucleosides as inhibitors of *Plasmodium falciparum* dUTPase. *ChemMedChem* **2011,** *6* (2), 309-320.

33. Carter, N. S.; Fairlamb, A. H., Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature* **1993,** *361* (6408), 173-176.

34. Mäser, P.; Sütterlin, C.; Kralli, A.; Kaminsky, R., A Nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* **1999,** *285* (5425), 242-244.

35. Matovu, E.; Stewart, M. L.; Geiser, F.; Brun, R.; Mäser, P.; Wallace, L. J. M.; Burchmore, R. J.; Enyaru, J. C. K.; Barrett, M. P.; Kaminsky, R.; Seebeck, T.; de Koning, H. P., Mechanisms of arsenical and diamidine uptake and resistance in *Trypanosoma brucei*. *Eukaryotic Cell* **2003,** *2* (5), 1003-1008.

36. Collar, C. J.; Al-Salabi, M. I.; Stewart, M. L.; Barrett, M. P.; Wilson, W. D.; de Koning, H. P., Predictive computational models of substrate binding by a nucleoside transporter. *J. Biol. Chem.* **2009,** *284* (49), 34028-34035.

37. Munday, J. C.; Tagoe, D. N.; Eze, A. A.; Krezdorn, J. A.; Rojas Lopez, K. E.; Alkhaldi, A. A.; McDonald, F.; Still, J.; Alzahrani, K. J.; Settimo, L.; de Koning, H. P., Functional analysis of drug resistance-associated mutations in the *Trypanosoma brucei* adenosine transporter 1 (TbAT1) and the proposal of a structural model for the protein. *Mol. Microbiol.* **2015,** *96* (4), 887-900.

38. de Koning, H. P.; Jarvis, S. M., Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. *Acta Trop.* **2001,** *80* (3), 245-250.

39. Ward, C. P.; Wong, P. E.; Burchmore, R. J.; de Koning, H. P.; Barrett, M. P., Trypanocidal furamidine analogues: influence of pyridine nitrogens on trypanocidal activity, transport kinetics, and resistance patterns. *Antimicrob. Agents Chemother.* **2011,** *55* (5), 2352-2361.

40. de Koning, H. P.; Anderson, L. F.; Stewart, M.; Burchmore, R. J. S.; Wallace, L. J. M.; Barrett, M. P., The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in African trypanosomes. *Antimicrob. Agents Chemother.* **2004,** *48* (5), 1515-1519.

41. Sanchez, M. A.; Tryon, R.; Green, J.; Boor, I.; Landfear, S. M., Six related nucleoside/nucleobase transporters from *Trypanosoma brucei* exhibit distinct biochemical functions. *J. Biol. Chem.* **2002,** *277* (24), 21499-21504.

42. Al-Salabi, M. I.; Wallace, L. J. M.; Lüscher, A.; Mäser, P.; Candlish, D.; Rodenko, B.; Gould, M. K.; Jabeen, I.; Ajith, S. N.; de Koning, H. P., Molecular interactions underlying the unusually high

adenosine affinity of a novel *Trypanosoma brucei* nucleoside transporter. *Mol. Pharmacol.* **2007,** *71* (3), 921-929.

43. Bridges, D. J.; Gould, M. K.; Nerima, B.; Mäser, P.; Burchmore, R. J. S.; de Koning, H. P., 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.* **2007,** *71* (4), 1098-1108.

44. Munday, J. C.; Eze, A. A.; Baker, N.; Glover, L.; Clucas, C.; Aguinaga Andrés, D.; Natto, M. J.; Teka, I. A.; McDonald, J.; Lee, R. S.; Graf, F. E.; Ludin, P.; Burchmore, R. J. S.; Turner, C. M. R.; Tait, A.; MacLeod, A.; Mäser, P.; Barrett, M. P.; Horn, D.; De Koning, H. P., *Trypanosoma brucei* aquaglyceroporin 2 is a high-affinity transporter for pentamidine and melaminophenyl arsenic drugs and the main genetic determinant of resistance to these drugs. *J. Antimicrob. Chemother.* **2014,** *69* (3), 651-663.

45. Eze, A. A.; Gould, M. K.; Munday, J. C.; Tagoe, D. N.; Stelmanis, V.; Schnaufer, A.; De Koning, H. P., Reduced mitochondrial membrane potential Is a Late adaptation of *Trypanosoma brucei brucei* to isometamidium preceded by mutations in the gamma subunit of the F1Fo-ATPase. *PLoS Neglected Trop. Dis.* **2016,** *10* (8), e0004791.

46. de Koning, H. P., Transporters in African trypanosomes: role in drug action and resistance. *Int. J. Parasitol.* **2001,** *31* (5-6), 512-522.

47. Ranjbarian, F.; Vodnala, M.; Alzahrani, K. J. H.; Ebiloma, G. U.; de Koning, H. P.; Hofer, A., 9-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl) adenine is a potent antitrypanosomal adenosine analogue that circumvents transport-related drug resistance. *Antimicrob. Agents Chemother.* **2017,** *61* (6), e02719.

48. Rodenko, B.; van der Burg, A. M.; Wanner, M. J.; Kaiser, M.; Brun, R.; Gould, M.; de Koning, H. P.; Koomen, G.-J., 2,N6-Disubstituted adenosine analogs with antitrypanosomal and antimalarial activities. *Antimicrob. Agents Chemother.* **2007,** *51* (11), 3796-3802.

49. Lin, C.; Hulpia, F.; da Silva, C. F.; Batista, D. d. G. J.; Van Hecke, K.; Maes, L.; Caljon, G.; Soeiro, M. N. C.; Van Calenbergh, S., Discovery of pyrrolo[2,3-*b*]pyridine (1,7-dideazapurine) nucleoside analogues as anti-*Trypanosoma cruzi* agents. *J. Med. Chem.* **2019,** *62* (19), 8847-8865.

50. Wallace, L. J. M.; Candlish, D.; De Koning, H. P., Different substrate recognition motifs of human and trypanosome nucleobase transporters: selective uptake of purine antimetabolites. *J. Biol. Chem.* **2002,** *277* (29), 26149-26156.

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