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Article

In Vitro and *In Vivo* Trypanocidal Efficacy of Nitrofuryl- and Nitrothienylazines

Helena D. Janse van Rensburg, David D. N'Da,* and Keisuke Suganuma*

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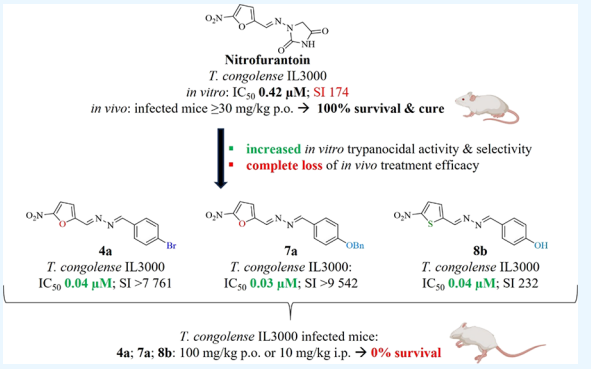
ABSTRACT: African trypanosomiasis is a vector-borne disease of animals and humans in the tsetse fly belt of Africa. *Trypanosoma congolense* ("nagana") is the most pathogenic trypanosome in livestock and causes high morbidity and mortality rates among cattle. In the absence of effective preventative vaccines, the management of trypanosomiasis relies on chemoprophylaxis and/or -therapy. However, the trypanocides in clinical use exhibit poor oral bioavailability and toxicity, and therapeutic failures occur because of resistant strains. Because nitrofurantoin displayed, in addition to its clinical use, promising antiparasitic activity, the current study was conducted to evaluate the *in vitro* trypanocidal activity and preliminary *in vivo* treatment efficacy of previously synthesized nitrofuranylazines. The trypanocidal activity of these nitrofuran derivatives varied among the evaluated trypanosome species; however, *T. congolense* strain IL3000 was more susceptible than other animal and human trypanosomes. The nitrofurylazines **4a** (IC_{50} 0.04 μM; SI > 7761) and **7a** (IC_{50} 0.03 μM; SI > 9542) as well as the nitrothienylazine **8b** (IC_{50} 0.04 μM; SI 232), with nanomolar IC_{50} values, were revealed as early antitrypanosomal leads. Although these derivatives showed strong trypanocidal activity *in vitro*, no *in vivo* treatment efficacy was observed in *T. congolense* IL3000 infected mice after both oral and intraperitoneal administration in a preliminary study. This was attributed to the poor solubility of the test compounds in the *in vivo* testing media. Indeed, a challenge in drug discovery is finding a balance between the physicochemical properties of a drug candidate, particularly lipophilicity and water solubility, and maintaining adequate potency to provide an effective dose. Hence, future chemical modifications may be required to generate lead-like to lead-like nitrofuranylazines that possess optimal physicochemical and pharmacokinetic properties while retaining *in vitro* and, ultimately, *in vivo* trypanocidal efficacy.



1. INTRODUCTION

Animal African trypanosomiasis (AAT, "nagana" in cattle) is a long-neglected tropical disease caused by multiple species and strains of the genus *Trypanosoma* (e.g., *T. brucei brucei* and *T. congolense*) that are cyclically transmitted by hematophagous tsetse flies (*Glossina* species) in sub-Saharan Africa.[1−6] These parasites cause a severe, often fatal disease in domestic animals including cattle, sheep, goats, and horses.[7] The same tsetse flies also transmit the causative agents of human African trypanosomiasis (HAT, "sleeping sickness"), namely, *T. b. gambiense* and *T. b. rhodesiense*.[2,3,8] Trypanosomiasis has plagued Africa for centuries,[9,10] in fact, during his extensive missionary travels and researches across Africa, David Livingstone noted that "fly disease" (as he called it) is a major hurdle to the development of the continent.[11] This observation was made in the 19th century and, unfortunately, still rings true today. Although the incidence of sleeping sickness has declined to less than 1000 reported cases per year (and eradication of the disease is earmarked for 2030),[12] nagana remains a threat to at least 50 million cattle that are at risk of infection.[13] Historically, Africa bore the greatest burden, but the disease has spread beyond the tsetse transmission zone, affecting animal husbandry in North and Northeast Africa, Latin America (except Chile), the Middle East, and Asia.[5,14] This is a result of the adaption of *T. evansi* parasites ("surra") to mechanical transmission by biting insects, such as horse (*Tabanus* species) and stable (*Stomoxys* species) flies,[4] and *T. equiperdum* parasites ("dourine") that are sexually transmitted among equines.[9,10,15]

The most pathogenic trypanosome for livestock is *T. congolense*, which contributes to high morbidity and mortality rates among cattle.[16] *T. congolense* parasites are divided into four subgroups, namely, Forest, Kilifi, Tsavo, and Savannah,[17] with the latter being the most virulent and clinically significant

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in cattle.[1,10] Notably, *T. congolense* IL3000 represents the Savannah subgroup.[18] Infected cattle are not suitable for meat and milk production (hence the name of the disease "N'gana" meaning "powerless/useless" in Zulu[19]), which hinder food security and eventually lead to socioeconomic problems.[16] Losses in terms of cattle production alone exceed USD 1 billion per year, whereas the total direct and indirect economic losses in terms of agricultural gross domestic product (GDP) are estimated at over USD 4 billion per year.[16] Therefore, the control of nagana is crucial to improving animal productivity.

Trypanosomes have developed mechanisms to continuously evade the host's immune system (while also preventing the host's premature death) by means of antigenic variation and the occurrence of intraspecies differences in variant surface glycoprotein (VSG) genes.[3,20] Despite the progress in vaccine development for *T. vivax* infection,[21] prevention through vaccination is not yet possible, and thus, disease management relies on vector control and chemoprophylaxis or -therapy.[21] Trypanocidal agents for ruminants, such as diminazene aceturate, isometamidium chloride, and homidium bromide (also known as ethidium bromide), have been in use since the 1950s and show poor oral bioavailability, toxicity, and therapeutic failures due to the presence of resistant strains.[10,16,22]

Recently, progress was made in the development of new therapeutics for sleeping sickness, with a new drug registered (fexinidazole, Figure 1) and another in advanced, late-stage

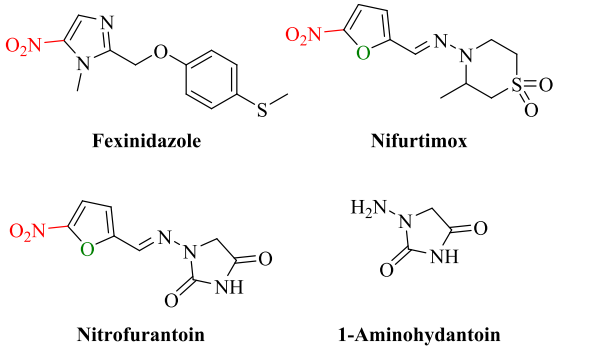

Figure 1. Structures of fexinidazole, nifurtimox, nitrofurantoin, and its azoreduction metabolite 1-aminohydantoin.

clinical trials (acoziborole).[8] Fexinidazole is the first orally active drug against both the peripheral and central nervous system stages of sleeping sickness caused by *T. b. gambiense*.[16] Similar to the other nitroheterocycles, fexinidazole is activated by parasite specific NADH-dependent nitroreductase I, a mitochondrial enzyme that catalyzes two-electron transfer, thereby reducing the nitro group to nitroso (−N═O), hydroxylamine (−NH−OH), and finally to amine ($-NH_2$).[8,23] Prodrugs like fexinidazole are considered magic bombs: once triggered by the transfer of electrons, it causes a redox cascade with haphazard, irreparable damage to any cell, but this is triggered inside the parasite only and not in the host.[23] Nitroheterocycles have been successfully used as oral drugs against different pathogens, including the causative agents of Chagas disease (*T. cruzi*) and sleeping sickness (*T. b. gambiense*). For example, nifurtimox (Figure 1) is used in the treatment of the second stage of sleeping sickness caused by *T. b. gambiense* as part of nifurtimox−eflornithine combination therapy, otherwise known as "NECT".[16] The potential toxicity phenomena associated with nitroheterocycles limited their use and even prohibited their inclusion in drug discovery programs. Nevertheless, the urgent need for novel trypanocidal agents and the approval of fexinidazole have led to renewed interest in these scaffolds.[24]

However, caution is advised not to develop too many nitroheterocycles against trypanosomatid diseases at once because the high dependency on prodrug activation by nitroreductase I can lead to drug resistance; thus, it is essential to determine the activation mechanism of new nitroheterocyclic compounds in the early stage of drug discovery.[24]

Interestingly, *Trypanosomas* are one of the very few genera that do not use the glutathione/glutathione reductase system for protection against oxidative stress but rather rely on trypanothione/trypanothione reductases.[25,26] Nitrofurans with aromatic and heterocyclic substituents have been found to inhibit *T. congolense* trypanothione reductase.[26]

Nitrofurantoin (Figure 1), characterized by a hydantoin ring with a nitro-substituted furanyl side chain, is administered orally for the prevention or treatment of urinary tract infections in humans as well as companion animals such as cats and dogs and, occasionally, horses (extra-label use).[27] Notably, the extra-label use of clinical nitrofurans in production animals is prohibited because of the residual effects (mutagenic, carcinogenic, and teratogenic) of these drugs.[28] The chemotherapeutic effects of clinical nitrofurans are linked to the 5-nitrofuran moiety, whereas the toxic properties (such as carcinogenicity and mutagenicity) are attributed to the azoreduction metabolite (1-aminohydantoin in the case of nitrofurantoin; Figure 1). Once cleaved off the parent drug, the azoreduction metabolite covalently binds to cellular proteins and persists *in vivo* for a certain time period.[29] Notably, 1-aminihydantoin was the least abundant clinical nitrofuran metabolite present in edible pig tissues, measured at 3−8 μg/kg, thus giving evidence of the short half-life of this metabolite.[30] Perhaps, a new compound that retains the 5-nitrofuran pharmacophore in its scaffold without the hydrazone side chain may hold the therapeutic benefit of nitrofurantoin and diminish its intolerable toxicity.

The design of bioisosteres is a creative approach to improve a molecule, including by enhancing potency, modulating physiochemical properties, addressing pharmacokinetic challenges, and reducing off-target liabilities.[31] As such, Saayman et al. (2023) incorporated classical bioisosteres, for example, ring equivalents (nitrofuran and nitrothiophene) and monovalent bioisosteres (such as Cl and Br), in the design of nitrofuranylazines **1a−9a** and **1b−9b** (Table 1).[32] Additionally, these nitrofuranylazines contain an azine (Table 1) instead of a hydrazone moiety (which is present in nifurtimox and nitrofurantoin; Figure 1). Hydrazones are characterized by the presence of an imine (C═N) linked to an amino (−NHR) moiety (−C═N−NHR), whereas azines have two "C−N" double bonds in conjugation with an "N−N" linker (−C═N−N═C−).[33] Both hydrazones and azines enhance electron communication between the substituting molecular fragments,[34] but azine tautomers were found to be more stable than hydrazone tautomers.[33] Additionally, azines can endow a molecule with apt chemical, physical, and biological properties.[33,35] N−N linked diamines have also demonstrated, among other, antibacterial,[36] antifilarial,[37] and anticancer[38] activities. Furthermore, Saayman and co-workers (2023) replaced the hydantoin ring of nitrofurantoin with an aromatic ring,[32] which offers various interaction modes with target proteins.[39] They

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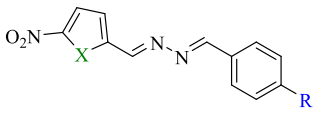
Table 1. Structures of Nitrofuryl- (1a−9a) and Nitrothienylazines (1b−9b)[a]



	X	R		X	R
1a	O	H	**1b**	S	H
2a	O	F	**2b**	S	F
3a	O	Cl	**3b**	S	Cl
4a	O	Br	**4b**	S	Br
5a	O	Me	**5b**	S	Me
6a	O	OMe	**6b**	S	OMe
7a	O	OBn	**7b**	S	OBn
8a	O	OH	**8b**	S	OH
9a	O	NO_2	**9b**	S	NO_2

[a]Me: methyl (CH_3); OMe: methoxy (OCH_3); OBn: benzyloxy ($OCH_2C_6H_5$).

hypothesized that linking nitrofuran or nitrothiophene and an aromatic ring via an azine bridge may well yield stable, safe, and biologically active molecules. The nitrofuran-based azines **4a**, **7a**, and **9a** (Table 1), respectively, showed the best antiamastigote activity against *Leishmania major* IR173 (**4a**: IC_{50} = 0.63 ± 0.02 μM; SI 159), *Leishmania donovani* 9515 (**7a**: IC_{50} = 0.25 ± 0.09 μM; SI 400), and *Trypanosoma cruzi* CL (**9a**: IC_{50} 0.78 ± 0.01; SI 21).[32]

Because these nitrofuranylazines showed activity against the causative agents of leishmaniasis and Chagas disease, which, like the African trypanosomes, are members of the Trypanosomatidae family, we evaluated the *in vitro* cytotoxic and trypanocidal activity of these previously synthesized nitrofuryl- (**1a−9a**) and nitrothienylazines (**1b−9b**) (Table 1) using mammalian cells and bloodstream forms of animal and human African trypanosomes, respectively, upon which we discussed the observed structure−activity relationships. Moreover, we assessed the preliminary *in vivo* treatment efficacy of hit drug candidates ($IC_{50} \leq 10$ μM; SI ≥ 10) against the animal trypanosome *T. congolense* in mice.

2. MATERIALS AND METHODS

2.1. Test Compounds. Nitrofuryl- (**1a−9a**) and nitrothienylazines (**1b−9b**) were previously synthesized by Saayman et al. (2023).[32] Test compounds were dissolved at a concentration of 10 mg/mL in dimethyl sulfoxide (DMSO) and stored at 4 °C until use.

2.2. *In Silico* Evaluation of Physiochemical and Pharmacokinetic Properties. The pharmacokinetics, lead-likeness, and medicinal chemistry friendliness of test compounds were predicted with the SwissADME web tool (http://www.swissadme.ch).[40]

2.3. *In Vitro* Evaluation of Cytotoxicity and Trypanocidal Activity. The cytotoxicity and trypanocidal activity of test compounds were determined *in vitro* using a 96-well plate format (with some modifications) previously described.[41,42] Madin−Darby bovine kidney (MDBK) cells were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. *T. b. brucei* GUTat3.1, *T. b. gambiense* IL1922, *T. b. rhodesiense* IL1501, *T. evansi* Tansui, and *T. congolense* IL3000 were provided by Dr. Hirumi. *T. equiperdum* IVM-t1 was established by Dr. K. Suganuma. Briefly, 50 μL of MDBK cells was cultured at a density of 1×10^4 cells/mL in a 96-well plate (Thermo Fisher Scientific) with 50 μL of the test compounds two times serially diluted at seven different concentrations (highest: 100 μg/mL; lowest: 0.39 μg/mL) and incubated at 37 °C for 72 h. Next, 10 μL of the Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) was added into each well, and the optical density (OD_{450}) before and after 4 h of incubation was measured at 450 nm using a GloMax-Multi+Detection System plate reader (Promega). Furthermore, 50 μL of bloodstream form trypanosomes was cultured at a density of 2.5×10^3 cells/mL (*T. b. brucei* GUTat3.1, *T. b. gambiense* IL1922, and *T. b. rhodesiense* IL1501), 1×10^4 cells/mL (*T. evansi* Tansui, *T. equiperdum* IVM-t1), or 1×10^5 cells/mL (*T. congolense* IL3000) in Nunc MicroWell 96-well optical bottom plates (Thermo Fisher Scientific, Waltham, MA, USA) using Hirumi's Modified Iscove's Medium-9, supplemented with 20% heat-inactivated fetal bovine serum,[43] exposed to 50 μL of various test compounds two times serially diluted at seven different concentrations (highest: 6.25 μg/mL; lowest: 0.008 μg/mL), and then incubated at 37 °C (33 °C for *T. congolense* IL3000) for 72 h. The condition of the trypanosomes was observed with phase-contrast microscopy after 72 h. Thereafter, 25 μL of the Cell-TiterGlo reagent (Promega, Madison, WI, USA) was added to each well, and bioluminescence was measured at 450 nm using a GloMax-Multi+Detection System plate reader (Promega). *In vitro* cytotoxicity and trypanocidal activity evaluations were replicated three times. The half-maximum cytotoxic concentration (CC_{50}, μM) of each test compound against MDBK cells and half-maximum inhibitory concentration (IC_{50}, μM) against trypanosomes were calculated by nonlinear regression (curve fit) using GraphPad Prism version 8 software (GraphPad, Inc., San Diego, CA, USA) and expressed as the mean ± standard deviation (SD).

2.4. *In Vivo* Evaluation of Treatment Efficacy. The experiment was approved by the Animal Ethics Committee of the Obihiro University of Agriculture and Veterinary Medicine (Approval No. 22-11). Healthy female 9 week old BALB/c mice (CLEA Japan, Inc., Tokyo, Japan) were used in this study. All mice had *ad libitum* access to normal chow and water. The virulent *T. congolense* IL3000 strain was propagated in a mouse and used for infection (these parasites were passaged once in the said mouse before the experiment). The experimental mice were intraperitoneally infected with 100 μL of *T. congolense* IL3000 in phosphate-buffered saline containing 10% glucose (PSG) at 1×10^3 cells/mouse. Mice were randomly allocated to four groups of two mice each as follows: group I served as the positive control (because both mice were infected but not treated), whereas group II (**4a**), III (**7a**), and IV (**8b**) mice were infected and treated with either 100 mg/kg of test compound **4a**, **7a**, or **8b** orally or 10 mg/kg of test compound **4a**, **7a**, or **8b** intraperitoneally. Treatment was initiated 4 days postinfection (d.p.i.) upon confirmation of parasitaemia by means of the wet smear technique and continued for 7 consecutive days. Treatments were newly prepared every day using 10% DMSO in corn oil for oral administration and 1% DMSO in phosphate buffered saline (PBS) for intraperitoneal administration. The number of trypanosomes in the peripheral blood (diluted to a suitable concentration with PSG) was assessed by using a cell counting chamber.

3. RESULTS

3.1. *In Silico* Evaluation of Physiochemical and Pharmacokinetic Properties. The pharmacokinetics, lead-

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Table 2. *In Vitro* Cytotoxicity and Trypanocidal Activity of Nitrofuryl- (1a−9a) and Nitrothienylazines (1b−9b)[e]

reference drug/compound #	cytotoxicity CC_{50} ± SD (μM)[a]	trypanocidal activity IC_{50} ± SD (μM)[b] (SI)[c]					
	MDBK	Tbb GuTat3.1	Tbg IL1922	Tbr IL1501	Teq IVM-t1	Tev Tansui	Tc IL3000
Nitrofurantoin							
	73.02 ± 36.15	1.51 ± 0.03 (48)	1.85 ± 0.23 (39)	1.69 ± 0.20 (43)	1.45 ± 0.03 (50)	1.67 ± 0.07 (44)	0.42 ± 0.05 (174)
Nitrofurylazines							
1a	11.59 ± 1.52	3.94 ± 2.08 (3)	4.00 ± 1.11 (3)	4.00 ± 1.09 (3)	3.49 ± 1.89 (3)	3.71 ± 2.04 (3)	0.19 ± 0.08 (61)
2a	33.92 ± 22.17	2.03 ± 0.95 (17)	2.48 ± 0.67 (14)	2.42 ± 0.40 (14)	2.46 ± 0.70 (14)	2.66 ± 1.02 (13)	0.18 ± 0.07 (188)
3a	>360.15	1.02 ± 0.43 (>353)	1.20 ± 0.24 (>300)	1.26 ± 0.15 (>286)	1.18 ± 0.17 (>305)	1.78 ± 0.46 (>202)	0.14 ± 0.05 (>2 572)
4a	>310.45	0.76 ± 0.35 (>408)	0.81 ± 0.20 (>383)	0.86 ± 0.23 (>361)	0.71 ± 0.18 (>437)	1.15 ± 0.16 (>270)	0.04 ± 0.01 (>7 761)
5a	>388.74	3.25 ± 1.94 (>120)	3.68 ± 1.43 (>106)	3.71 ± 1.30 (>105)	4.04 ± 0.53 (>96)	5.43 ± 1.08 (>72)	0.18 ± 0.05 (>2 160)
6a	41.14 ± 3.15	2.68 ± 1.37 (15)	3.43 ± 1.20 (12)	2.88 ± 0.51 (14)	3.15 ± 0.53 (13)	4.06 ± 1.50 (10)	0.22 ± 0.08 (187)
7a	>286.25	0.50 ± 0.35 (>573)	0.46 ± 0.07 (>622)	0.55 ± 0.10 (>520)	0.52 ± 0.09 (>550)	0.62 ± 0.04 (>462)	0.03 ± 0.01 (>9 542)
8a	18.44 ± 2.51	3.89 ± 2.14 (5)	5.47 ± 0.23 (3)	4.81 ± 1.10 (4)	5.01 ± 1.18 (4)	6.83 ± 2.66 (3)	0.20 ± 0.07 (92)
9a	24.56 ± 15.79	1.29 ± 0.38 (19)	1.50 ± 0.28 (16)	1.31 ± 0.04 (19)	1.24 ± 0.46 (20)	1.74 ± 0.74 (14)	0.17 ± 0.06 (144)
Nitrothienylazines							
1b	10.37 ± 1.97	0.30 ± 0.10 (35)	0.34 ± 0.01 (31)	0.35 ± 0.02 (30)	0.33 ± 0.09 (31)	0.42 ± 0.02 (25)	0.10 ± 0.04 (104)
2b	87.96 ± 66.83	0.46 ± 0.20 (191)	0.57 ± 0.08 (154)	0.59 ± 0.07 (149)	0.57 ± 0.11 (154)	0.67 ± 0.02 (131)	0.06 ± 0.03 (1 466)
3b	>340.45	0.84 ± 0.42 (>405)	1.04 ± 0.23 (>327)	0.95 ± 0.27 (>358)	1.23 ± 0.16 (>277)	1.34 ± 0.02 (>254)	0.14 ± 0.06 (>2 432)
4b	>295.70	0.82 ± 0.45 (>361)	1.01 ± 0.25 (>293)	0.94 ± 0.31 (>315)	1.04 ± 0.35 (>384)	1.83 ± 0.54 (>162)	0.12 ± 0.03 (>2 464)
5b	>365.88	2.09 ± 1.21 (>175)	2.50 ± 0.81 (>146)	2.07 ± 0.99 (>177)	3.39 ± 0.44 (>108)	5.17 ± 1.58 (>232)	0.35 ± 0.10 (>1 045)
6b	>345.65	1.05 ± 0.48 (>329)	1.29 ± 0.20 (>268)	1.30 ± 0.21 (>177)	2.10 ± 0.41 (>165)	2.65 ± 0.42 (>232)	0.22 ± 0.04 (>1 571)
7b	>273.67	4.88 ± 4.20 (>56)	4.60 ± 0.73 (>59)	4.10 ± 1.13 (>67)	10.51 ± 3.76 (>26)	12.30 ± 2.96 (>22)	0.28 ± 0.08 (>977)
8b	9.26 ± 2.36	0.33 ± 0.12 (28)	0.43 ± 0.14 (22)	0.37 ± 0.05 (25)	0.50 ± 0.09 (19)	0.61 ± 0.02 (15)	0.04 ± 0.01 (232)
9b	>328.64	0.91 ± 0.51 (>361)	0.97 ± 0.19 (>339)	0.93 ± 0.23 (>353)	1.01 ± 0.50 (>325)	1.41 ± 0.20 (>233)	0.06 ± 0.02 (>5 477)
Reference drugs[d]							
eflornithine	-	38.56 ± 9.88	36.66 ± 12.87	45.99 ± 17.07	-	57.21 ± 17.56	16.13 ± 2.93
nifurtimox	-	4.66 ± 19.70	4.58 ± 2.38	4.35 ± 1.59	-	2.62 ± 1.40	1.06 ± 0.22
pentamidine	-	0.041 ± 0.0023	0.014 ± 0.0031	0.029 ± 0.0062	0.0013 ± 0.0003	0.00097 ± 0.00019	0.33 ± 0.05
suramin	-	0.066 ± 0.0052	0.064 ± 0.0018	0.076 ± 0.011	0.038 ± 0.014	0.38 ± 0.058	7.17 ± 0.87
diminazene aceturate	-	-	-	-	0.011 ± 0.0029	-	0.109 ± 0.026

[a]Half-maximal cytotoxic concentration (IC_{50}, μM) represented as the mean ± standard deviation (SD), three biological replicates. [b]Half-maximal inhibitory concentration (IC_{50}, μM) represented as the mean ± standard deviation (SD), four biological replicates. [c]Selectivity index (SI): CC_{50} of MDBK/IC_{50} of trypanosome. [d]IC_{50} values obtained from the literature.[42,51,52]. [e]MDBK: Madin−Darby bovine kidney cells; Tbb: *Trypanosoma brucei brucei*; Tbg: *Trypanosoma brucei gambiense*; Tbr: *Trypanosoma brucei rhodesiense*; Teq: *Trypanosoma equiperdum*; Tev: *Trypanosoma evansi*; Tc: *Trypanosoma congolense*; qualifies as trypanocidal hit if $IC_{50} \leq 10$ μM and SI ≥ 10.[53]

likeness, and medicinal chemistry friendliness of nitrofuryl- (**1a−9a**) and nitrothienylazines (**1b−9b**) are summarized in Appendix A, Tables S1−3 and Figures S1−4.

The absorption and permeation of a drug upon oral administration are more likely when it has the following physiochemical properties: MW < 500, $\log P_{o/w} < 5$, N or O < 10, and NH or OH < 5.[44] Overall, the nitro-based azine derivatives **1a−9a** and **1b−9b** complied with these rules and were thought to be orally bioavailable. The polar surface area (PSA) of a drug that is transported by the transcellular route also influences oral absorption (PSA ≤ 120 Å) as well as its brain penetration (PSA 60−70 Å).[45] Based on the PSA of these azines, **1a−9a** and **1b−8b** were all expected to have high gastrointestinal (GI) absorption except for **9b** (PSA 144.60 Å). None of these azines (**1a−9a** and **1b−9b**) were predicted to permeate the blood−brain barrier (BBB) due to their relatively large PSA (83.68−144.60 Å). Additionally, if N + O < 5 and log *P* − (N + O) > 0, a drug has a greater probability of

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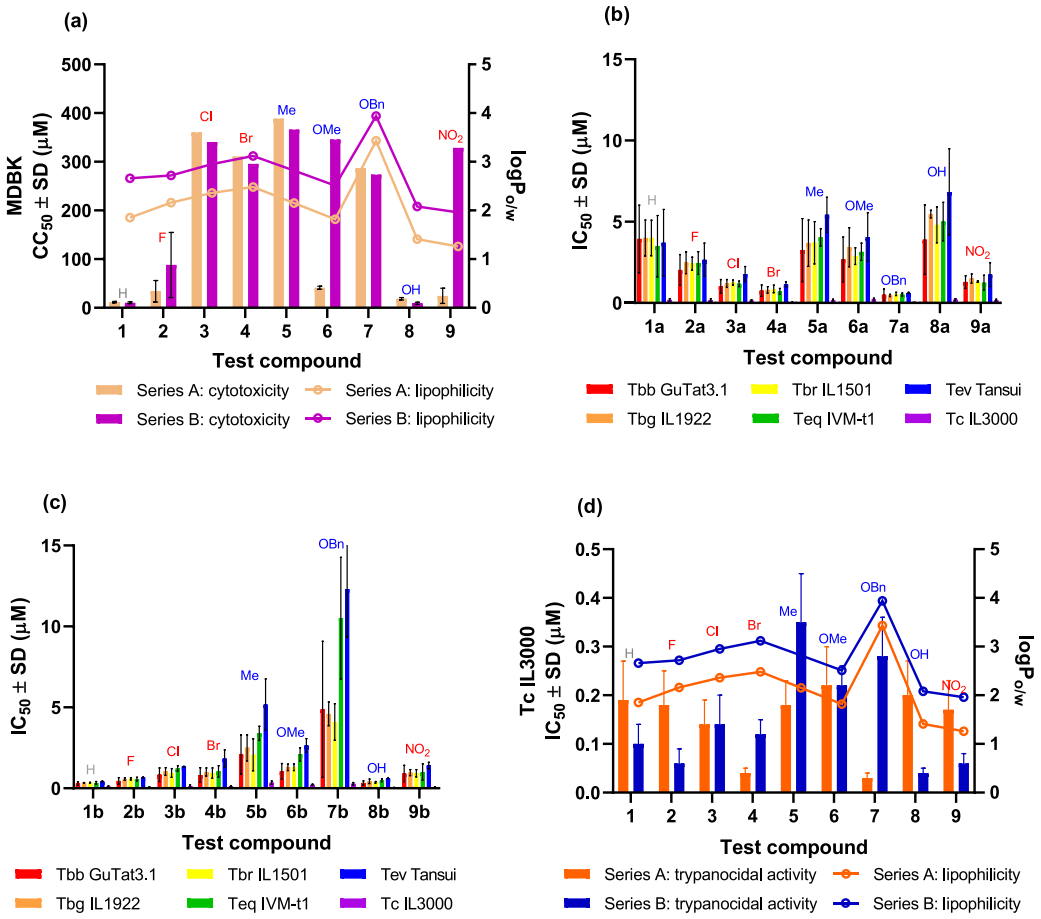

Figure 2. (a) Nitrofuran- (series A) and nitrothiophene-based (series B) azine derivatives' cytotoxicity (CC_{50} ± SD, μM) toward Madin−Darby bovine kidney (MDBK) cells. (b) Comparison of nitrofuryl- (**1a−9a**), and (c) nitrothienylazines' (**1b−9b**) trypanocidal activity (IC_{50} ± SD, μM) against *T. b. brucei* GuTat3.1 (Tbb GuTat3.1), *T. b. gambiense IL1922* (Tbg IL1922), *T. b. rhodesiense* (Tbr IL1501), *T. equiperdum* (Teq IVM-t1), *T. evansi* Tansui (Tev Tansui), and *T. congolense* IL3000 (Tc IL3000). (d) Comparison of series A and B's trypanocidal activity (IC_{50} ± SD, μM) against *T. congolense* IL3000 (Tc IL3000) and their lipophilicity ($\log P_{o/w}$). EWG (Br, Cl, F, NO_2) indicated in red, neutral group (H) in gray, and EDG (Me, OH, OMe, OBn) in blue.

entering the brain.[46] Azines **1a−9a** and **1b−9b** did not comply to either of these rules and thus were not expected to be active in the central nervous system (CNS). Interestingly, CNS penetration is now considered a requirement for therapeutics targeting sleeping sickness; however, this is not the case for nagana.[47]

All in all, derivatives **1a−9a** and **1b−8b** were considered drug-like (i.e., a molecule with the potential to be an oral drug with respect to bioavailability) according to five different rule-based filters.[44,48] Notably, there were no mentionable differences between the physiochemical properties of veterinary drugs and human drugs, with 85% of veterinary drugs falling within human drug-like parameters.[49] Furthermore, derivatives **2a−6a, 8a−9a, 1b, 6b,** and **8b−9b** were considered lead-like.[50] This concept is similar to drug-likeness but focuses on physicochemical properties that define a good lead, that is, a molecular entity suitable for optimization.[40]

3.2. *In Vitro* Evaluation of Cytotoxicity and Trypanocidal Activity. The cytotoxicity and trypanocidal activity of the reference drug nitrofurantoin and the nitrofuryl- (**1a−9a**) and nitrothienylazines (**1b−9b**) are summarized in Table 2.

3.2.1. Cytotoxicity. Nitrofurylazines **3a−5a** and **7a** showed low basal toxicity toward mammalian MDBK cells ($CC_{50} \geq 100$ μM),[54] whereas **1a−2a, 6a,** and **8a−9a** were moderately toxic (10 μM $\leq CC_{50} \leq$ 50 μM)[55,56] (Figure 2a). The nitrothienylazines **3b−7b** and **9b** also showed low toxicity,[54] whereas **2b** was weakly toxic (50 μM$\leq CC_{50} \leq$ 100 μM),[55] **1b** was moderately toxic,[55,57] and **8b** was highly toxic ($CC_{50} \leq$ 10 μM)[55,57,58] (Figure 2a). The nitrofuryl- **1a−9a** and **1b−2b** and nitrothienylazines **7b−8b** previously showed a similar toxicity profile against animal-derived Vero and human-derived THP-1 cells.[32]

3.2.2. Trypanocidal Activity. The synthesized azines **1a−9a** (Figure 2b) and **1b−9b** (Figure 2c) generally showed hit trypanocidal activity against the evaluated trypanosomes ($IC_{50} \leq$ 10 μM, SI ≥ 10),[53] with the best activity against the animal trypanosome *T. congolense* IL3000 (Figure 2d). Although **1a, 2a, 6a, 8a, 9a, 1b,** and **8b** presented with moderate to high toxicity toward mammalian cells, because of their relatively good activity against trypanosomes, these azines were deemed intrinsic as seen from favorable selectivity indices (SI ≥ 10) and thus qualify as trypanocidal hits despite their toxicity (Table 2). Compared to *T. congolense* (IC_{50} 0.03−0.35 μM), the trypanocidal activity of the azines decreased almost 10- to 35-fold across the board against the phylogenetically related *T. brucei* complex organisms (IC_{50} 0.30−5.47 μM) as well as *T. equiperdum* (IC_{50} 0.33−10.55 μM) *and T. evansi* (IC_{50} 0.42−12.30 μM). The best selective activity against *T. congolense* was

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shown by nitrofuranylazine **7a** closely followed by **4a**, also in the furan subseries, and nitrothienylazine **8b**. Although **8b** had an IC_{50} value comparable to that of **4a**, it was 33 times less selective toward *T. congolense* than **4a**. The nitrofuranylazine **1b** showed the best activity against *T. brucei* complex organisms, as well as *T. equiperdum* and *T. evansi* (IC_{50} 0.30−0.42 μM).

Notably, the nitrofuranylazines had solubility issues in both DMSO and growth medium, forming quickly separated suspensions that made uniform sampling challenging. This resulted in substantial standard deviations (SDs) for several test compounds (Figure 2).

3.3. *In Vivo* Evaluation of Treatment Efficacy. Group I (untreated) positive control mice showed high levels of parasitemia (Figure 3a) and died 9 and 8 d.p.i., respectively

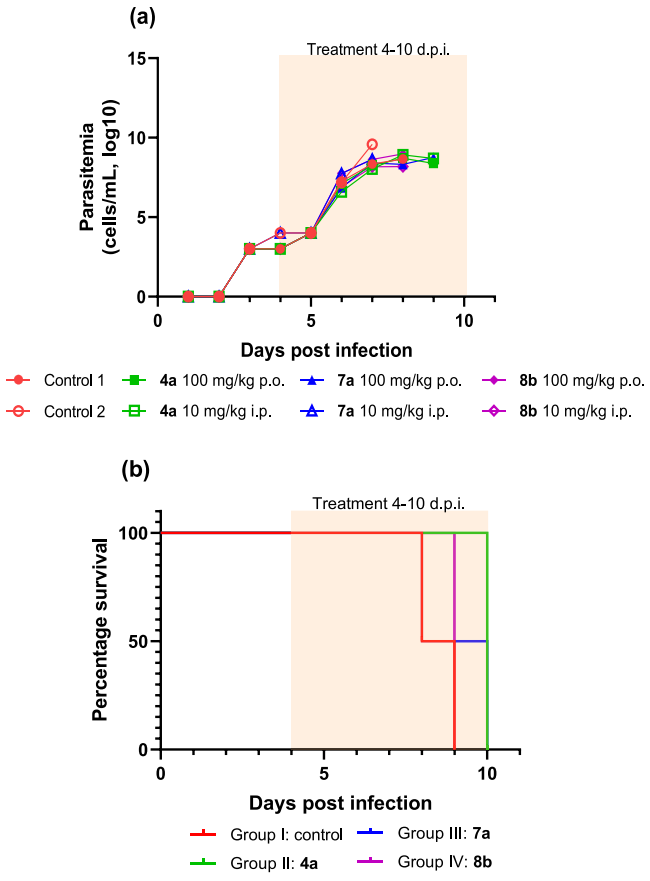

Figure 3. *In vivo* evaluation of treatment efficacy of nitrofuryl- (**4a**, **7a**) and nitrothienylazines (**8b**). (a) Parasitemia (cells/mL, log10) in mice infected with *T. congolense* IL3000 and left untreated (controls) or treated with either 100 mg/kg of **4a**, **7a**, or **8b** orally or 10 mg/kg of **4a**, **7a**, or **8b** intraperitoneally from 4 d.p.i. (b) Survival of mice infected with *T. congolense* IL3000 and left untreated (group I: control) or treated with **4a** (group II), **7a** (group III), or **8b** (group IV). Group II to IV mice died before treatment could be completed. d.p.i.: days postinfection; p.o.: *per os*; i.p. intraperitoneal.

(Figure 3b). Group II and group III mice treated with either 100 mg/kg of test compound **4a** or **7a** orally or 10 mg/kg of test compound **4a** intraperitoneally died 10 d.p.i., whereas the group III mouse treated with 10 mg/kg of test compound **7a** intraperitoneally died 8 d.p.i. (Figure 3b). Group IV mice treated with either 100 mg/kg of test compound **8b** orally or 10 mg/kg of test compound **8b** intraperitoneally died 9 d.p.i. (Figure 3b). The preliminary evaluation of the azines' *in vivo* treatment efficacy revealed no significant suppression of parasitemia (Figure 3a) and/or prolonged survival of mice in groups II to IV compared to group I (Figure 3b), and group II to IV mice all died before treatment could be completed. These results did not warrant any further *in vivo* studies using more mice per group.

4. DISCUSSION

The *in vitro* cytotoxic and trypanocidal activities of nitrofuryl- (**1a**−**9a**) and nitrothienylazines (**1b**−**9b**) were evaluated using mammalian cells and bloodstream forms of trypanosomes, respectively. Because the nitrofuranylazines showed the best trypanocidal activity against the animal trypanosome *T. congolense* IL3000 (Figure 2), structure−activity relationships (SARs) were observed relative to the said trypanosome. Differences in drug sensitivity may result from variances in the parasites' capacity to internalize,[59] activate,[60] and/or metabolize[61] drug candidates. Moreover, based on the promising *in vitro* trypanocidal activity and selectivity of derivatives **4a**, **7a**, and **8b** (Table 2), these azines were selected for *in vivo* treatment efficacy assessment in *T. congolense* IL3000 infected mice.

4.1. Structure−Activity Relationships. Lipophilicity is a key physiochemical property linking drug potency, toxicity, and pharmacokinetic properties such as water solubility and membrane permeability.[62] Thus, the SAR of the nitrofuranylazines was analyzed in relation to their predicted lipophilicity, or $\log P_{o/w}$, (and indirectly, the X and R substituents' electronic effect and strength) and trypanocidal activity.

Generally, the sulfur-containing nitrothiophenes (**1b**−**9b**: $\log P_{o/w}$ 1.96−3.94) were more lipophilic than the oxygen-containing nitrofurans (**1a**−**9a**: $\log P_{o/w}$ 1.26−3.43, Figure 2d) because the S atom in the thiophene ring is less electronegative, and consequently less polar, than the O atom in the furan ring.[63] However, this augmented lipophilicity did not always translate to superior activity; for example, the less lipophilic nitrofurylazines **4a**, **5a**, and **7a** exhibited more potent activity than their more lipophilic nitrothienyl counterparts **4b**, **5b**, and **7b** (Figure 2d). This suggests that other physical properties such as the electronic effect and strength of the R substituents on the aromatic ring may act as modulators of the trypanocidal activity of these compounds. Increased lipophilicity is generally associated with the improved permeation of a molecule through a biological membrane and thus improved biological activity,[64] exemplified by the thiophene derivatives **1b**−**2b** and **8b**−**9b** that showed increased lipophilicity and better activity than their furan counterparts **1a**−**2a** and **8a**−**9a** (Figure 2d). Additionally, Rando and colleagues (2008) suggested that because the sulfur's free d atomic orbitals in the nitrothienyl ring can accommodate more electrons than permitted by the octet theory, and it being adjacent to a nitro-linked carbon, these orbitals could accept the nitro group's electrons, thus leading to the increased susceptibility of the said group to reduction by parasite specific nitroreductase I, ultimately increasing trypanocidal activity.[65] Interestingly, azines **3a** and **3b** showed identical IC_{50} values (Figure 2a) and similar CC_{50} values (Figure 2d) even though the furan and thiophene heterocyclic synthons differ by their chalcogen atoms as well as their lipophilicity (**3a**: log*P* 2.36 versus **3b**: log*P* 2.95). Azines **6a** and **6b** also showed identical IC_{50} values and different log*P* values (Figure 2d); however,

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furan **6a** (log*P* 1.82) was moderately toxic toward mammalian cells, and thiophene **6b** (log*P* 2.51) showed low basal toxicity and high selective activity (Figure 2a). In this instance, higher lipophilicity was not associated with higher toxicity.[66]

The electronic effect and strength of the R substituents on the aromatic ring may be divided, in order of increasing strength, into a neutral group (H), electron-withdrawing groups (EWG: Br < Cl < F < NO_2), and electron-donating groups (EDG: Me < OH < OMe < OBn). The neutral nitrothiophene **1b** (R = H) showed greater lipophilicity and trypanocidal activity than the corresponding nitrofuran **1a** (Figure 2d), reiterating that the S atom in the thiophene ring and the O atom in the nitrofuran ring impacted lipophilicity[63] and, in turn, increased lipophilicity and improved potency.[67] Notably, these azines were both moderately toxic (10 μM ≤ CC_{50} ≤ 50 μM); however, toxicity toward mammalian cells generally decreased with substitution of the neutral hydrogen atom with either EWG or EDG (Figure 2a). For the EWG-containing nitrofuranylazines **2a**−**4a** and **9a**, lipophilicity decreased as the strength of the EWG increased (because the larger the difference in electronegativity between two atoms is, the more polar is the bond); expectedly, the activity decreased (Figure 2d) and, unexpectedly, toxicity increased with decreased lipophilicity (Figure 2a). For the EWG-containing nitrothienylazines **2b**−**4b** and **9b**, lipophilicity again decreased as the strength of the EWG increased, but contrary to their nitrofuran counterparts **2a**−**4a** and **9a**, activity increased (Figure 2d) and toxicity decreased (Figure 2a). For the EDG-containing azines (**5a**−**8a** and **5b**−**8b**), no conclusive SAR could be observed. However, the nitrofuranylazine **7a** containing the strongest EDG showed the highest lipophilicity and best trypanocidal activity (as well as low toxicity toward mammalian cells). Comparison of **7a** to the weaker nonpolar methoxy derivative **6a** and the polar hydroxy azine **8a** revealed that as the strength of the EDG increased, lipophilicity and trypanocidal activity increased, but cytotoxicity decreased. The corresponding nitrothiophene derivatives **8b**, **6b**, and **7b** showed a similar trend; as the strength of the EDG increased, lipophilicity again increased and cytotoxicity decreased, but in this series, trypanocidal activity decreased. The nitrothiophene derivative **8b** containing a polar hydroxy group showed the best trypanocidal activity of the nitrothienylazine subseries and, like its nitrofuran counterpart **8a**, the highest cytotoxicity (Figure 2d).

As summarized in Figure 4, when the R substituent was neutral and the X substituent's electron negativity (EN) increased (S < O), lipophilicity (represented by the log$P_{o/w}$ value) as well as trypanocidal activity decreased (in other words, the IC_{50} value increased). For both the nitrofuryl- and nitrothienylazines, lipophilicity decreased with an increased EWG strength. However, the nitrofuranylazines' trypanocidal activity decreased (exemplified by increased IC_{50} values) and cytotoxicity increased (exemplified by decreased CC_{50} values), whereas the nitrothienylazines' trypanocidal activity increased and cytotoxicity decreased. For both the nitrofuran and nitrothiophene derivatives, lipophilicity increased with increased EDG strength, and cytotoxicity decreased. However, contrasting effects were observed for the trypanocidal activity. Indeed, activity within the nitrofuranylazine subseries improved with an increased EDG strength, whereas that of the nitrothienylazines decreased.

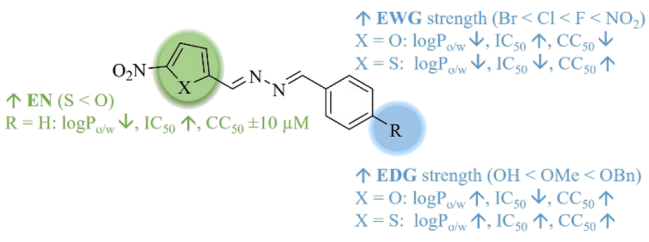

Figure 4. Summary of structure−activity relationships. ↑: increased; ↓: decreased; log$P_{o/w}$: log of partition coefficient of solute between octanol and water, indicator of lipophilicity; IC_{50}: half-maximum inhibitory concentration for trypanosomes; CC_{50}: half-maximum cytotoxic concentration for mammalian cells; EN: electron negativity; EWG: electron-withdrawing groups; EDG: electron-donating groups.

The discussed SAR demonstrated that, for the nitrofuranylazines at least, the relative electronic and lipophilic contributions of the chalcogen atom (O or S) and the electronic nature of the R substituent (EWG, neutral group, or EDG) to the entire structure influence trypanocidal activity and cytotoxicity; in other words, a single structural feature did not govern the potency of these azines. Although SARs were discussed with regards to *T. congolense* IL3000, similar observations were made for the *T. brucei* complex organisms as well as *T. equiperdum* and *T. evansi*.

4.2. Treatment Efficacy. The nitrofuryl- (**1a**−**9a**) and nitrothienylazines (**1b**−**9b**) exhibited better trypanocidal activity against *T. congolense* IL3000 than the clinical antibiotic nitrofurantoin. The azines **4a**, **7a**, **2b**, **8b**, and **9b** also displayed improved activity when compared to the commonly used veterinary trypanocide diamizene aceturate (IC_{50} 0.109 μM).[52] Diamizene aceturate is an aromatic diamidine consisting of two aminodiphenyl moieties linked by a triazene bridge, similar to the azine bridge present in the investigated test compounds. The nitrofurylazines **4a** and **7a** as well as the nitrothienylazine **8a** showed the most promising trypanocidal activity against *T. congolense* IL3000 of all of the test compounds and were selected for further investigation *in vivo*.

Previously, a 100% survival and cure were achieved with a dose of nitrofurantoin ≥30 mg/kg in *T. congolense* IL3000 infected mice,[68] and therefore, this preliminary data served as a benchmark to compare the *in vivo* treatment efficacy of the structurally related compounds **4a**, **7a**, and **8b**. Compared to nitrofurantoin, the test compounds **4a**, **7a**, and **8b** showed increased *in vitro* trypanocidal activity against *T. congolense* IL3000 and decreased toxicity against MDBK cells. However, this activity could not be translated to the *in vivo* treatment efficacy in *T. congolense* IL3000 infected mice after oral or intraperitoneal administration of the test compounds in a preliminary study. Notably, the oral dose of **4a**, **7a**,and **8b** (100 mg/kg) was three times higher than the minimum effective dose (30 mg/kg) of nitrofurantoin, yet no marked decrease in parasitemia was observed. Therefore, these azines were not further explored in additional *in vivo* studies with more mice per group.

This lack of efficacy is most probably due to the poor solubility of these compounds in *in vivo* testing media. The nitrofuranylazines were thought to be orally bioavailable based on Lipinski's "rule of five"; nevertheless, adhering to these rules is no guarantee that a molecule will be drug-like and that no oral bioavailability problems will be encountered.[69] In addition to their increased *in vitro* trypanocidal activity against *T. congolense* IL3000, these compounds also possessed increased lipophilicity compared with the reference drug. Lipophilicity

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impacts not only potency but also water solubility.[70] Nitrofurantoin had a $\log P_{o/w}$ value of −0.66, where the negative value signifies that nitrofurantoin has a higher affinity for the aqueous phase (it is more hydrophilic), whereas **4a** (2.48), **7a** (3.43), and **8b** (2.08) had positive $\log P_{o/w}$ values that denote a higher concentration in the lipid phase (more lipophilic). Generally, a molecule with a $\log P_{o/w}$ value between 3 and 5 displays high membrane permeability but low water solubility, leading to variable oral absorption *in vivo*.[62] The idea that better *in vitro* potency will lead to a more effective therapeutic is often embedded in early drug discovery schemes; however, to be a successful drug candidate, potent activity must be accompanied by desirable physicochemical properties.[71]

Munsimbwe and colleagues (2021) previously encountered a similar problem when they evaluated a series of *N*-alkyl analogues of nitrofurantoin against human and animal trypanosomes. They found that analogues containing 11- and 12-carbon aliphatic chains showed promising *in vitro* trypanocidal activity against *T. congolense* IL3000 (11: 0.011 ± 0.0035 μM, SI > 21,978; 12: 0.012 ± 0.0045 μM, SI > 21,322); however, *in vivo* experiments involving oral and intraperitoneal administration of the selected analogues showed no treatment efficacy.[51] Their results suggested that nitrofurantoin analogues with high hydrophilicity were required for *in vivo* experiments to determine if these analogues are promising leads for the development of trypanocides. Indeed, a significant challenge in drug discovery is finding a balance between the constraints of the physicochemical properties, such as lipophilicity and water solubility, of the drug candidate and maintaining adequate potency to provide an effective dose.[71]

Water is a ubiquitous solvent in chemistry and biology;[72] therefore, it is no surprise that the aqueous solubility of compounds plays a key role in various domains; for example, poorly soluble compounds not only create problems for *in vitro* and *in vivo* assays in drug discovery but also place a significant burden on drug development.[73] Traditionally, low solubility was considered a drug development issue, and it was addressed in a later phase by pharmaceutical scientists through formulation approaches.[69] Undeniably, drug candidates with insufficient solubility have a higher risk of attrition and lead to higher costs during the drug development phase.[73]

Because derivatives **2a**−**6a**, **8a**−**9a**, **1b**, **6b**, and **8b**−**9b** are considered lead-like, that is, molecular entities suitable for optimization,[50] desirable physiochemical properties (like high water solubility) may be attained by chemical modifications, such as disruption of molecular planarity and symmetry to reduce crystal packing and/or introducing solubilizing groups at certain positions on the core structure.

5. CONCLUSIONS

In view of the antiparasitic activity of the nitrofuranylazines against *Leishmania* species as well as against the causative agent of American trypanosomiasis (or Chagas disease), namely, *T. cruzi*, the current study was conducted to evaluate the *in vitro* trypanocidal activity and *in vivo* treatment efficacy of these derivatives against African trypanosomes. Nitrofuryl- (**1a**−**9a**) and nitrothienylazines (**1b**−**9b**) generally showed hit trypanocidal activity against the evaluated trypanosomes ($IC_{50} \leq 10$ μM, SI ≥ 10), with the highest activity against the animal trypanosome *T. congolense* IL3000. The best activity against *T. congolense* was achieved by nitrofurylazine **7a** closely followed by **4a**, also in the furan subseries, and nitrothienylazine **8b**. Although derivatives **4a**, **7a**, and **8b** showed strong trypanocidal activity *in vitro*, no *in vivo* treatment efficacy was observed in *T. congolense* IL3000 infected mice after both oral and intraperitoneal administration in a preliminary study. This was attributed to the poor solubility of the test compounds in the *in vivo* testing media. Poorly soluble compounds not only complicate *in vitro* and *in vivo* assays in drug discovery but also place a significant burden on drug development. Hence, chemical modifications to lead-like nitrofuryl- (**2a**−**6a** and **8a**−**9a**) and nitrothienylazines (**1b**, **6b**, and **8b**−**9b**) may optimize the physicochemical and pharmacokinetic properties while retaining *in vitro* and, ultimately, *in vivo* trypanocidal efficacy.

HIGHLIGHTS

- Synthesized nitrofuranylazines were evaluated against various trypanosome species.
- *T. congolense* trypanosomes were more susceptible to nitrofuranylazines than others
- Nitrofuranylazines were more active *in vitro* than nitrofurantoin.
- No *in vivo* treatment efficacy was observed in *T. congolense* infected mice.
- Poor water solubility impeded the efficacy of the promising azines.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06508.

Pharmacokinetics, lead-likeness, and medicinal chemistry friendliness of nitrofuryl- and nitrothienylazines (PDF)

AUTHOR INFORMATION

Corresponding Authors

David D. N'Da − *Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa*; Email: david.nda@nwu.ac.za

Keisuke Suganuma − *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan*; orcid.org/0000-0001-5809-8811; Email: k.suganuma@obihiro.ac.jp

Author

Helena D. Janse van Rensburg − *Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa*; orcid.org/0000-0001-5181-9428

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06508

Author Contributions

Keisuke Suganuma: Conceptualization; Methodology; Formal analysis and investigation; Writing - review and editing; Funding acquisition; Resources. David D. N'Da: Conceptualization; Methodology; Formal analysis and investigation; Writing - review and editing; Funding acquisition; Resources. Helena D. Janse van Rensburg: Formal analysis and

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investigation; Writing - original draft preparation; Writing - review and editing. K. Suganuma and D.D. N'Da contributed equally to this work.

Notes

The authors declare no competing financial interest.

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