9-(2'-Deoxy-2'-Fluoro- β -D-Arabinofuranosyl) Adenine Is a Potent Antitrypanosomal Adenosine Analogue That Circumvents Transport-Related Drug Resistance

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ABSTRACT Current chemotherapy against African sleeping sickness, a disease caused by the protozoan parasite *Trypanosoma brucei*, is limited by toxicity, inefficacy, and drug resistance. Nucleoside analogues have been successfully used to cure *T. brucei*-infected mice, but they have the limitation of mainly being taken up by the P2 nucleoside transporter, which, when mutated, is a common cause of multidrug resistance in *T. brucei*. We report here that adenine arabinoside (Ara-A) and the newly tested drug 9-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl) adenine (FANA-A) are instead taken up by the P1 nucleoside transporter, which is not associated with drug resistance. Like Ara-A, FANA-A was found to be resistant to cleavage by methylthioadenosine phosphorylase, an enzyme that protects *T. brucei* against the antitrypanosomal effects of deoxyadenosine. Another important factor behind the selectivity of nucleoside analogues is how well they are phosphorylated within the cell. We found that the *T. brucei* adenosine kinase had a higher catalytic efficiency with FANA-A than the mammalian enzyme, and *T. brucei* cells treated with FANA-A accumulated high levels of FANA-A triphosphate, which even surpassed the level of ATP and led to cell cycle arrest, inhibition of DNA synthesis, and the accumulation of DNA breaks. FANA-A inhibited nucleic acid biosynthesis and parasite proliferation with 50% effective concentrations (EC₅₀s) in the low nanomolar range, whereas mammalian cell proliferation was inhibited in the micromolar range. Both Ara-A and FANA-A, in combination with deoxycoformycin, cured *T. brucei*-infected mice, but FANA-A did so at a dose 100 times lower than that of Ara-A.

KEYWORDS 9-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl) adenine, FANA-A, *Trypanosoma brucei*, adenosine kinase, drug resistance, methylthioadenosine phosphorylase, nucleoside transporters, trypanosome

Trypanosoma brucei is transmitted by tsetse flies and causes African sleeping sickness in humans and nagana in cattle (1, 2). The disease is characterized by two stages. Initially, the parasites are restricted to circulating in the blood and lymph systems, but in the advanced stages of the disease, they are also found in the cerebrospinal fluid, which leads to coma and death if the patient is not treated. There is no existing vaccine, and available treatments are specific to the disease stage and the parasite subspecies. High toxicity, complex administration protocols, and drug resistance all contribute to the urgent need for new therapies.

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T. brucei lacks the pathway for *de novo* purine biosynthesis and is absolutely dependent on exogenous purines that are taken up from the body fluids of its hosts (3). As a consequence, the trypanosomes have evolved an array of efficient nucleoside and nucleobase transporters for purine uptake (4). The efficient transporters and downstream purine salvage enzymes can be exploited by developing antitrypanosomal substrate analogues that utilize the powerful salvage systems in *T. brucei* and subsequently kill the parasite by interfering with nucleotide-dependent reactions and/or nucleic acid biosynthesis.

It is not only ribonucleosides that are salvaged by *T. brucei*. The parasite is also able to salvage some deoxyribonucleosides, which are taken up by high-affinity nucleoside transporters (5, 6) and are subsequently phosphorylated by thymidine kinase and adenosine kinase (7–10). Thymidine kinase phosphorylates thymidine and deoxyuridine (7), whereas adenosine kinase is able to salvage adenosine and deoxyadenosine (9). It has been shown that higher levels of dATP accumulate in *T. brucei* than in mammalian cells after treatment with high concentrations of deoxyadenosine in the growth medium and that this accumulation is followed by *T. brucei* growth inhibition and lysis (11, 12). The dATP increase is less prominent at lower concentrations of deoxyadenosine, because *T. brucei* methylthioadenine phosphorylase (*TbMTAP*) cleaves this substrate into adenine and deoxyribose-1-phosphate, thereby rescuing the parasite from the cytotoxicity of deoxyadenosine (11). At higher concentrations of deoxyadenosine, this protective system is inhibited by its reaction product, adenine, which allows the deoxynucleoside to be phosphorylated by adenosine kinase instead, leading to the subsequent buildup of toxic levels of dATP in the cell (11). *TbMTAP* is relevant for drug development, because adenosine kinase substrate analogues need to be resistant to *TbMTAP* cleavage in order to be effective against the parasites.

Nucleoside/deoxynucleoside analogues are commonly used as drugs for cancer therapy and antiviral treatments (13). One advantage of using nucleoside analogues against second-stage sleeping sickness is that many of them can cross the blood-brain barrier via transporters used for the uptake of nucleosides needed by the brain (14). *T. brucei* bloodstream forms express two different purine nucleoside transporters, P1 and P2 (5, 15). The *T. brucei* P2 transporter has received much attention because of its role in multidrug resistance. It is involved in the uptake of diamidines and melaminophenyl arsenicals, which are two of the main groups of antitrypanosomal agents known to be effective against *T. brucei* (5, 15). The P2 transporter is particularly important for the uptake of diminazene aceturate (Berenil), a diamidine drug used against nagana in cattle (16). Experience using this drug for cattle has shown that the trypanosomes rapidly become resistant (16), and specific resistance mutations in the P2 transporter have been experimentally verified (17). Moreover, the P2 transporter has been shown to be the sole transporter for the furamidine trypanocides (18), which until recently were in clinical trials against sleeping sickness (19), and it has also been implicated as one of the main *T. brucei* transporters for melarsoprol and pentamidine (15, 20). A main reason for the easy acquisition of resistance is that the transporter is encoded by a single gene, *TbAT1* (5, 21). Mutations of this gene can mediate drug resistance with no major loss in purine nucleoside transport, which can still occur by P1 transporter activity as well as by the various nucleobase transporters (22). In contrast, P1 nucleoside transporters are encoded by at least half a dozen genes spread throughout the genome, and the different genetic variants of the P1 transporter seem to have similar substrate specificities (23, 24). It is therefore unlikely that mutations or the deletion of only one of them could cause resistance to a nucleoside analogue.

Most of the antitrypanosomal adenosine/deoxyadenosine analogues developed so far, including cordycepin, 2-fluorocordycepin, and tubercidin, are taken up mainly by the P2 nucleoside transporter (25, 26), which poses a problem with respect to cross-resistance to existing therapies. Our aim in this study was to identify novel antitrypanosomal adenosine analogues that are taken up by the P1 transporter and thus, due to the many genetic variants, are less likely to become ineffective as a result of gene mutation. Other criteria for an efficient antitrypanosomal nucleoside analogue exploit-

FIG 1 Deoxyadenosine and adenosine analogues containing 2' substituents oriented as in Ara-A. The 2' position is indicated in the left structure. Adenosine, which is not included in the figure, has its 2'-hydroxyl group pointing in the same direction as in 2'-C-methyladenosine.

ing the adenosine kinase-mediated salvage pathway are that it should be resistant to cleavage by *Tb*MTAP and should be a good substrate of *T. brucei* adenosine kinase.

RESULTS

Selection of adenosine analogues. One of the requirements for an adenosine kinase substrate analogue to be efficient against *T. brucei* is resistance to cleavage within the cell. Previous experiments have shown that adenine arabinoside (Ara-A) is a very poor substrate for the *Tb*MTAP enzyme, which cleaves deoxyadenosine, and that the Ara-A sensitivity of *Tb*MTAP knockdown trypanosomes was unaltered, indicating that the intracellular cleavage of Ara-A by *Tb*MTAP is insignificant (11). In addition to being cleavage resistant, Ara-A has good antitrypanosomal activity in the submicromolar range (9, 11). Thus, we tested the cleavage resistance and antitrypanosomal activities of other deoxyadenosine and adenosine analogues that have different 2' substituents pointing in the same direction as the 2'-OH group in Ara-A (Fig. 1). Two such compounds are commercially available: 9-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl) adenine (FANA-A), a deoxyadenosine analogue with a fluorine atom at this position, and 2'-C-methyladenosine, an adenosine analogue with a methyl group at this position (Fig. 1). In addition, we also tested two variants of FANA-A containing a halogen at the 2-position of the base to make them resistant to the adenosine deaminase activity present in mammalian serum. These were 2-fluoro-FANA-A (2F-FANA-A), a kind gift from Jack Secrist at the Southern Research Institute, and clofarabine, an anticancer agent used clinically (27). The advantage of the deamination-resistant drugs is that they can be used as single agents in chemotherapy, whereas other adenosine analogues generally need to be combined with adenosine deaminase inhibitors such as deoxycofornycin (dCF) in order to be stable in circulation.

Resistance to cleavage by *Tb*MTAP. The selected compounds were tested in enzyme assays with *Tb*MTAP. As shown in Table 1, all of the new compounds were even more resistant to cleavage than Ara-A. In all four cases, the enzyme activity was below

TABLE 1 Cleavage activities catalyzed by *Tb*MTAP and cell extracts

Substrate (100 μM)	Cleavage activity (μmol · min ⁻¹ · mg ⁻¹)	
	By recombinant <i>Tb</i> MTAP	In cell extracts
Methylthioadenosine	4.3 ± 0.2	Not analyzed
Deoxyadenosine	16.7 ± 0.7	Not analyzed
Adenosine	11.8 ± 0.3	0.16 ± 0.05
Ara-A	0.0027 ± 0.0004	<0.0002
FANA-A	0.0006 ± 0.0002	<0.0003
2F-FANA-A	<0.0002	<0.0002
Clofarabine	<0.00006	<0.0001
2'-C-methyladenosine	0.0007 ± 0.00006	<0.0003

FIG 2 Effects of FANA-A on *T. brucei* TC221 proliferation, nucleotide pools, and nucleic acid biosynthesis. (A) Inhibition of *T. brucei* TC221 proliferation as a function of FANA-A concentration. (B) Pools of ATP (■), FANA-A triphosphate (▽), FANA-A diphosphate (△), UTP (▼), and GTP (▲) in *T. brucei* cells treated with various concentrations of FANA-A. (C) Effect of FANA-A on DNA biosynthesis. The background is ~20 CPM. (D) Effect of FANA-A on RNA biosynthesis. All experiments were performed with 2 μM dCF in the growth medium, and the results are based on at least three independent experiments with standard errors.

or very close to the detection limit of the experiment. For comparison, the activities with the native substrates methylthioadenosine, deoxyadenosine, and adenosine were >1,000 times higher than those with any of the nucleoside analogues evaluated. We also tested the cleavage of the different drugs in *T. brucei* cell extracts in order to determine if the drugs were recognized by other nucleoside cleavage activities in *T. brucei*. The main purine nucleoside cleavage activity in *T. brucei* cells is inosine-adenosine-guanosine nucleoside hydrolase (IAG-NH), an enzyme that efficiently cleaves purine ribonucleosides but has very low activity on deoxyribonucleosides (28). The cleavage activity in cell extracts was below the detection limit for all of the nucleoside analogues tested, whereas the activity with adenosine, which was used as the positive control in the experiment, was ~1,000 times higher than the detection limit (Table 1).

FANA-A is an efficient inhibitor of *T. brucei* proliferation. Among the *TbMTAP*-resistant adenosine analogues, FANA-A had by far the greatest effect on *T. brucei* proliferation, with a 50% effective concentration (EC_{50}) of 2.8 nM (Fig. 2A; Table 2). The EC_{50} of 2'-C-methyladenosine was 300 nM, which is in a range similar to that reported for Ara-A (9, 11). Both FANA-A and 2'-C-methyladenosine were also very selective, as indicated by the much higher EC_{50} s against the mammalian reference cell lines (Table 2), with FANA-A achieving selectivity indexes of 1,900 and 790 (compared to BALB/3T3 and

TABLE 2 Inhibition of the proliferation of *T. brucei* TC221, mouse BALB/3T3, and human HL-60 cells by adenosine analogues

Nucleoside analogue ^a	EC_{50} (μM)			Selectivity index
	<i>T. brucei</i>	BALB/3T3	HL-60	
FANA-A (+dCF)	0.0028 ± 0.0002	5.4 ± 1.2	2.2 ± 0.1	1,900, 790 ^b
2F-FANA-A	0.56 ± 0.11	10 ± 5		18
Clofarabine	75 ± 17	1.24 ± 0.03		0.017
2'-C-methyladenosine (+dCF)	0.30 ± 0.07	11 ± 7		37

^aFANA-A and 2'-C-methyladenosine were given together with 2 μM deoxycytosine (dCF).

^bThe selectivity index of FANA-A was 1,900 compared to BALB/3T3 cells and 790 compared to HL-60 cells.

HL-60 cells, respectively). However, the addition of fluoro or chloro substituents at the 2-position of the nucleobase resulted in reduced effectiveness against *T. brucei* proliferation. This effect can be attributed to the fact that these compounds are poor substrates of adenosine kinase, as predicted by previous molecular modeling studies with this enzyme (26). Clearly, the 2-position substitutions are poorly tolerated by adenosine kinase, and this is directly correlated to the size of the substituent, because the chloro-substituted analogue clofarabine (2-chloro-FANA-A) displayed very low anti-trypanosomal activity, and the mammalian cells were much more sensitive than the trypanosomes.

FANA-A is phosphorylated in *T. brucei* cells and inhibits DNA synthesis. In mammalian cells, it is well-established that fludarabine, clofarabine, cladribine, and other nucleoside analogues lacking a 2'-OH group oriented as in ribonucleotides are phosphorylated in the cell and subsequently inhibit DNA synthesis indirectly via inhibition of ribonucleotide reductase and directly via inhibition of DNA polymerase (29). Inhibition of ribonucleotide reductase is in most cases due to the fact that the analogue mimics dATP as an overall negative regulator of activity, a type of regulation that *T. brucei* lacks (12). Nucleotide pool analysis of FANA-A-treated *T. brucei* cells showed that the parasites accumulated high levels of intracellular FANA-A 5'-diphosphates and triphosphates, which increased above the level of ATP and became the dominant nucleotides in the cell (Fig. 2B). The concomitant reduction in the ATP pool, which was previously observed with deoxyadenosine-treated and Ara-A-treated trypanosomes (11, 12), was only modest in the case of FANA-A, indicating that this is probably not a significant factor contributing to the growth inhibition, which occurs at FANA-A concentrations much lower than those needed to reduce ATP levels. A radioactive tracer experiment showed that FANA-A blocked nucleic acid biosynthesis in the parasites (Fig. 2C and D). When parasites were grown in the presence of [³H]uracil and various concentrations of FANA-A, the incorporation of radiolabel in DNA was strongly inhibited, even at a FANA-A concentration of 0.1 μ M (Fig. 2C). At higher concentrations of the drug, RNA synthesis was also inhibited (Fig. 2D). In principle, the results in Fig. 2C and D could also be indirect effects caused by altered metabolism of the uracil that we used as a probe or by FANA-ATP competing with ATP-dependent reactions in the cell. However, the low concentrations of FANA-A and the short time (1 h) needed to produce an effect on DNA synthesis are strong indications of specificity. For comparison, much higher concentrations were needed to obtain a high level of FANA-ATP accumulation in the parasite (Fig. 2B). In addition, FANA-A does not resemble uracil in structure, and UTP levels were normal in cells treated with the analogue (Fig. 2B). Unfortunately, the effect on the deoxynucleoside triphosphate (dNTP) pools could not be determined accurately in these experiments, because the FANA-A nucleotides interfered with the high-performance liquid chromatography (HPLC) analysis. Therefore, more experiments were needed to confirm that the inhibition of DNA synthesis was a direct effect and not a cause of altered dNTP pools (see below).

We performed a series of experiments to assess whether FANA-A directly inhibits DNA synthesis (Fig. 3). Visual inspection of cultured *T. brucei* by fluorescence microscopy of 4',6-diamidino-2-phenylindole (DAPI)-stained cells showed that the cell cycle distribution of trypanosomes treated with 0.2 μ M FANA-A was similar to that of control cells during the initial hours after treatment. However, the treated cells gradually started to accumulate in the G₁ stage with one nucleus and one kinetoplast (increasing from 60% of the cells in the G₁ stage at the beginning of treatment to >90% at 24 h) (Fig. 3A). Concomitantly, the percentages of cells in all other cell cycle stages decreased. At the FANA-A concentration used, DNA synthesis was nearly completely inhibited (Fig. 2C), explaining why the change in cell cycle distribution takes 15 h even though a normal cell cycle lasts only 6 h. The accumulation of cells in the G₁ stage means that they cannot pass the restriction point to reenter S phase. For comparison, there was no change in cell cycle distribution in *T. brucei* cells treated with dCF only (see Fig. S1 in the supplemental material). In order to determine if the altered

FIG 3 Cell cycle distribution and DNA fragmentation in FANA-A-treated *T. brucei* Lister 427 cells. (A) Cell cycle distribution in *T. brucei* cells treated with 0.2 μM FANA-A and 2 μM dCF (open symbols) and in untreated cells (filled symbols). The stage of the cell cycle was determined on the basis of the numbers of nuclei (N) and kinetoplasts (K) and the presence of a furrow ingression. Cells were divided into 1N1K (circles), 1N2K (triangles), early 2N2K (inverted triangles), and late 2N2K cells (in which cytokinesis had been initiated) (squares). The ordinate is split into two segments for legibility. Results are averages for two independent experiments with standard errors. (B) Percentage of DNA fragmentation as determined by the TUNEL assay on *T. brucei* cells grown in the absence (control) or presence of 2 μg/ml phleomycin (Phleo), 0.2 μM FANA-A, or a combination of 0.2 μM FANA-A and 2 μM dCF. Results are averages for three independent experiments with standard errors. Asterisks indicate *P* values from a Student *t* test where the results for treated and untreated cells were compared (*, *P* < 0.05; ***, *P* < 0.001).

cell cycle in FANA-A-treated *T. brucei* is due to DNA damage, we performed a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay for the detection of double-strand breaks. FANA-A-treated parasites accumulated DNA breaks, and this effect was enhanced if the FANA-A was protected from deamination by coadministration of dCF (Fig. 3B; see also Fig. S2 in the supplemental material). The extent of DNA breaks was almost as great as with phleomycin, which was used as a positive control in the experiment. The induction of DNA breaks indicates that FANA-A interferes directly with the accuracy of DNA replication.

Adenosine kinase activity. The first phosphorylation step is often rate limiting for the formation of nucleoside analogue diphosphates and triphosphates in the cell (30). In order to determine the reason for the selectivity of FANA-A against *T. brucei*, the recombinant adenosine kinase activity with FANA-A as the substrate was compared to that of human adenosine kinase (Fig. 4). Mammalian and *T. brucei* adenosine kinases are known to be dependent on phosphate ions for optimal enzyme activity when adenosine is the substrate (9, 31). With FANA-A, the phosphate stimulation was fairly modest and was already saturated at 5 mM phosphate with both enzymes (Fig. 4A). An experiment with adenosine was also included for the human enzyme in order to highlight the difference in activity from FANA-A. With adenosine, the effect of phosphate ions was much stronger, and the activity continued to increase over the whole

FIG 4 *T. brucei* and human adenosine kinase activities. (A) Enzyme activity as a function of phosphate concentration with *T. brucei* and human adenosine kinases. The substrates were 100 μM FANA-A with the *T. brucei* adenosine kinase (■) and 100 μM FANA-A (□) or 100 μM adenosine (△) with the human adenosine kinase. (B) Enzyme activity as a function of FANA-A concentration with *T. brucei* (■) and human (□) adenosine kinases. *T. brucei* adenosine kinase activity with 2F-FANA-A is shown for comparison (◆). All experiments were performed with 3 mM ATP as the cosubstrate. The graphs show averages from at least three independent experiments with standard errors.

TABLE 3 Effects of FANA-A and 2F-FANA-A on the proliferation of *T. brucei* cells of different strains^a

Strain	EC ₅₀ (μM) ^b			
	Pentamidine	FANA-A	FANA-A (+dCF)	2F-FANA-A
Lister 427	0.0030 ± 0.0002	0.120 ± 0.001	0.014 ± 0.001	0.47 ± 0.09
B48	0.65 ± 0.03****	0.079 ± 0.005**	0.0072 ± 0.0004***	0.094 ± 0.016***
B48 + P2	0.049 ± 0.003****	0.060 ± 0.001*	0.0049 ± 0.0004*	0.11 ± 0.03 (NS)
TC221		0.25 ± 0.06	0.0028 ± 0.0002 ^c	0.56 ± 0.11 ^c

^aThe strains tested were Lister 427 (parent strain), P2 knockout cells (strain B48), B48 cells complemented with the P2 nucleoside transporter (strain B48 + P2), and *T. brucei* strain TC221. Pentamidine was used as the positive control.

^b*P* values from Student *t* tests were obtained by comparing B48 with Lister 427 cells and B48 + P2 with B48 cells (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; NS, not significant).

^cThese values are taken from Table 2.

range of phosphate concentrations tested (Fig. 4A). The main conclusion from a drug development perspective is that FANA-A is a better substrate for the *T. brucei* adenosine kinase than for the human enzyme, and this conclusion is valid regardless of the phosphate concentration. The two enzymes were also studied using a fixed phosphate concentration of 5 mM and various substrate concentrations in order to obtain Michaelis-Menten parameters (Fig. 4B). The *T. brucei* adenosine kinase activity had both a higher *k*_{cat} than the human enzyme (3.5 ± 0.3 s⁻¹ versus 0.99 ± 0.04 s⁻¹, calculated per polypeptide) and a slightly more favorable *K*_m (55 ± 16 μM versus 96 ± 11 μM). 2F-FANA-A was also tested on the *T. brucei* enzyme and was found to be a much less favorable substrate (*k*_{cat} = 0.151 ± 0.006 s⁻¹; *K*_m = 84 ± 10 μM), explaining why the sensitivity of the trypanosome cells to this compound was 2 orders of magnitude lower than their sensitivity to FANA-A (Table 2).

FANA-A is taken up via the P1 nucleoside transporter family in *T. brucei*.

Exhaustive characterization of purine transport activities in *T. brucei* has shown that the P1 and P2 transporters are the only transporters that facilitate the uptake of adenosine or its analogues in bloodstream forms of the parasite (4, 5, 25, 32). By comparing the sensitivity of *T. brucei* cells (Lister 427, the parent strain) to FANA-A with those of multidrug-resistant cells (strain B48) that lack both the P2 transporter and the high-affinity pentamidine transporter (HAPT) (33) and the same cells carrying a plasmid for stable expression of the P2 gene (strain B48 + P2) (17), it was possible to determine if the P2 nucleoside transporter plays a significant role in FANA-A uptake. As shown in Table 3, sensitivity to pentamidine, which is known to be taken up by both the P2 nucleoside transporter and the HAPT (later identified as the aquaporin *TbAQP2* [34]), was dramatically reduced in the B48 strain and was partially restored when the P2 transporter was reintroduced. A complete reversion was not expected, because the cells still lacked the HAPT. In contrast, sensitivity to FANA-A or 2F-FANA-A was not decreased in the multidrug-resistant cells, indicating that the P2 transporter is not important for the uptake of the drugs. Instead, a slight but significant increase in sensitivity could be observed for the P2 knockout compared to the parent strain, possibly because of compensatory upregulation of the P1 transporter in the absence of the P2 transporter. The reintroduction of the P2 transporter on a high-expression plasmid (B48 + P2 cells) caused only an ~30% increase in FANA-A (+dCF) sensitivity over that of the B48 strain. This indicates either that FANA-A is a very poor substrate for the P2 transporter or that uptake through the P1 transporter is already so efficient that it does not limit the antitrypanosomal effects of FANA-A.

Interestingly, Table 3 also shows that there was an order-of-magnitude difference in FANA-A sensitivity between experiments in the absence of dCF and those in the presence of dCF. This indicates that the heat inactivation procedure used to inactivate enzymes in the serum for the preparation of growth medium does not seem to be sufficient to disable all of the adenosine deaminase activity. This conclusion was verified in experiments where the deamination of deoxyadenosine in cell-free growth medium was checked by HPLC. Deoxyadenosine was nearly completely converted to de-

FIG 5 HPLC profiles showing the deamination of 1 mM deoxyadenosine (A) and FANA-A (B) when incubated in HMI-9 medium. The medium contained 10% heat-inactivated fetal bovine serum, and the measurements were performed after 48 h of incubation at 37°C. The chromatograms are shown in a staggered view where the baselines are shifted vertically to fit several traces in each plot.

oxynosine if it was incubated at 37°C for 48 h in growth medium containing heat-inactivated serum (Fig. 5A). In the presence of dCF, there was no deamination of either deoxyadenosine or FANA-A (Fig. 5A and B). Due to the lack of a reference compound for the deaminated form of FANA-A, we monitored deamination by the disappearance of the FANA-A peak. The results shown in Fig. 5 are just a few examples from several experiments where the incubation time, substrate concentration, and/or source of serum was varied (commercially available heat-inactivated sera were also tested). A general conclusion was that heat inactivation has only a modest effect on adenosine deaminase. Deamination was negligible in the first hours of incubation but became a severe problem after 24 h.

The roles of the P1 and P2 nucleoside transporters for FANA-A uptake were also tested in assays for the ability of the drug to inhibit [^3H]adenosine uptake in trypanosomes (Table 4; Fig. 6). As expected, FANA-A was a good competitor for the P1 transporter, with a K_i value similar to the K_m of adenosine itself. The result with the P2 transporter was much more surprising. The K_i value was 10 times lower than that with adenosine, indicating that it seems to be a substrate with superior binding affinity (with a difference in the Gibbs free energy of binding [ΔG°] of 5.7 kJ/mol), suggesting that a 2'-fluorine pointing upward in a Haworth projection actually contributes to the binding interactions with the transporter, although a hydroxy moiety at that position is not favored ($K_i = 1.9 \mu\text{M}$ for Ara-A). It has been shown previously that a downward-directed hydroxyl group at the 2' position is also not favored, because deoxyadenosine has greater affinity than adenosine (5) (Table 4). Thus, we also tested the stereoisomer of FANA-A, 2'-F-2'-deoxyadenosine, and found that it had a binding affinity almost as high as that of FANA-A (Table 4). Thus, a fluorine at the 2' position, in either direction, seems to be favorable for high-affinity binding to the P2 transporter. In conclusion, FANA-A interacts with both types of transporters efficiently, although the P1 transporter alone appears to be sufficient for the antitrypanosomal effect.

TABLE 4 Adenosine transporter assay

Compound	P1 transporter		P2 transporter	
	K_m or K_i (μM) ^a	ΔG° (kJ/mol)	K_m or K_i (μM) ^a	ΔG° (kJ/mol)
Adenosine	0.41 ± 0.08	-36.8	0.92 ± 0.06	-34.5
FANA-A	0.48 ± 0.11	-36.1	0.09 ± 0.02	-40.2
2'-F-deoxyadenosine	0.06 ± 0.01	-41.1	0.19 ± 0.03	-38.3
Ara-A	ND		1.9 ± 0.5	-32.6
Deoxyadenosine ^b	0.19 ± 0.02	-38.3	0.23 ± 0.04	-37.9

^a K_m values are given for adenosine. The other values are K_i values (inhibition constants) determined by competition against [^3H]adenosine transport. ND, not determined.

^bValues for deoxyadenosine, from the 1999 work of de Koning and Jarvis (5), are included for comparison.

FIG 6 Transport of [3 H]adenosine by *T. brucei* bloodstream forms. (A) Transport of 0.1 μ M [3 H]adenosine by the B48 cell line over 30 s, in the absence (first data point) or presence of unlabeled adenosine (▲) or FANA-A (□) at the indicated concentrations. (B) Transport of adenosine by B48 cells expressing the P2 transporter (B48 + P2) in the presence of 1 mM inosine (in order to block P1 transport activity) and either unlabeled adenosine (▲) or FANA-A (□). The experiment was performed similarly to that for which results are shown in panel A. Data in both panels are representative of results from at least three independent experiments with standard errors.

In vivo experiments. Black C57BL/6 mice were infected with *T. brucei* TC221 bloodstream forms, and treatment was initiated with the test drug (Ara-AMP or FANA-A) injected intraperitoneally in combination with dCF as soon as the parasites were detectable in the blood. Because Ara-A has low aqueous solubility, it was given in the form of Ara-AMP in these experiments. Ara-AMP is a prodrug, which, upon dephosphorylation to Ara-A in the blood, is able to cross the cell membrane (35). As shown in Table 5, all the mice treated with either of the two drug combinations were cured. The level of parasites in the blood decreased below the detection limit within a day after the FANA-A treatment was initiated and remained the same during the rest of the treatment and for an additional 60 days of drug-free follow-up, demonstrating that all of the mice were cured. Similar results were obtained for the Ara-AMP treatment, except that it sometimes took 2 days for the parasite levels to drop below the detection limit. No obvious side effects were observed with the FANA-A treatment, whereas the Ara-AMP treatment produced some weight loss, which was recovered in all mice after the treatment was finished. On average, the Ara-AMP-treated mice showed a $13\% \pm 2\%$ (standard error) difference between the starting weight and the lowest weight during treatment. No weight loss was observed in the FANA-A-treated mice. Control groups, which received dCF or phosphate-buffered saline (PBS) only, were sacrificed 3 days after the treatment was initiated due to heavy parasite loads. Because the dosage protocol described here was 100% curative, it is likely that lower dosages or fewer administrations might still be effective, and the lower limits required for cure remain to be determined. Importantly, the mice treated with FANA-A did not suffer any overt toxic effects from the current protocol.

DISCUSSION

To date, the antitrypanosomal adenosine analogues developed against *T. brucei* are taken up primarily by the P2 transporter (25, 26, 36). However, because mutations in this transporter are strongly associated with resistance to current treatments (37–40), we propose that adenosine analogues that are instead taken up by the P1 transporter, or by both the P1 and P2 transporters, should be prioritized in the search for new

TABLE 5 Effect of intraperitoneal FANA-A or Ara-AMP treatment on *T. brucei*-infected mice

Drug	Dose (mg/kg) ^a	Cure rate
Ara-AMP (+dCF)	2 (+0.25 dCF)	10/10
FANA-A (+dCF)	200 (+0.25 dCF)	10/10
dCF	0.25	0/5
PBS only		0/7

^aThe indicated dose was given both in the morning and in the evening on days 1, 3, and 5.

FIG 7 Metabolism and mechanism of action of FANA-A in *T. brucei*. The P2 nucleoside transporter is not included in the figure because the P1 transporter is sufficient to give maximal growth inhibition by FANA-A. dCF, deoxycoformycin; ADA, adenosine deaminase; MTAP, methylthioadenosine phosphorylase; AK, adenosine kinase.

agents against *T. brucei*. In this study, we have identified FANA-A as an example of such an agent. FANA-A inhibited *T. brucei* proliferation in the low nanomolar range, and multidrug-resistant trypanosomes lacking the P2 transporter were equally sensitive to the drug as the parent strain, showing that the P1 transporter alone is sufficient for the uptake of the drug. Yet the uptake assays showed that FANA-A very strongly inhibits adenosine uptake by both the P1 and P2 transporters. FANA-A contains both the P2 binding $\text{H}_2\text{N}-\text{C}(\text{R}^1)=\text{N}-\text{R}^2$ “amidine motif,” which is either incorporated into or attached to an aromatic ring (38, 41), and the P1 binding motif, including the N-7- and N-3 atoms of the base as well as the 3'- and 5'-hydroxyl groups of the ribose (5, 23). Thus, FANA-A provides a perfect fit for the recognition motifs of both *T. brucei* nucleoside transporters. The observation that the P1 and P2 transporters both display very high affinity for FANA-A is consistent with the P2 knockout experiments showing that this transporter is not important for the trypanocidal activity of FANA-A. One possibility is that FANA-A binds efficiently to this transporter but that its translocation rate is very slow (low V_{max}/K_m for the substrate), possibly because of a slow off-rate linked to the unusually high binding affinity, but this can be assessed only by using radiolabeled FANA-A (which was not available) as the substrate. The alternative explanation is that uptake by the P1 transporters, which are encoded by several genes, is already sufficient, and that toxicity is limited by the rate of phosphorylation by adenosine kinase or the rate of incorporation into DNA, rather than the rate of uptake.

The sensitivity of the parasites to FANA-A seems to be dependent on many factors, including the efficiency of uptake and phosphorylation, resistance to cleavage, and effect on DNA biosynthesis (Fig. 7). The affinity of FANA-A for the P1 transporter is as high as that of adenosine, which is one of the main physiological substrates of this transporter, and as mentioned above, it is likely that the P2 transporter adds more uptake capacity. The initial phosphorylation of FANA-A, which is carried out by adenosine kinase, is also efficient, with a catalytic efficiency (k_{cat}/K_m) six times higher for the *T. brucei* enzyme than for the human enzyme, although it should be remembered that phosphorylation of substrates in the cell is dependent both on the catalytic efficiency of the enzyme and on its physiological concentration and that mammalian cells also have deoxycytidine kinase, which can phosphorylate deoxyadenosine analogues in the cytosol (30). The efficient uptake and phosphorylation of FANA-A were confirmed in *T. brucei* cells, which accumulated concentrations of FANA-ATP higher than the endogenous level of ATP. Another major factor behind the efficient accumulation of FANA-ATP is the fact that FANA-A is not recognized by *Tb*MTAP and other cleavage activities in the cell

and is thereby protected from degradation. The ultimate effect of FANA-A is that it interferes with DNA synthesis and causes DNA breaks. For nucleoside analogues, this is a very strong indication that they are incorporated into DNA. DNA breaks have been observed previously in mammalian cells treated with F-Ara-A, which was found to be incorporated into DNA, where it acted as a structural chain terminator (27). In contrast to classical chain terminators, F-Ara-A does not lack the 3'-OH group. Instead, it is the structure of the DNA that is altered in such a way that the 3' end cannot be extended, leading to breakage-sensitive single-stranded regions in the replicated DNA (27, 42). While delayed nonobligate chain terminators allow the DNA polymerase to add a few nucleotides after the incorporation site, F-Ara-A, clofarabine (which resembles FANA-A), and many other 2'-modified nucleoside analogues are pseudo-obligate, which means that they resemble classical terminators in that the 3' end is not extended further (42, 43).

Experience so far has shown that most adenosine analogues tested cross the blood-brain barrier to various degrees (44, 45), and Ara-A given intravenously has been used successfully to cure herpes simplex brain infections in humans (46). However, the neurological side effects reported for a few Ara-A-treated patients (47) and the need to use the phosphorylated form of the drug to make it soluble limit its applicability and appear to make FANA-A a more interesting drug candidate. Neither FANA-A nor Ara-A can be used as a single agent in therapy; both need to be combined with dCF, which is also called pentostatin, in order to be protected from deamination by adenosine deaminase in the mammalian blood (Fig. 7). dCF is known to cross the blood-brain barrier in monkeys (48), making it suitable for use in second-stage sleeping sickness. A possible problem with dCF is that it has been reported to be teratogenic, but only if given to pregnant mice on days 7 to 8 (49). However, a later study that compared mice and rabbits found that this was a mouse-specific effect, although this has to be verified in other animal models as well if dCF is going to be used in pregnant sleeping sickness patients (50). Nevertheless, it is still an advantage if FANA-A can be modified in such a way that it can be used as a single agent instead. One such modified analogue is 2F-FANA-A, which we found was 20 times more selective against *T. brucei* than against the mammalian fibroblasts used as reference. However, this level of selectivity is quite modest compared to that of FANA-A, and we are currently experimenting with other ways of making the molecule resistant to adenosine deaminase. In conclusion, FANA-A is a promising candidate for further drug development and demonstrates that nucleoside analogues can be created that are taken up principally by the P1 nucleoside transporter, thereby avoiding the drug resistance problems associated with uptake by the P2 nucleoside transporter.

MATERIALS AND METHODS

TbMTAP cleavage assay. TbMTAP assays were performed as described previously with the recombinantly expressed and purified enzyme (11). Briefly, the enzyme was diluted in a buffer containing 50 mM potassium acetate, 50 mM Tris-HCl (pH 7.4), and 0.05% Tween 20 prior to use in the enzyme assay. Enzyme activity was checked by incubating the enzyme with the compound in question in the presence of 50 mM KH_2PO_4 (adjusted to pH 7.4 with KOH) for 30 min at 37°C. The reaction was stopped by incubation at 100°C for 2 min, and the reaction products were analyzed by HPLC as described previously (11) to determine if the compound had been cleaved by TbMTAP. The amount of enzyme was adjusted for each substrate to give an assay that was linear over the 30-min incubation period.

***T. brucei* and mammalian cell culture.** *T. brucei* bloodstream forms were cultured at 37°C under a humidified 5% CO_2 atmosphere in HMI-9 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Thermo Fisher Scientific [Gibco], Waltham, MA, USA) (51). Several clonal *Trypanosoma brucei brucei* cell lines were used, all derivatives of the Lister 427 strain (http://tryps.rockefeller.edu/trypsru2_pedigrees.html). These include the Lister 427 strain in H. P. de Koning's laboratory, TC221 in A. Hofer's laboratory (a previous gift from F. Opperdoes, Universite Catholique Louvain, Brussels, Belgium), and the multidrug-resistant B48 cell line in which the TbAT1/P2 gene first had been deleted by homologous recombination (TbAT1-KO) (52) and the knockout cells had subsequently been continuously exposed to increasing levels of pentamidine *in vitro* (33). Reintroduction of the TbAT1 gene in this line created B48 + P2 (17), resulting in a higher-level and more stable expression of the P2 transporter, which appears to be downregulated *in vitro* when it is expressed only from its original locus (18). Mouse BALB/3T3 fibroblasts (ATCC CCL-163) were cultivated as monolayers at 37°C under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, glutamine (0.584 g/liter), and 10 ml/liter of 100× penicillin and streptomycin (Thermo Fisher Scientific [Gibco]). Human promyelocytic leukemia cells (HL-60 cells; ATCC CCL-240)

were grown as suspensions of cells under conditions similar to those for BALB/3T3 cells, the only difference being that RPMI medium (Thermo Fisher Scientific [Gibco]) was used instead of Dulbecco's modified Eagle's medium.

Drug sensitivity assays. FANA-A and other test compounds were serially diluted in HMI-9 medium, and 100 μ l was added to each well in two rows on a 96-well plate. Cultures of bloodstream-form *T. brucei* were diluted to 2×10^5 cells/ml in HMI-9 medium, and 100 μ l of cells was added to all wells. The plate was incubated at 37°C under 5% CO₂ for 48 h, after which 20 μ l of 0.5 mM resazurin sodium salt (Sigma) in phosphate-buffered saline (PBS) was added to each well, and the plate was incubated for a further 24 h. Fluorescence was measured with 540-nm excitation and 590-nm emission using an Infinite M200 microplate reader (Tecan Group, Männedorf, Switzerland). EC₅₀s were calculated after plotting the fluorescence against the test compound concentration using a sigmoid curve with a variable slope (Prism, version 5.0; GraphPad Software, La Jolla, CA, USA).

Nucleotide pool measurement. NTP and dNTP pools from *T. brucei* TC221 cells were extracted as described previously (12). Briefly, 10^8 *T. brucei* cells were lysed with a trichloroacetic acid-MgCl₂ solution and centrifuged, and the supernatant was subsequently extracted twice with Freon-trioctylamine to remove the trichloroacetic acid. The resulting nucleotide extract (20 μ l) was loaded onto a 100-mm by 4.6-mm ACE 3 AQ column (Advanced Chromatography Technologies, Aberdeen, Scotland) run at 1 ml/min. The mobile phase consisted of 11.3% (vol/vol) methanol, 80 mM KH₂PO₄, and 2 mM tetrabutylammonium bisulfate, and the final solution was adjusted to pH 6 with KOH. NTPs as well as FANA-A diphosphates and triphosphates were quantified by comparing the peak heights to the results with a standard nucleotide solution. This HPLC protocol was optimized for the determination of the FANA-A nucleotides and cellular NTPs, whereas the levels of cellular dNTPs were too low to be separated reliably from minor background peaks.

Measurement of [5,6-³H]uracil incorporation into *T. brucei* nucleic acids. Logarithmically growing *T. brucei* TC221 cells (1.8×10^8 to 2×10^8 cells) were collected by centrifugation at $1,800 \times g$ for 2 min and were resuspended in 10 ml fresh medium. The resuspended cells were incubated with 2 μ M deoxycytosine (dCF) for 30 min before treatment with various concentrations of FANA-A for 1 h in the presence of 10 μ Ci [5,6-³H]uracil (Moravak Biochemicals Inc., Brea, CA). ³H-labeled nucleic acids from *T. brucei* were extracted and precipitated, and the amounts of radioactivity in the RNA and DNA were measured as described (9).

Assessing cell cycle progression in *T. brucei*. The cell cycle progression of bloodstream-form *T. brucei* (strain Lister 427) treated with FANA-A was assessed by visualizing the DNA in the nuclei and kinetoplasts by use of the fluorescent dye DAPI (4',6-diamidino-2-phenylindole), followed by microscopic analysis as described previously (53). Briefly, 50 μ l of a trypanosome suspension (5×10^5 cells/ml) was spread onto a glass microscope slide and was left to dry before methanol fixation overnight at 20°C. The slides were then rehydrated with 1 ml PBS for 10 min and were then allowed to dry (but not totally). One drop of Vectashield antifade mounting medium with DAPI (Vector Laboratories Inc., USA) was added to the slides and was spread out before being covered with the coverslip. The slides were then viewed under UV light using a Zeiss Axioplan microscope with a Hamamatsu digital camera and OpenLAB software. For each sample, 500 cells were counted and scored for their DNA configuration as 1N1K, 1N2K, 2N2K (early), or 2N2K (late) (where N stands for nucleus, K stands for kinetoplast, "early" means that there is no ingression furrow, and "late" means that cell division has started, with an obvious ingression furrow). Samples were taken at 0, 4, 8, 12, 16, and 24 h, and untreated cultures were used as controls.

Assessment of DNA integrity. DNA fragmentation after exposure of *T. brucei* bloodstream forms (Lister 427) to FANA-A (with or without dCF) was determined as described previously (54). Drug-free and phleomycin (Sigma)-treated cells were used as negative and positive controls, respectively. Briefly, the cell density was adjusted to 1×10^6 cells/ml, and the cells were incubated with or without the test compounds for 12 h. The cells were fixed in 5 ml of 1% (wt/vol) paraformaldehyde in PBS, then centrifuged at $400 \times g$ for 5 min, and washed twice in 5 ml PBS. Finally, 5 ml of 70% ethanol was added to the sample, which was kept at -20°C for at least 1 h. The sample was then centrifuged for 5 min at $400 \times g$. The pellets were resuspended in 1 ml of transporter assay buffer (see below) and were centrifuged for 5 min at $400 \times g$. The resuspension-centrifugation step was performed twice to remove any residual ethanol. The presence of DNA strand breaks in the FANA-A-treated trypanosomes was observed by flow cytometry using the APO-BrdU TUNEL assay kit supplied by Life Technologies (catalog no. A23210) according to the manufacturer's protocol, where double-stranded breaks are extended by terminal deoxynucleotide transferase in the presence of dNTPs and bromodeoxyuridine (BrdU) triphosphate, followed by immunolabeling of BrdU in DNA.

Adenosine kinase assay. Recombinant *T. brucei* adenosine kinase was expressed and purified as described previously but using a concentration of 100 mM imidazole to elute the protein in the nickel-nitrilotriacetic acid (NTA) agarose chromatography step (9). The enzyme assay mixture contained 3 mM ATP, 6.4 mM MgCl₂, 100 mM KCl, and 50 mM Tris-HCl (pH 7.6). Various concentrations of potassium phosphate (from a 100 mM KH₂PO₄-K₂HPO₄ [pH 7.6] stock solution) and a substrate (usually FANA-A) were included as described in the figure legends. The enzyme (0.05 μ g/reaction) was diluted prior to use in the assay with a buffer consisting of 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 0.1 mM dithiothreitol, and 0.05% (vol/vol) Tween 20. The 50- μ l assay mixture was incubated at 37°C for 10 min, and the reactions were stopped by heating the sample for 2 min at 100°C. The reaction product was separated from the substrate, ATP, and other UV-absorbing compounds by HPLC using a 50-mm by 2.1-mm Ultracore 2.5 SuperHexylPhenyl column (Advanced Chromatography Technologies) and a mobile phase containing 65 mM KH₂PO₄, 2 mM tetrabutylammonium bisulfate, and 7% (vol/vol) methanol adjusted to pH 6 with KOH. The flow rate was 0.4 ml/min, and the injection volume was 5 μ l.

The short form of human adenosine kinase (55) was expressed, purified, and assayed in a manner similar to that described for the *T. brucei* enzyme except that the induction was performed for 10 h at 30°C (instead of 3 h at 37°C). The His tag removal step was skipped in the purification protocol, because it is known that the tag does not influence enzyme activity (55). The enzyme assay conditions were the same as those for the *T. brucei* enzyme, and no adjustment to the amount of enzyme in micrograms was necessary, because the molecular masses of the *T. brucei* and human (tagged) adenosine kinases are very similar (38 and 39 kDa, respectively). The bacteria expressing the human adenosine kinase (55) were part of a collection of mammalian adenosine kinases, bacteria, and antibodies obtained as a kind gift from James Bibb at the University of Texas Southwestern Medical Center. All adenosine kinase assays were verified to be in the linear range with respect to time and protein amount.

Uptake assays. Uptake assays were performed exactly as described previously (32, 56). Bloodstream forms of *T. brucei* cultures were harvested, washed with assay buffer (33 mM HEPES, 98 mM NaCl, 4.6 mM KCl, 0.55 mM CaCl₂, 0.07 mM MgSO₄, 5.8 mM NaH₂PO₄, 0.3 mM MgCl₂, 23 mM NaHCO₃, and 14 mM glucose [pH 7.3]), and resuspended at 10⁸ cells/ml. The cells were incubated for 30 s with 0.1 μM [2,8-³H]adenosine in the presence or absence of competitive substrates. The uptake assay for the P1 transporter was performed with the *T. brucei* B48 knockout strain, which lacks the P2 transporter. The uptake assay for the P2 transporter was performed with the same strain transfected with an expression construct carrying the P2 gene (B48 + P2), and in this case, the experiment was performed in the presence of 1 mM inosine to saturate the P1 transporter. After the 30-s incubation with [³H]adenosine and competitors, the incubation was stopped with ice-cold unlabeled substrate (1 mM in assay buffer), and the product was centrifuged through oil (13,000 × *g* for 1 min). This incubation time is well within the linear range of uptake as shown for both bloodstream and procyclic forms of *T. brucei* (57). The cell pellet was transferred to a scintillation tube, and radioactivity was counted by a scintillation counter.

Adenosine deaminase assays. The presence of adenosine deaminase in the culture medium was measured by incubating the medium with as much as 1 mM adenosine or FANA-A for as long as 48 h at 37°C. The sample was centrifuged through a 3-kDa-cutoff filter (Nanosep 3K Omega; Pall Corp., Port Washington, NY) and was diluted 40 times in water. Subsequently, 5 μl of the diluted sample was analyzed by HPLC. Chromatography was performed on a 50-mm by 2.1-mm ACE Ultracore 2.5 SuperC₁₈ column (Advanced Chromatography Technologies, Aberdeen, United Kingdom) using an aqueous mobile phase containing 7% methanol and 42 mM ammonium acetate adjusted to pH 6 with acetic acid. The flow rate was 0.4 ml/min.

Mouse experiments. *T. brucei* TC221 bloodstream forms (20,000 cells) were injected intraperitoneally into black C57BL/6 mice. After confirmation of the presence of parasites in the blood (generally 3 days after injection), treatment started with an intraperitoneal injection of PBS supplemented with 2 mg FANA-A/kg of body weight in combination with 0.25 mg/kg dCF. Similar experiments were also performed with 200 mg/kg Ara-AMP in combination with dCF. The doses were given twice daily on every alternate day for a total of 5 days (i.e., in the morning and afternoon on days 1, 3, and 5). Control mice were treated either with dCF mixed in PBS or with PBS only. Blood samples were regularly collected from the tail and were checked for the presence of *T. brucei* cells, and the mice were considered to be cured if they were parasite free for 60 days after the last dose. The mouse experiments were approved by the animal review board of the Court of Appeal of Northern Norrland in Umeå (decision 74-15) and were performed according to national legislation and Umeå University guidelines.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02719-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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