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EXPERIMENTAL THERAPEUTICS

In Vitro, *Ex Vivo*, and *In Vivo* Activities of Diamidines against *Trypanosoma congolense* and *Trypanosoma vivax*

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ABSTRACT African animal trypanosomosis (AAT) is caused by the tsetse fly-transmitted protozoans *Trypanosoma congolense* and *T. vivax* and leads to huge agricultural losses throughout sub-Saharan Africa. Three drugs are available to treat nagana in cattle (diminazene diaceturate, homidium chloride, and isometamidium chloride). With increasing reports of drug-resistant populations, new molecules should be investigated as potential candidates to combat nagana. Dicationic compounds have been demonstrated to have excellent efficacy against different kinetoplastid parasites. This study therefore evaluated the activities of 37 diamidines, using *in vitro* and *ex vivo* drug sensitivity assays. The 50% inhibitory concentrations obtained ranged from 0.007 to 0.562 μg/ml for *T. congolense* and from 0.019 to 0.607 μg/ml for *T. vivax*. On the basis of these promising results, 33 of these diamidines were further examined using *in vivo* mouse models of infection. Minimal curative doses of 1.25 mg/kg of body weight for both *T. congolense*- and *T. vivax*-infected mice were seen when the diamidines were administered intraperitoneally (i.p.) over 4 consecutive days. From these observations, 15 of these 33 diamidines were then further tested *in vivo*, using a single bolus dose for administration. The total cure of mice infected with *T. congolense* and *T. vivax* was seen with single i.p. doses of 5 and 2.5 mg/kg, respectively. This study identified a selection of diamidines which could be considered lead compounds for the treatment of nagana.

KEYWORDS chemotherapy, diamidines, nagana, *Trypanosoma*, *in vitro* drug sensitivity assays, *in vivo* animal models

Trypanosoma congolense (subgenus *Nannomonas*) and *Trypanosoma vivax* (subgenus *Dutonella*) are transmitted by tsetse flies (*Glossina* species) and remain the two main causative agents of African animal trypanosomosis (AAT), resulting in estimated economic losses of between $1 billion and $5 billion per annum throughout sub-Saharan Africa (1). Other causes of animal trypanosomosis include *Trypanosoma evansi*, *T. equiperdum*, and, to some extent, *T. brucei brucei*. Like *T. evansi* (which is found worldwide and which causes the disease surra), *T. vivax* is also found outside the African tsetse fly belt, adapting itself well to the South American continent via mechanical transmission. The chemotherapeutic treatment surrounding the control of *T. congolense* and *T. vivax* infections (nagana in cattle) within Africa has relied predominantly on three drugs, namely, diminazene diaceturate, homidium chloride, and isometamidium chloride; isometamidium chloride is often administered prophylactically, as well as for the treatment of infections. Numerous reports (2–7) have clearly demonstrated that drug resistance has become a serious hindrance to the effective control of AAT in Africa. With such a small repertoire of potential drugs, limited success with the production of

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vaccines for use in the future, and the continued movement of animals to and from tsetse fly-infested areas, alternative chemotherapeutic agents are urgently required.

The activities and efficacies of diamidine (dicationic) compounds against a panel of different kinetoplastid parasites have previously been investigated (8–10), and these compounds have been demonstrated to have good efficacy against a variety of pathogens (11). Diamidines were also investigated for their *in vitro* activities and efficacies against *T. evansi* in animal models (12–14). With such promising data, it seemed appropriate to ascertain the potential activity that related diamidines (and their analogues) could exert against *T. congolense* and *T. vivax*. A selection of compounds was made on the basis of the *in vitro* activities of the compounds against *T. brucei*-related species found previously (8, 13). Hence, the aim of this study was to evaluate the *in vitro*, *ex vivo*, and *in vivo* (mouse) efficacies of 37 diamidine compounds against both *T. congolense* and *T. vivax* strains.

RESULTS

In total, the activities of two standard drugs and 37 diamidine compounds against a susceptible *T. congolense* strain (IL-3000) were investigated *in vitro*. The 50% inhibitory concentrations (IC_{50}s; in micrograms per milliliter) of each compound were obtained for three separate assay incubation times: 40, 48, and 72 h. These IC_{50}s are shown in Table 1, together with those of the two standard drugs, diminazene aceturate and isometamidium chloride. In summary, the *in vitro* IC_{50}s of the standard drugs for IL-3000 for assay incubation times of 40, 48, and 72 h were observed to be 0.278, 0.076, and 0.066 μg/ml, respectively, for diminazene and 0.0014, 0.0004, and 0.0003 μg/ml, respectively, for isometamidium. The IC_{50}s of the 37 diamidine compounds ranged from 0.039 to 2.721 μg/ml for the 40-h assay, from 0.010 to 0.875 μg/ml for the 48-h assay, and from 0.007 to 0.562 μg/ml for the 72-h assay. In general, the IC_{50}s consistently decreased as the incubation time increased. A similar trend was seen for the two standard drugs diminazene and isometamidium. The influence of the incubation time on the IC_{50} results could clearly be seen across the 40-, 48-, and 72-h *in vitro* assays with *T. congolense*. The IC_{50}s did not differ greatly between the 48- and 72-h *in vitro* assays with *T. congolense*, with 31 of the 37 compounds tested showing less than 2-fold decreases in their IC_{50}s. Furthermore, the remaining 6 of the 37 compounds tested showed less than a 3-fold decrease in their IC_{50}s. In contrast, the IC_{50}s produced in the 40- and 72-h *in vitro* assays with *T. congolense* demonstrated a much wider range, with an up to 8-fold decrease in IC_{50}s being seen between the 40- and 72-h assay durations.

In comparison, all 37 diamidine compounds and the two standard drugs were investigated *ex vivo* using *T. congolense* (STIB 736/IL-1180) and *T. vivax* (STIB 719/ILRAD 560) strains, neither of which is currently adapted to axenic culture conditions. The *ex vivo* assay was adapted from the [^{3}H]hypoxanthine incorporation assay (15) and was performed at 40 h for all compounds. The IC_{50}s obtained for both strains are shown in Table 1. The *ex vivo* IC_{50}s of the standards, diminazene and isometamidium, against both parasite strains were similar, namely, 0.095 and 0.0004 μg/ml, respectively, for *T. congolense* and 0.076 and 0.0008 μg/ml, respectively, for *T. vivax*. The IC_{50}s of the 37 diamidine compounds tested against *T. congolense* ranged from 0.012 to 1.793 μg/ml, whereas the IC_{50}s against *T. vivax* ranged from 0.019 to 0.607 μg/ml. The IC_{50}s obtained for *T. vivax* were generally lower than those obtained for the *T. congolense* strain.

Subsequently, diminazene and isometamidium, together with 33 of the original 37 diamidine compounds, were further investigated for their *in vivo* efficacies against *T. congolense* and *T. vivax* in mouse models of infection. Four diamidine compounds had to be excluded from the *in vivo* experiments due to the discontinuation of product availability. The *in vivo* efficacy and the results of dose-response assays in which infected mice were intraperitoneally (i.p.) treated with the compounds on 4 consecutive days are shown in Table 2. For diminazene, 100% of *T. congolense*-infected mice were cured by doses of 20, 10, and 5 mg/kg of body weight given i.p. on 4 consecutive days, but only 75% (3/4) could be cured by a dose of 2.5 mg/kg. In comparison, 100% of *T. vivax*-infected mice could be cured only with a diminazene dose of 20 mg/kg given i.p.

TABLE 1 *In vitro* and *ex vivo* IC_{50}s of two standard drugs and 37 novel diamidine compounds for *T. congolense* and *T. vivax* strains for various assay incubation times

		IC_{50} (μg/ml)				
		In vitro assays with *T. congolense* IL-3000			*Ex vivo* assays	
Compound identity	Chemical family	40 h	48 h	72 h	*T. congolense* STIB 736 (40 h)	*T. vivax* STIB 719 (40 h)
Diminazene	Triazene diamidine	0.278	0.076	0.066	0.095	0.076
Isometamidium	Triazene amidine	0.0014	0.0004	0.0003	0.0004	0.0008
DB 75	Diphenylfuran	0.327	0.146	0.084	0.166	0.068
DB 283	Diphenylpyrimidine	0.842	0.225	0.187	0.212	0.100
DB 320	Diphenylpyrrole	0.532	0.218	0.200	0.210	0.139
DB 346	Biphenyl	0.119	0.061	0.036	0.041	0.048
DB 820	Pyridylfuran	0.316	0.203	0.187	0.206	0.195
DB 829	Pyridylfuran	0.510	0.204	0.198	0.212	0.166
DB 867	Pyridylfuran	0.258	0.179	0.173	0.222	0.062
DB 1052	Thiazole	1.725	0.533	0.562	0.815	0.607
DB 1055	Benzimidazole	0.373	0.115	0.051	0.053	0.040
DB 1192	Indole	0.244	0.088	0.069	0.091	0.073
DB 1307	Thiophene	0.465	0.209	0.188	0.247	0.089
DB 1406	Diphenylpyrimidine	0.698	0.216	0.197	0.259	0.147
DB 1854	Indole	0.039	0.010	0.007	0.012	0.032
DB 1866	Thiophene	0.086	0.021	0.019	0.053	0.019
DB 1870	Indole	0.087	0.019	0.019	0.058	0.066
DB 1893	Indole	0.204	0.062	0.058	0.089	0.178
DB 1903	Indole	0.261	0.060	0.033	0.044	0.023
DB 1915	Biphenylbenzanilide	0.476	0.187	0.065	0.101	0.074
DB 1917	Biphenylbenzanilide	2.721	0.729	0.420	1.193	0.032
DB 2017	Thiazolothiazole	0.094	0.040	0.034	0.016	0.101
DB 2175	Diphenylether	0.505	0.208	0.169	0.236	0.114
DB 2179	Bifuran	0.685	0.237	0.194	0.190	0.031
DB 2180	Selenophene	0.445	0.144	0.177	0.363	0.105
DB 2190	Thiazole	0.520	0.183	0.153	0.175	0.155
7 SAB 038	Benzofuran	0.198	0.074	0.059	0.084	0.052
10 SAB 078	Benzofuran	2.623	0.875	0.413	1.793	0.130
12 SAB 081	Benzofuran	0.623	0.220	0.118	0.703	0.062
13 SAB 017	Benzofuran	0.272	0.122	0.067	0.080	0.040
13 SAB 089	Benzimidazole	0.139	0.073	0.026	0.035	0.027
16 DAP 095	Isoxazole	0.309	0.155	0.072	0.220	0.071
17 SAB 085	Triazole	0.116	0.051	0.052	0.166	0.121
18 SAB 023	Triazole	0.426	0.186	0.174	0.241	0.153
19 DAP 025	Naphthylene	0.336	0.197	0.130	0.215	0.189
24 SMB 001	Dithiophene	0.184	0.124	0.066	0.070	0.048
27 DAP 060	Dipyridylphenyl	0.248	0.147	0.068	0.222	0.176
28 DAP 010	Dipyridylphenyl	0.606	0.226	0.195	0.586	0.089
32 DAP 022	Pyridyloxazole	0.071	0.027	0.016	0.043	0.033

on 4 consecutive days, while only 2/4 mice (50%) could be cured at doses of 10, 5, and 2.5 mg/kg. The minimal curative doses of isometamidium in *T. congolense*- and *T. vivax*-infected mice were observed to be 0.03125 mg/kg and 0.0625 mg/kg, respectively, when the drug was given i.p. on 4 consecutive days.

In summary, 16 of the 33 diamidine compounds investigated were found to provide a full cure (4/4) in *T. congolense*-infected mice when they given at the 5-mg/kg dose i.p. on 4 consecutive days. Eleven of the 33 diamidine compounds tested were found to provide at least a 75% curative efficacy (3/4) at the 2.5-mg/kg dose, and just 3 of the 33 diamidine compounds tested were found to be at least 75% curative when they were given at the 1.25-mg/kg dose i.p. on 4 consecutive days. In comparison, 15 of the 33 diamidine compounds investigated were found to provide a full cure (4/4) in *T. vivax*-infected mice when they were given at the 5-mg/kg dose i.p. on 4 consecutive days. Just 7 of the 33 diamidine compounds tested were found to provide at least 75% curative efficacy (3/4) when they were given at the 2.5-mg/kg dose, and just 4 of the 33 diamidine compounds tested were found to be at least 75% curative (3/4) when they

TABLE 2 *In vivo* efficacy and dose-response for two standard drugs and 33 novel diamidine compounds given on 4 consecutive days i.p. in mouse models of *T. congolense* and *T. vivax* infection

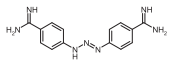
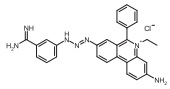
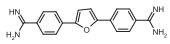
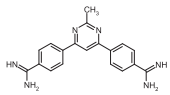
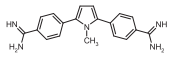
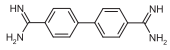
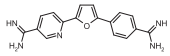
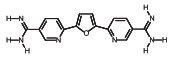
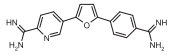
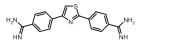
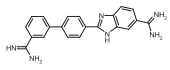
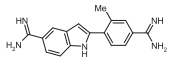
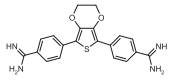
Compound identity	Dose tested (mg/kg)	*T. congolense* (STIB 736/IL-1180)		*T. vivax* (STIB 719/ILRAD 560)		Chemical structure
		No. of mice cured/no. infected	Relapse day[a]	No. of mice cured/no. of mice infected	Relapse day	
Diminazene	20	4/4	NA[b]	4/4	NA	
	10	4/4	NA	2/4	40	
	5	4/4	NA	2/4	6	
	2.5	3/4	11	2/4	1	
Isometamidium	0.0625	4/4	NA	4/4	NA	
	0.03125	4/4	NA	0/4	7	
	0.015625	3/4	41	0/4	7	
DB 75	5	4/4	NA	4/4	NA	
	2.5	3/4	14	4/4	NA	
	1.25	2/4	4	4/4	NA	
	0.625	0/4	4	0/4	2	
DB 283	5	0/4	2	3/4	17	
DB 320	5	0/4	11	1/4	6	
DB 346	5	0/4	12	0/4	2	
DB 820	2.5	4/4	NA	4/4	NA	
	1.25	2/4	8	4/4	NA	
	0.625	0/4	1	0/4	1	
DB 829	1.25	4/4	NA	4/4	NA	
	0.625	0/4	3	0/4	1	
DB 867	5	4/4	NA	4/4	NA	
	2.5	4/4	NA	0/4	3	
	1.25	3/4	8	0/4	1	
DB 1052	5	0/4	3	2/4	9	
DB 1055	5	0/4	5	0/4	7	
DB 1192	5	0/4	7	0/4	9	
DB 1307	5	0/4	1	0/4	7	

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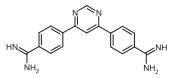
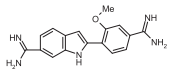
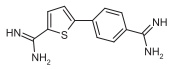
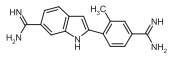
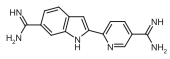
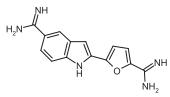
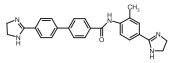
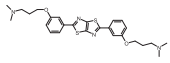
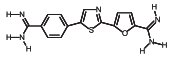
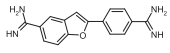
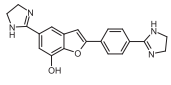
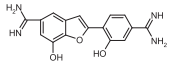
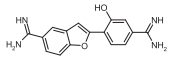
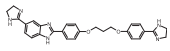
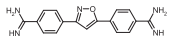
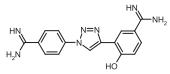
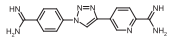
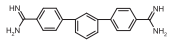
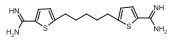
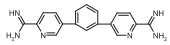
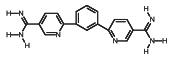
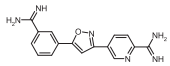
TABLE 2 (Continued)

Compound identity	Dose tested (mg/kg)	*T. congolense* (STIB 736/IL-1180)		*T. vivax* (STIB 719/ILRAD 560)		Chemical structure
		No. of mice cured/no. infected	Relapse day[a]	No. of mice cured/no. of mice infected	Relapse day	
DB 1406	5	4/4	NA	4/4	NA	
	2.5	0/4	10	4/4	NA	
	1.25	0/4	1	2/4	12	
DB 1854	5	4/4	NA	4/4	NA	
	2.5	3/4	44	2/4	18	
DB 1866	5	2/4	9	1/4	11	
DB 1870	5	4/4	NA	0/4	7	
	2.5	4/4	NA	0/4	3	
	1.25	3/4	15	0/4	1	
DB 1893	5	4/4	NA	1/4	10	
	2.5	4/4	NA	0/4	2	
	1.25	0/4	5	0/4	1	
DB 1903	5	0/4	6	0/4	3	
DB 1917	5	0/4	2	3/4	10	
DB 2017	5	0/4	2	0/4	1	
DB 2190	5	2/4	15	0/4	6	
7 SAB 038	5	4/4	NA	4/4	NA	
	2.5	3/4	11	2/4	21	
10 SAB 078	5	0/4	3	0/4	3	
12 SAB 081	5	0/4	8	4/4	NA	
	2.5	0/4	1	4/4	NA	
	1.25	0/4	1	3/4	39	
13 SAB 017	5	4/4	NA	4/4	NA	
	2.5	0/4	12	0/4	11	

(Continued on next page)

TABLE 2 (Continued)

Compound identity	Dose tested (mg/kg)	*T. congolense* (STIB 736/IL-1180) No. of mice cured/no. infected	*T. congolense* (STIB 736/IL-1180) Relapse day[a]	*T. vivax* (STIB 719/ILRAD 560) No. of mice cured/no. of mice infected	*T. vivax* (STIB 719/ILRAD 560) Relapse day	Chemical structure
13 SAB 089	5	4/4	NA	4/4	NA	
	2.5	3/4	18	4/4	NA	
	1.25	1/4	5	0/4	9	
16 DAP 095	5	2/4	14	4/4	NA	
	2.5	0/4	10	2/4	16	
17 SAB 085	5	4/4	NA	1/4	2	
	2.5	4/4	NA	0/4	1	
	1.25	0/4	1	0/4	1	
18 SAB 023	5	4/4	NA	4/4	NA	
	2.5	0/4	12	3/4	7	
19 DAP 025	5	4/4	NA	4/4	NA	
	2.5	0/4	8	0/4	9	
24 SMB 001	5	0/4	6	4/4	NA	
	2.5	0/4	1	0/4	5	
27 DAP 060	5	4/4	NA	4/4	NA	
	2.5	0/4	8	0/4	4	
28 DAP 010	5	3/4	38	3/4	9	
	2.5	3/4	15	0/4	7	
32 DAP 022	5	4/4	NA	2/4	10	
	2.5	2/4	28	0/4	5	

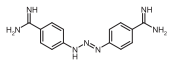
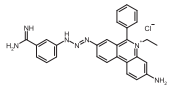
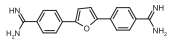
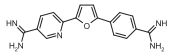
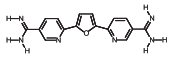
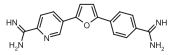
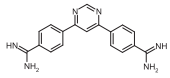
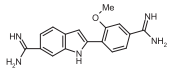
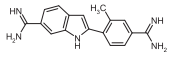
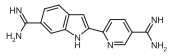
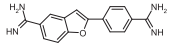
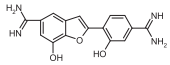
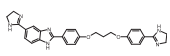
[a]Relapse day, the day on which the mice were monitored, beginning from the day after final drug administration.
[b]NA, not applicable, as no relapse was seen during the complete 60-day monitoring phase.

were given at the 1.25-mg/kg dose i.p. on 4 consecutive days. The compound providing 100% curative efficacy for both *T. congolense*- and *T. vivax*-infected mice when it was given at the minimal curative dose of 1.25 mg/kg i.p. on 4 consecutive days was compound DB 829.

Consequently, the two standard drugs and 15 of the 33 diamidine compounds previously tested *in vivo* were additionally examined for their curative efficacy when they were given to *T. congolense*- and *T. vivax*-infected mice as a single bolus treatment dose i.p. The resulting *in vivo* efficacy data can be viewed in Table 3. The minimal dose showing a 100% curative efficacy of diminazene against both parasites when it was given i.p. as a single bolus dose was observed to be 10 mg/kg. A single bolus dose of 5 mg/kg given i.p. was found to have insufficient efficacy (1/4 or 0/4) against both trypanosome species. For isometamidium, the minimal curative dose showing a 100% rate of cure for *T. congolense*- and *T. vivax*-infected mice was 0.25 mg/kg given i.p. A single bolus dose of 0.125 mg/kg given i.p. cured 3 out of 4 *T. vivax*-infected mice but only 1 out of 4 *T. congolense*-infected mice.

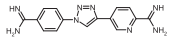
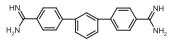
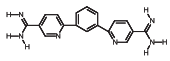
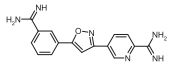
In summary, 4 of the 15 diamidine compounds evaluated were found to provide the full cure (4/4) of both *T. congolense*- and *T. vivax*-infected mice when given as a single bolus dose of 10 mg/kg i.p. Two of the 15 diamidine compounds tested were found to provide at least a 75% cure (3/4) of both *T. congolense*- and *T. vivax*-infected mice when

TABLE 3 *In vivo* efficacy and dose-response for two standard drugs and 15 novel diamidine compounds given as a single bolus dose i.p. in mouse models of *T. congolense* and *T. vivax* infection

		T. congolense (STIB 736/IL-1180)		*T. vivax* (STIB 719/ILRAD 560)		
Compound identity	Dose tested (mg/kg)	No. of mice cured/no. infected	Relapse day[a]	No. of mice cured/no. infected	Relapse day	Chemical structure
Diminazene	20	4/4	NA[b]	4/4	NA	
	10	4/4	NA	4/4	NA	
	5	1/4	11	0/4	6	
	2.5	0/4	12	0/4	1	
Isometamidium	1	4/4	NA	4/4	NA	
	0.5	4/4	NA	4/4	NA	
	0.25	4/4	NA	4/4	NA	
	0.125	1/4	11	3/4	14	
	0.0625	0/4	8	0/4	6	
DB 75	10	4/4	NA	4/4	NA	
	5	4/4	NA	4/4	NA	
	2.5	0/4	6	4/4	NA	
	1.25	0/4	1	0/4	7	
DB 820	10	4/4	NA	4/4	NA	
	5	0/4	10	1/4	19	
DB 829	10	4/4	NA	4/4	NA	
	5	3/4	14	3/4	7	
DB 867	10	4/4	NA	0/4	7	
	5	0/4	8	0/4	1	
DB 1406	10	4/4	NA	4/4	NA	
	5	0/4	3	0/4	3	
DB 1854	10	2/4	8	2/4	4	
	5	2/4	1	2/4	1	
DB 1870	10	4/4	NA	2/4	14	
	5	3/4	33	1/4	3	
	2.5	0/4	11	0/4	1	
DB 1893	10	4/4	NA	2/4	7	
	5	0/4	10	0/4	2	
7 SAB 038	10	0/4	11	2/4	17	
	5	0/4	4	2/4	5	
12 SAB 081	10	0/4	1	4/4	NA	
	5	0/4	1	4/4	NA	
	2.5	0/4	1	4/4	NA	
	1.25	0/4	1	0/4	3	
13 SAB 089	10	0/4	8	2/4	10	
	5	0/4	6	2/4	5	

(Continued on next page)

TABLE 3 (Continued)

Compound identity	Dose tested (mg/kg)	*T. congolense* (STIB 736/IL-1180) No. of mice cured/no. infected	*T. congolense* (STIB 736/IL-1180) Relapse day[a]	*T. vivax* (STIB 719/ILRAD 560) No. of mice cured/no. infected	*T. vivax* (STIB 719/ILRAD 560) Relapse day	Chemical structure
18 SAB 023	10	0/4	9	4/4	NA	
	5	0/4	8	2/4	1	
19 DAP 025	10	1/4	8	2/4	17	
	5	0/4	7	2/4	7	
28 DAP 010	10	2/4	12	4/4	NA	
	5	0/4	8	0/4	3	
32 DAP 022	10	1/4	15	0/4	7	
	5	0/4	8	0/4	2	

[a]Relapse day, the day on which the mice were monitored, beginning from the day after final drug administration.
[b]NA, not applicable, as no relapse was seen during the complete 60-day monitoring phase.

they were given as a single bolus dose of 5 mg/kg i.p. None of the diamidine compounds tested were found to provide a cure when they were given to *T. congolense*-infected mice as a single bolus dose of 2.5 mg/kg i.p. However, 2 of the 15 diamidine compounds were found to be 100% curative when they were given to *T. vivax*-infected mice as a single bolus dose of 2.5 mg/kg. The minimal curative doses providing 100% cure of *T. congolense*- and *T. vivax*-infected mice were therefore single bolus doses of 5 and 2.5 mg/kg given i.p., respectively.

DISCUSSION

The aim of this study was to determine the activities of diamidine compounds (and their analogues) against the animal-pathogenic parasites *Trypanosoma congolense* and *T. vivax*. By leveraging such chemical classes of molecules, previously found to be efficacious against a variety of similar kinetoplastid organisms, and the in-depth knowledge already gained from such investigations, the pursuit of more effective, alternative chemotherapeutic agents for the treatment of nagana can efficiently be explored. Both *T. congolense* and *T. vivax* originate from subgenera different from those of other trypanosome species, such as *T. brucei brucei*, *T. brucei rhodesiense*, and *T. evansi*. The current inability to continuously culture bloodstream forms of *T. vivax* under full axenic conditions still presents a severe hindrance to the accurate evaluation of new potential chemotherapeutic molecules with activities against these organisms. By establishing an *ex vivo* hypoxanthine assay for determination of the drug sensitivity of *T. vivax* with optimized assay duration, trypanosome concentration, and temperature parameters, the first reported set of novel compounds with activities against the bloodstream forms of *T. vivax* according to IC_{50}s indicating susceptibility has been achieved. Work is already under way to improve this *ex vivo* approach by establishing a stable and reproducible alamarBlue assay for the drug sensitivity of *T. vivax*, which will enhance the time efficiency and cost-effectiveness of the current *ex vivo* hypoxanthine test for drug susceptibility.

Diamidines are known to take 48 to 72 h to fully exert their biological and chemotherapeutic potency, so the decrease in the IC_{50}s for *T. congolense* IL-3000 obtained across the 40-, 48-, and 72-h alamarBlue assays was expected. This trend was similarly observed for the standard drugs diminazene (a diamidine) and isometamidium (an amidine). In comparison, the IC_{50}s for *T. congolense* STIB 736/IL-1180 determined in the *ex vivo* assays were lower than the IC_{50}s determined in the *in vitro* assays for. Both assays used incubation times of 40 h. The [^{3}H]hypoxanthine assay could be run only for

40 h, since at 48 and 72 h the parasites were no longer viable. Neither *T. congolense* STIB 736/IL-1180 nor *T. vivax* STIB 719/ILRAD 560 is adapted to axenic culture and thus can be maintained in culture medium only for up to 42 h. Nevertheless, the IC_{50}s for both STIB 736/IL-1180 and STIB 719/ILRAD 560 obtained at 40 h in the *ex vivo* assays correlated well with those obtained for *T. congolense* IL-3000 in the alamarBlue assay.

Since the target animals for an alternative chemotherapeutic agent for the treatment of nagana are ruminants, in particular, cattle, the desired drug candidate should be able to be administered effectively via the intramuscular (i.m.) route. In mouse models of infection, i.m. administration is rather cumbersome; therefore, an i.p. route of compound administration was used. Two of the standard drugs, diminazene and isometamidium, were assessed separately in established mouse models of *T. congolense* and *T. vivax* infection to determine their effectiveness. Once a reference profile for the standard drugs was established, the diamidine molecules were comparatively assessed for their curative potential on the basis of a 4-day consecutive treatment schedule. The ideal target product profile (TPP) of a new drug for the treatment of nagana should have an optimized treatment regimen, preferably with a single application, since a 4-day treatment schedule would be impractical for rural field settings. Consequently, the top 15 most efficacious diamidines identified in the 4-day treatment schedule in the *in vivo* mouse models were further examined by application of only a single bolus dose.

Special attention has to be given to the problem of cross-resistance to the standard drugs diminazene aceturate (a diamidine) and isometamidium (an amidine). New diamidines have to be able to overcome this cross-resistance. This could be shown by using the knockout line *T. brucei* AT1 (which is missing the transporter responsible for the uptake of many diamidines), which showed a level of sensitivity to several diamidines comparable to that of a reference *T. b. rhodesiense* strain and a drug-sensitive *T. evansi* strain (13). The use of drug-resistant *T. congolense* and *T. vivax* isolates should be envisaged for any further studies with diamidine molecules.

In summary, the process described here highlights that the following compounds are potential candidates for evaluation in preclinical studies as treatments for infections caused by (i) both *T. congolense* and *T. vivax* trypanosome species (DB 75, DB 820, DB 829, DB 1406, 19 DAP 025, 28 DAP 010, and 13 SAB 089), (ii) *T. congolense* only (DB 867, DB 1854, DB 1870, DB 1893, 17 SAB 085 and 32 DAP 022), and (iii) *T. vivax* only (12 SAB 081 and 18 SAB 023). Having identified several lead diamidines in this study, the next step will be to investigate these compounds in a ruminant (e.g., goat) model of infection to assess their viability as candidates for the clinical treatment of *T. congolense* and *T. vivax* infections. Cross-resistance should also be investigated by employing drug-resistant isolates of *T. congolense* and *T. vivax*.

MATERIALS AND METHODS

Trypanosome stocks. The IL-3000 *T. congolense* strain was originally derived from the Trans Mara I strain, which was isolated from a bovine (within the Trans Mara region of Kenya) in 1966 (16). The IL-3000 derivative grows well as bloodstream forms in axenic culture and was thus used as the *T. congolense* reference strain in all *in vitro* drug sensitivity assays in this study. The STIB 736/IL-1180 *T. congolense* strain is a clone originally derived from the STIB 212 *T. congolense* strain, which was isolated from a lion in the Serengeti National Park of Tanzania in 1971 (17). The STIB 736/IL-1180 strain was used for all *ex vivo* and *in vivo* experiments performed with *T. congolense* in this study. Both *T. congolense* strains used in this study belong to the savannah subgenotype family. The STIB 719/ILRAD 560 *T. vivax* strain originated from the Y486 *T. vivax* strain, isolated from a naturally infected bovine in 1976 in Zaria, Nigeria (18). The Y486 *T. vivax* strain could be cultivated as bloodstream forms over a feeder layer (19), which is not appropriate for drug-screening purposes. Axenic cultivation is still not possible today. To our knowledge, strains derived from the *T. vivax* Y486 strain are the only *T. vivax* strains that can be successfully propagated in rodent models and are representative of West African *T. vivax* strains. The STIB 719/ILRAD 560 *T. vivax* strain was therefore used in all *ex vivo* and *in vivo* experiments carried out in this study.

Mice. Female NMRI mice weighing between 19 and 22 g were used for all *in vivo* experiments. Mice were specific pathogen free (SPF) and were housed in standard Macrolon type II cages at 22°C with a relative humidity of 60 to 70%. The mice received pelleted food and water *ad libitum*. All *in vivo* experiments were carried out in compliance with the regulations set out by the Swiss Federal Veterinary Office.

Standard trypanocidal drugs. Diminazene aceturate (catalog number D-7770; Sigma, St. Louis, MO, USA), isometamidium chloride (Trypamidium-Samorin; Merial, France), and homidium chloride (No-

vidium; Merial, France) were used as the standard trypanocidal drugs in the *in vitro*, *ex vivo*, and *in vivo* experiments performed in this study.

Diamidine test compounds. All the diamidine test compounds investigated had previously been synthesized in the laboratories of David W. Boykin (Georgia State University, Atlanta, GA, USA) and Richard R. Tidwell (University of North Carolina, Chapel Hill, NC, USA) with the aim of obtaining structural diversity, chemical stability, and a low cost of goods. For the *in vivo* experiments evaluating the activities of the diamidine test compounds against *T. congolense* and *T. vivax*, the diamidine test compounds were selected according to their previously demonstrated *in vivo* efficacies against *T. brucei*-related species and their absence of acute toxicity in previous experiments (8, 12). All selected compounds showed greater than 75% *in vivo* efficacy against *T. b. rhodesiense* or *T. evansi* and no acute *in vivo* toxicity at cumulative doses of up to 100 mg/kg of body weight given intraperitoneally (i.p.); an exception to this was the parent compound DB 75, where acute toxicity in mice was seen at a cumulative dose of 20 mg/kg of body weight given i.p.

Culture media. Bloodstream-form trypanosomes of *T. congolense* (IL-3000) were cultured in Iscove's modified Dulbecco's medium (IMDM; catalog number I3390; Sigma, St. Louis, MO, USA) supplemented with 3 g/liter $NaHCO_3$ and 200 mM L-glutamine. The medium was then further supplemented by adding 1% of a 1.2 mM stock of 2-mercaptoethanol, 1% of a stock consisting of 5 mM bathocuproindisulfate, 150 mM L-cysteine HCl, 100 mM pyruvate, 50 mM hypoxanthine, 16 mM thymidine, and 20% heat-inactivated bovine serum. The complete medium was used for *T. congolense* (IL-3000) cultivation, as well as for all *in vitro* antitrypanosomal assay procedures. Bloodstream-form trypanosomes of non-culture-adapted *T. congolense* (STIB 736/IL-1180) and *T. vivax* (STIB 719/ILRAD 560) were supported in IMDM (catalog number I3390; Sigma, St. Louis, MO, USA) supplemented with 3 g/liter $NaHCO_3$. The medium was then further supplemented by adding 1% of a stock consisting of 5 mM bathocuproindisulfate, 150 mM L-cysteine HCl, 100 mM pyruvate, 16 mM thymidine, 200 mM L-glutamine, and 20% heat-inactivated bovine serum. The complete medium was used for all *ex vivo* [^{3}H]hypoxanthine incorporation assays with *T. congolense* and *T. vivax*.

Radioactive hypoxanthine. Radioactively labeled hypoxanthine ([8-^{3}H]hypoxanthine; catalog number TRK74; Amersham Biosciences UK Limited, Buckinghamshire, United Kingdom) was used for the *ex vivo* [^{3}H]hypoxanthine (40-h) incorporation assays with *T. congolense* and *T. vivax*.

Stock solutions and dilutions. A 10-mg/ml stock solution was prepared for each compound (dissolved in 100% dimethyl sulfoxide [DMSO]) and was stored frozen at −20°C. From these stock solutions, further stock solutions and compound dilutions were made for use in the various *in vitro T. congolense* cell viability assays and the *T. congolense* and *T. vivax ex vivo* incorporation assays using the appropriate culture medium as a solvent. Compound dilutions were prepared fresh on the day of the respective assays. For the *in vivo* mouse experiments, a 10-mg/ml stock solution was similarly prepared for each diamidine test compound, which was dissolved in sterile distilled water, containing 10% DMSO. Further dilutions depending on the dose being tested were made from these stock solutions. Stock solutions and dilutions of the standard trypanocidal drugs were prepared in sterile distilled water. All stock solutions and dilutions for the *in vivo* mouse experiments were made fresh on the day of administration and for each individual *in vivo* experiment.

***In vitro* antitrypanosomal assay.** The IC_{50}s of the test compounds for *T. congolense* (IL-3000) were determined using the alamarBlue assay (20), but with modified incubation times of 40, 48, and 72 h. Trypanosome densities were calculated using a cell counter and analyzer system (CASY; Schärfe System, Reutlingen, Germany), and the trypanosomes were diluted accordingly. Trypanosome seeding densities of 2×10^5/ml, 1×10^5/ml, and 1×10^5/ml in culture medium were used for the 40-, 48-, and 72-h alamarBlue assays, respectively. All assay plates were incubated at 34°C with 5% CO_2 for the time period being tested (24, 44, and 68 h), before the plates were removed from the incubator and 10 μl of resazurin dye (12.5 mg in 100 ml phosphate-buffered saline; catalog number 33934; Aldrich/Fluka, Buchs, Switzerland) was added to each well. The plates were then further incubated for 16, 4, and 4 h respectively, under the same conditions described above. Thereafter, the assay plates were read using a fluorescence reader (SpectraMax, Gemini XS; Bucher Biotec, Basel, Switzerland) at excitation and emission wavelengths of 536 and 588 nm, respectively. The data generated were analyzed using SOFTmax Pro software (version 5.2) to determine the IC_{50}s. All *in vitro* experiments were performed in duplicate in three independent assay runs for each compound.

***Ex vivo* [^{3}H]hypoxanthine incorporation assay.** The exact procedure for the *ex vivo* [^{3}H]hypoxanthine incorporation assay has been described previously (15) but was slightly modified for use in this study. Briefly, 50 μl of culture medium containing no hypoxanthine was added to each well of a 96-well microtiter plate, except for the first two and last two wells of the last column (to which 100 μl was added instead to act as a negative control) and all the wells in the first column. The drugs were applied at 75-μl volumes (containing two times the highest drug concentration) into the empty wells of the first column, corresponding to the required starting concentration of each drug being tested. Thereafter, 25-μl volumes were removed from the first column using a multichannel pipette and mixed with the contents in the wells in the next column. Again, 25 μl was removed from the second column and placed into the next column, and the contents were mixed several times. This step was repeated until the 11th column was reached. The final 25 μl from this 11th column was then discarded. This process created a 3-fold serial drug dilution across the microtiter plate.

Cardiac puncture of a highly parasitemic NMRI (female) mouse that had previously been infected with the corresponding *T. congolense* or *T. vivax* strain was performed. The blood collected was then mixed with phosphate-buffered saline with glucose (PSG; 6:4) in a 1:2 ratio, and the mixture was centrifuged for 12 min at 70 × *g* to separate the blood cells from the trypanosomes. After centrifugation,

the supernatant containing the trypanosomes was carefully transferred to a fresh tube, and the trypanosome concentration was determined using a Neubauer chamber. The trypanosome density was adjusted to provide starting concentrations of 2×10^6/ml and 2×10^5/ml for *T. congolense* and *T. vivax*, respectively. A 50-μl volume of this trypanosome suspension was then added to all 96 wells, with the exception of the 4 negative-control wells in the last column. The plates were then incubated in a humidified atmosphere containing 5% CO_2 at 34°C for *T. congolense* or 37°C for *T. vivax*. After 24 h of incubation, the plates were removed, and a solution of 1 μCi of radioactive hypoxanthine in 20 μl of culture medium was placed into each well. The plates were returned to the incubator for a further 16-h incubation period under the same conditions described above. After a complete incubation time of 40 h, the plates were removed and the contents of the wells were harvested on glass fiber filters using a 96-well harvester (model 1290-004 Betaplate; Berthold Technologies GmbH, Regensdorf, Switzerland). Thereafter, the radioactivity counts were measured using a liquid scintillation counter (model 1205 Betaplate; Berthold Technologies GmbH, Regensdorf, Switzerland). The data obtained were further analyzed by transferring them into a standard operating protocol template in a graphics program (Microsoft Excel) for determination of the IC_{50}s. All *ex vivo* experiments were performed in duplicate in three independent assay runs for each compound.

***In vivo* mouse efficacy experiments.** NMRI (female) mice were arranged into groups of four before being independently infected with either 10^5 or 10^4 parasites in 0.25 ml of PSG in a ratio of 6:4 for *T. congolense* (STIB 736/IL-1180) or *T. vivax* (STIB 719/ILRAD 560), respectively. Infection in all experiments was performed from stabilated blood, stored frozen in liquid nitrogen, using the i.p. route. For all experiments of the efficacies of the compounds against *T. congolense*, a parasitemia of 10^6 per ml blood was allowed to develop over 168 h (7 days), before treatment was administered i.p. on days 7 to 10 postinfection. Comparatively, for all experiments of the efficacies of the compounds against *T. vivax*, a parasitemia of 10^6 per ml blood was allowed to develop over 72 h (3 days), before treatment was administered i.p. on days 3 to 6 postinfection. Thereafter, the level of parasitemia in the mice was monitored using a tail blood examination technique until day 60 posttreatment. This lengthy follow-up period posttreatment was carried out to account for any possible relapses during the experiments. Parasitemia was checked twice a week for the first month and then once per week for the remaining month. Thereafter, any surviving and aparasitemic mice were considered cured. Untreated (control) mice infected with *T. congolense* and *T. vivax* survived, on average, for 11 or 6 days postinfection, respectively.

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