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In vitro anti-trypanosomal activity of synthetic nitrofurantoin-triazole hybrids against *Trypanosoma* species causing human African trypanosomosis

Anna Seetsi[1] | **David D. N'da**[2] | **Nthatisi Molefe-Nyembe**[3] | **Keisuke Suganuma**[4] | **Tsepo Ramatla**[1] | **Oriel Thekisoe**[1]

[1]Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

[2]Centre of Excellence for Pharmaceutical Sciences (PHARMACEN), North-West University, Potchefstroom, South Africa

[3]Department of Zoology and Entomology, University of the Free State, Phuthaditjhaba, South Africa

[4]OIE Reference Laboratory for Surra, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

Correspondence
Tsepo Ramatla, Unit for Environmental Sciences and Management, North-West University, Potchefstroom 2521, South Africa.
Email: ra21205450@gmail.com; 21205450@mynwu.ac.za

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[Correction added on 9 August 2023, after first online publication: The middle initial of the second author was added in this version.]

Abstract

Human African trypanosomosis (HAT) which is also known as sleeping sickness is caused by *Trypanosoma brucei gambiense* that is endemic in western and central Africa and *T. b. rhodesiense* that is endemic in eastern and southern Africa. Drugs used for treatment against HAT first stage have limited effectiveness, and the second stage drugs have been reported to be toxic, expensive, and have time-consuming administration, and parasitic resistance has developed against these drugs. The aim of this study was to evaluate the anti-trypanosomal activity of nitrofurantoin-triazole hybrids against *T. b. gambiense* and *T. b. rhodesiense* parasites in vitro. This study screened 19 synthesized nitrofurantoin-triazole (NFT) hybrids on two strains of human trypanosomes, and cytotoxicity was evaluated on Madin-Darby bovine kidney (MDBK) cells. The findings in this study showed that an increase in the chain length and the number of carbon atoms in some n-alkyl hybrids influenced the increase in anti-trypanosomal activity against *T. b. gambiense* and *T. b. rhodesiense*. The short-chain n-alkyl hybrids showed decreased activity compared to the long-chain n-alkyl hybrids, with increased activity against both *T. b. gambiense* and *T. b. rhodesiense*. Incorporation of additional electron-donating substituents in some NFT hybrids showed increased anti-trypanosomal activity than to electron-withdrawing substituents in NFT hybrids. All 19 NFT hybrids tested displayed better anti-trypanosomal activity against *T. b. gambiense* than *T. b. rhodesiense*. The NFT hybrid no. 16 was among the best performing hybrids against both *T. b. gambiense* (0.08 ± 0.04 μM) and *T. b.rhodesiense* (0.11 ± 0.06 μM), and its activity might be influenced by the introduction of fluorine in the para-position on the benzyl ring. Remarkably, the NFT hybrids in this study displayed weak to moderate cytotoxicity on MDBK cells. All of the NFT hybrids in this study had selectivity index values ranging from 18 to greater than 915, meaning that they were up to 10–100 times fold selective in their anti-trypanosomal activity. The synthesized NFT hybrids showed strong selectivity >10 to *T. b. gambiense* and *T. b. rhodesiense*, which indicates that they qualify from the initial selection criteria for potential hit drugs.

Abbreviations: HAT, human African trypanosomosis; NFT, nitrofurantoin; MDBK, madin-darby bovine kidney; CNS, central nervous system; DMEM, dulbecco's modified eagle's medium; HI-FBS, heat-inactivated fetal bovine serum; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; IC50, 50% inhibition concentration of the cells/parasites; CCK-8, Cell Counting Kit-8.

KEYWORDS
anti-trypanosomal activity, nitrofurantoin-triazole hybrids, *T. b. rhodesiense*, *Trypanosoma brucei gambiense*

1 | INTRODUCTION

Human African trypanosomosis (HAT), also known as sleeping sickness, is caused by *Trypanosoma brucei* sub-species parasites transmitted by flies of the genus *Glossina* in sub-Saharan Africa [1]. *Glossina fuscipes* and *G. palpalis* are the main vectors for transmission of HAT [2]. There are two forms of HAT: chronic western and central African HAT caused by *T. b. gambiense*, whereby symptoms develop from months to years before the first stage progresses to the second stage of the central nervous system (CNS), before death [3]. The second type is the acute eastern and southern African HAT caused by *T. b. rhodesiense* infection that takes weeks to months from the first stage to the second stage of the disease prior to death [4].

Treatment of HAT following a positive diagnosis relies solely on chemotherapy [5]. Clinical anti-HAT drugs have been associated with major challenges [6], disadvantages [7], and limitations [8], leading to therapeutic failures [9]. Pentamidine and suramin, which are drugs used to treat the first stage of HAT, have limited effectiveness against the first stage of *T. b. gambiense* and *T. b. rhodesiense* [5]. The second-stage drugs eflornithine and melarsoprol have been reported to have toxic effects and be ineffective against *T. b. rhodesiense*; additionally, they are expensive, involve time-consuming parenteral administration, and most importantly, increase parasitic resistance [10]. Therefore, there is an urgent need for the development of new trypanocides. However, developing effective trypanocides is a daunting task as it depends on the *Trypanosoma* sub-species and the diagnostic stage of the disease [11].

Drugs containing nitrogen functional groups have long been used in medicinal chemistry and have been reported to be effective against diseases such as cancer, leishmaniasis, malaria, trypanosomosis, and tuberculosis [12]. Nitroaromatic or nitroheteromatic analogs induce therapeutic effects through diverse mechanisms of the bioreduction activation process [13]. There has been a modification of these analogs to enhance efficacy and reduce toxic effects. Nitrofurantoin is one of the most widely used nitrofurans that possess antimicrobial activity. It is commonly used to treat cystitis, kidney infections, and urinary tract infections [14].

Synthesis and modification of derivatives are crucial to obtaining drugs with favorable pharmacokinetic and pharmaceutical properties. Different types of candidate drugs with similar structure and functionality are used to investigate their mode of action and wide range of biological activity. There is a continuous need to develop efficient antitrypanosomal drugs for HAT that are cost effective, safe and can also cater for non-parenteral/oral administration. In this study, synthesized nitrofurantoin-triazole (NFT) hybrids [15] were tested against *T. b. gambiense* and *T. b. rhodesiense* parasites in vitro.

2 | MATERIALS AND METHODS

2.1 | Chemistry

A series of NFT hybrids containing nitrofuran and 1,2,3-triazole pharmacologically active scaffolds were synthesized in low to high yields (30%–80%), as described by Zuma et al. [15]. The NFT hybrids used in this study are shown in Table 1 together with their chemical structures.

2.2 | In vitro cultivation of mammalian cells

Madin-Darby bovine kidney cells (MDBK; NBL-1) were cultured and maintained in GIT medium supplemented with Dulbecco's modified eagle's medium (DMEM; Sigma Aldrich, Tokyo, Japan), 20% heat-inactivated fetal bovine serum (HI-FBS), and 1% penicillin streptomycin (Thermo Fisher Scientific, Yokohama, Japan). The cells were incubated at 37°C in 5% CO2, and the medium was replaced with a fresh medium every 3 to 4 days. When cells were approximately 80% confluent, they were passaged by removing the medium from the cell culture flask and washed once with 5 mL phosphate-buffered saline (PBS); 250 μL of pre-heated trypsin was added to dislodge adherent cells from the cell culture flask with a 5 mL pipette, then incubated for 1 min at 37°C. The cell suspension was completely sucked out from the cell culture flask, and approximately 11 mL of fresh medium was added to the cells as previously described by Nefertiti et al. [16].

2.3 | In vitro cultivation of parasites

Bloodstream forms of *T. b. gambiense* (IL1923) and *T. b. rhodesiense* (IL2343) were cultivated in HMI-9 medium supplemented with Iscove's modified Dulbeccos's medium (IMDM: Sigma-Aldrich, Tokyo, Japan), 20% HI-FBS, 0.1 mM bathocuproine sulfonic acid (Sigma-Aldrich, Tokyo, Japan), 1% penicillin streptomycin (Thermo Fisher Scientific, Yokohama, Japan), 1 mM pyruvic acid sodium salt, 2 mM L-cysteine (Sigma-Aldrich, Tokyo, Japan), 1 mM sodium

TABLE 1 The structural composition of synthesized nitrofurantoin-triazole hybrids.

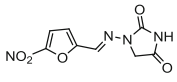
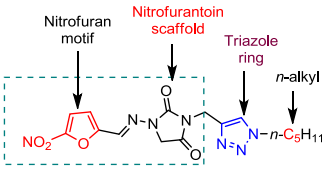
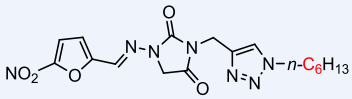
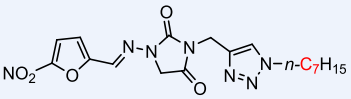
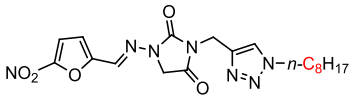
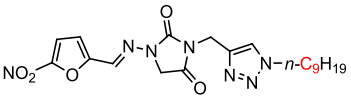
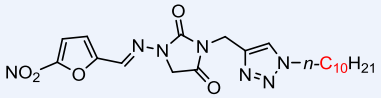
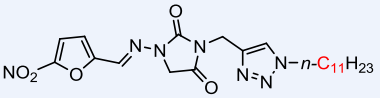
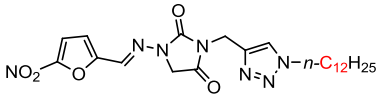
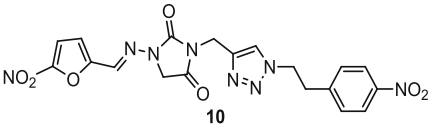
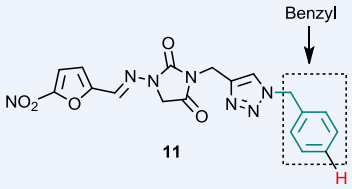
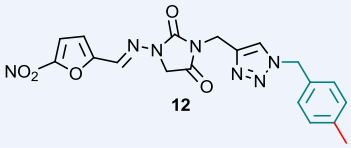
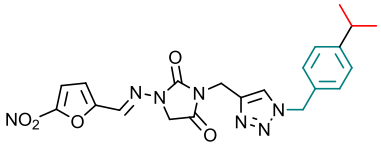
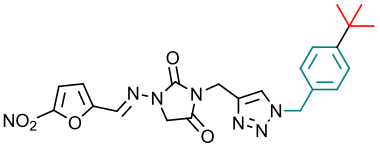
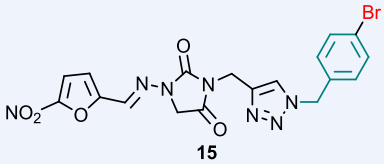
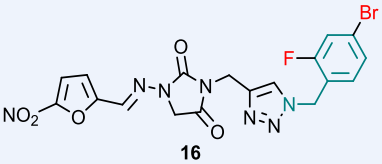
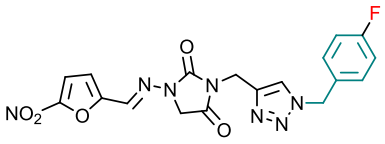
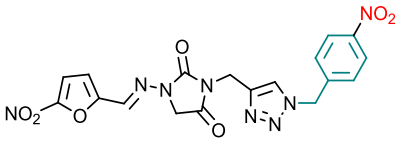
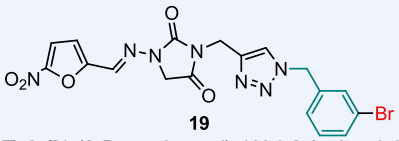
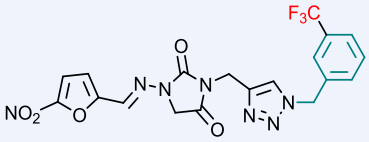
Chemical structure	Chemical structure
 1 - Nitrofurantoin (*E*)-1-{[(5-Nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 2 (*E*)-1-{[(5-Nitrofuran-2-yl)methylene]amino}-3-[(1-pentyl-1*H*-1,2,3-triazol-4-yl)methyl]imidazolidine-2,4-dione
 3 (*E*)-3-[(1-Hexyl-1*H*-1,2,3-triazol-4-yl)methyl]-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 4 (*E*)-3-[(1-Heptyl-1*H*-1,2,3-triazol-4-yl)methyl]-1-{[(5-nitrofuran −2-yl)methylene]amino}imidazolidine-2,4-dione
 5 (*E*)-1{([(5-Nitrofuran-2-yl)methylene]amino}-3-[(1-octyl-1*H*-1,2,3-triazol-4-yl)methyl]imidazolidine-2,4-dione	 6 (*E*)-1-{[(5-Nitrofuran-2-yl)methylene]amino}-3-[(1-nonyl-1*H*-1,2,3-triazol-4-yl)methyl]imidazolidine-2,4-dione
 7 (*E*)-3-[(1-Decyl-1*H*-1,2,3-triazol-4-yl)methyl]-1-{[(5-nitrofuran-2-yl) methylene]amino}imidazolidine-2,4-dione	 8 (*E*)-1-{[(5-Nitrofuran-2-yl)methylene]amino}-3-[(1-undecyl -1*H*-1,2,3-triazol-4-yl)methyl]imidazolidine-2,4-dione
 9 (*E*)-3-[(1-Dodecyl-1*H*-1,2,3-triazol-4-yl)methyl]-1-([(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 10 (*E*)-1-{[(5-Nitrofuran-2-yl)methylene]amino}-3-{[1-(4-nitrophenethyl)-1*H*-1,2,3-triazol-4-yl]methyl}imidazolidine-2,4-dione
 11 (*E*)-3-[(1-Benzyl-1*H*-1,2,3-triazol-4-yl)methyl]-1-{[(5-nitrofuran-2-yl) methylene]amino}imidazolidine-2,4-dione	 12 (*E*)-3-{[1-(4-Methylbenzyl)-1*H*-1,2,3-triazol-4-yl]methyl}-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione
 13 (*E*)-3-[(1-(4-Isopropylbenzyl)-1*H*-1,2,3-triazol-4-yl]methyl)-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 14 (*E*)-3-{{1-[4-(*tert*-Butyl)benzyl]-1*H*-1,2,3-triazol-4-yl}methyl}-1-{[(5-nitrofuranyl)methylene]amino}imidazolidine-2,4-dione
 15 (*E*)-3-{[1-(4-Bromobenzyl)-1*H*-1,2,3-triazol-yl]methyl}-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 16 (*E*)-3-{[1-(4-Bromo-2-fluorobenzyl)-1*H*-1,2,3-triazol-4 yl]methyl}-1-{[(5-nitrofuranyl)methylene]amino}imidazolidine-2,4-dione

TABLE 1 (Continued)

Chemical structure	Chemical structure
 **17** (*E*)-3-{[1-(4-Fluorobenzyl)-1*H*-1,2,3-triazol-4-yl]methyl}-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 **18** (*E*)-3-{[1-(4-Nitrobenzyl)-1*H*-1,2,3-triazol-4-yl]methyl}-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione.
 **19** (*E*)-3-{[1-(3-Bromobenzyl)-1*H*-1,2,3-triazol-4-yl]methyl}-1-{[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione	 **20** (*E*)-1-{[(5-Nitrofuran-2-yl)methylene]amino}-3-{{1-[3(trifluoromethyl)benzyl]-1*H*-1,2,3-triazol-4-yl}methyl}imidazolidine-2,4-dione

hypoxanthine, 16 mM thymidine (HT supplement: Thermo Fisher Scientific, Yokohama, Japan), 0.0001% 2-mercaptoethanol, 10 μg/L insulin, 5.5 μg/L transferrin, and 6.7 ng/L sodium selenite (ITS-X Supplement: Thermo Fisher Scientific, Yokohama, Japan). The cultures were placed in a humidified atmosphere containing 5% CO_2 at 37°C for in vitro screening, and the cultures were maintained by subculturing with fresh medium every second day [17, 18].

2.4 | In vitro anti-trypasomal activity

The assay was conducted by adding 50 μL of each nitrofurantoin hybrid in 2% dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Osaka, Japan) to HMI-9 medium at various concentrations (25 to 0.003 ng/mL). The nitrofurantoin hybrids and commercial reference drugs, suramin, pentamidine, nifurtimox, eflornithine, and diminazene aceturate, were added to the Nunc® MicroWell 96-well optical bottom plate (Thermo Fisher Scientific, Yokohama, Japan) and incubated at 37°C in a humidified atmosphere. Parasite viability was observed under a light microscope, and parasites were counted manually after every 24 h using a counting chamber. Fifty microliters of *T. b. gambiense* and *T. b. rhodesiense* parasites were inoculated at 5×10^3, added to Nunc® MicroWell 96-well optical bottom plates containing nitrofurantoin hybrids, and incubated for 24, 48, and 72 h at 37°C. The effect of nitrofurantoin hybrids on viability was evaluated by adding 23 μL of CellTiter Glo™Luminescent Cell Viability Assay (Promega Japan, Tokyo, Japan) to the plates, which were shaken for 2 min at 500 rpm by an MS3 basic plate shaker (IKA® Japan, Osaka, Japan) to facilitate cell lysis and release intracellular ATP; the plates were further incubated for 10 min at room temperature. Absorbance was measured using a GloMax®-Multi Detection System plate reader (Promega, Japan), and the experiments were conducted independently in triplicate as reported previously by Suganuma et al. [19] and Suganuma et al. [20].

2.5 | In vitro cytotoxicity evaluation

Cytotoxicity evaluation of nitrofurantoin hybrids was conducted using the MDBK cell line (Obihiro University of Agriculture and Veterinary Medicine, Japan) as described by Li et al. [21]. Approximately 1×10^5 cells/mL were seeded in a 96-well microtiter plate (Thermo Fisher Scientific, Yokohama, Japan); 50 μL of the cells were exposed to nitrofurantoin hybrids at varying concentrations (100 μg/mL to 0.80 μg/mL) and incubated for 72 h at 37°C in a 5% CO_2 incubator. Cell viability was detected by an optical microscope and Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) by adding 10 μL of CCK-8 solution to each well. After the incubation period, the plates were read using a GloMax®-Multi Detection System plate reader (Promega, Japan) at 450 nm. The 96-well plates were incubated again after the initial absorbance reading at 37°C for 4 h; subsequently, the plates were read for the second time, and the experiments were each repeated three times [18]. Selectivity index which is expressed as the ratio of 50% cytotoxic concentration (IC_{50}) of the cells over the inhibitory concentration (IC_{50}) of the trypanosome parasites to determine the nitrofurantoin-triazole hybrids that are active against the parasites but nontoxic to the mammalian cells [22].

2.6 | Statistical analysis

The activity of each hybrid was expressed as IC_{50} ± S. D., which is the concentration of each hybrid that induced 50% inhibition of the parasites for experiments

performed in triplicate. Nonlinear regression (curve fit) graphs were performed using GraphPad PRISM version 5 software (GraphPad Software, Inc., CA, USA). Cytotoxicity of the NFT hybrids was analyzed by Cell Counting Kit-8 (CCK-8) and measured by 50% cytotoxic concentration (IC_{50}) on MDBK cells and SI, which is the ratio of trypanosome inhibition to host cell toxicity. The SI was calculated to allow for the possible identification of compounds with high efficacy and low cytotoxicity. This parameter reflects the quantity of the compound that is active against the pathogen but is not toxic toward the host cells [22]. Clog*P* was calculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009) as log (coctanol/water). SI, which is expressed as the ratio of the 50% cytotoxic concentration (IC_{50}) of the cells versus the IC_{50} of the parasites, is used to determine the NFT hybrids that are active against the parasites but nontoxic to the cells. SI = minimum toxic concentration (μg/mL)/minimum inhibitory concentration (μg/mL).

3 | RESULTS

3.1 | In vitro anti-trypanosomal activity

Anti-trypanosomal activity of 19 synthesized NFT-triazole hybrids against *T. b. gambiense* (IL1922), and *T. b. rhodesiense* (IL1501) are shown in (Tables 2 and 3), respectively. The activities were expressed as IC_{50} ± S.D., which is the concentration of each hybrid that induced 50% inhibition of the parasites. The nonlinear regression curves were also calculated to show the IC50 of anti-trypanosomal activity of NFT hybrids against *T. b. gambiense* (Figure S1A–S) and *T. b. rhodesiense* (Figure S2A–S). The hybrids were moderately active in comparison to reference drugs against *T. b. gambiense* and *T. b. rhodesiense* (Tables 2 and 3). On the other hand, all of the tested NFT hybrids in this study were more potent against *T. b. gambiense* and *T. b. rhodesiense* than the reference drug nifurtimox, respectively (Tables 2 and 3).

TABLE 2 Anti-trypanosomal activity of nitrofurantoin-triazole hybrids against *T. b. gambiense* and cytotoxicity on MDBK cells.

Compound	cLog*P*[a]	Anti-trypanosomal activity, IC_{50} (μM) (mean ± SD) Tbg (IL1922)	Cytotoxicity, IC_{50} (μM) MDBK	SI[b]
Nitrofuratoin **1**	−0.22	1.62 ± 0.28	–	–
2	1.62	0.39 ± 0.16	102.75 ± 19.01	263.05
3	2.07	0.45 ± 0.20	66.57 ± 44.62	146.49
4	2.51	1.04 ± 0.26	210.51 ± 41.91	202.68
5	2.96	1.14 ± 0.45	159.77 ± 78.97	140.26
6	3.40	0.38 ± 0.14	125.11 ± 2.01	331.70
7	3.85	0.32 ± 0.12	195.35 ± 43.74	611.75
8	4.29	0.09 ± 0.03	63.13 ± 30.48	706.71
9	4.74	0.07 ± 0.03	59.73 ± 12.25	832.53
10	1.81	0.35 ± 0.09	71.04 ± 9.99	201.83
11	1.58	0.12 ± 0.06	54.16 ± 5.84	435.41
12	2.09	0.18 ± 0.12	112.95 ± 12.47	621.71
13	2.82	0.12 ± 0.04	71.04 ± 24.56	587.11
14	3.12	0.10 ± 0.03	90.31 ± 13.56	915.45
15	2.35	0.23 ± 0.11	111.00 ± 53.06	474.21
16	1.52	1.03 ± 0.41	22.20 ± 12.11	21.65
17	2.49	0.26 ± 0.09	38.73 ± 9.55	147.21
18	1.72	0.08 ± 0.04	23.67 ± 5.26	298.47
19	2.35	0.14 ± 0.05	53.96 ± 32.58	377.69
20	2.46	0.40 ± 0.06	138.56 ± 44.20	346.38
Suramin	–	0.06 ± 0.002	–	–
Pentamidine	–	0.01 ± 0.003	–	–
Nifurtimox	–	4.59 ± 2.38	–	–
Eflornithine	–	16.13 ± 2.93	–	–
Diminazene aceturate	–	0.02 ± 0.004	–	–

Note: Nitrofurantoin 1 is reported as a drug by Munsimbwe et al. (2021).
Abbreviations: MDBK, Madin-Darby bovine kidney; nd, not done; SI, selectivity index; Tbg, *Trypanosoma brucei gambiense*.
[a]Calculated from ChemDraw Ultra 12.
[b]SI_1 = IC_{50} MDBK/IC_{50} Tbg; nd.

TABLE 3 Anti-trypanosomal activity of nitrofurantoin-triazole hybrids against *T. b. rhodesiense* and cytotoxocity on MDBK cells.

Compound	cLog*P*[a]	Anti-trypanosomal activity, IC_{50} (μM) (mean ± SD) Tbr (IL1501)	Cytotoxicity, IC_{50} (μM) MDBK	SI[b]
Nitrofuratoin **1**	−0.22	1.42 ± 0.25	–	–
2	1.62	0.55 ± 0.25	102.75 ± 19.01	188.06
3	2.07	1.08 ± 0.97	66.57 ± 44.62	61.66
4	2.51	1.75 ± 0.89	210.51 ± 41.91	120.10
5	2.96	1.60 ± 0.61	159.77 ± 78.97	99.59
6	3.40	0.53 ± 0.29	125.11 ± 2.01	234.89
7	3.85	0.45 ± 0.20	195.35 ± 43.74	436.83
8	4.29	0.27 ± 0.21	63.13 ± 30.48	231.84
9	4.74	0.15 ± 0.09	59.73 ± 12.25	410.11
10	1.81	0.57 ± 0.22	71.04 ± 9.99	124.19
11	1.58	0.17 ± 0.07	54.16 ± 5.84	327.11
12	2.09	0.46 ± 0.41	112.95 ± 12.47	246.05
13	2.82	0.36 ± 0.29	71.04 ± 24.56	197.98
14	3.12	0.37 ± 0.32	90.31 ± 13.56	244.30
15	2.35	0.63 ± 0.59	111.00 ± 53.06	175.02
16	1.52	1.19 ± 0.29	22.20 ± 12.11	18.68
17	2.49	0.72 ± 0.57	38.73 ± 9.55	54.07
18	1.72	0.11 ± 0.06	23.67 ± 5.26	220.54
19	2.35	0.50 ± 0.45	53.96 ± 32.58	108.25
20	2.46	17.46 ± 1.54	138.56 ± 44.20	79.99
Suramin	–	0.08 ± 0.01	–	–
Pentamidine	–	0.03 ± 0.006	–	–
Nifurtimox	–	4.36 ± 1.59	–	–
Eflornithine	–	45.99 ± 17.07	–	–
Diminazene aceturate	–	0.05 ± 0.009	–	–

Note: Nitrofurantoin 1 is reported as a drug by Munsimbwe et al. (2021).
Abbreviations: MDBK, Madin-Darby bovine kidney; nd, not done; SI, selectivity index; Tbr, *Trypanosoma brucei rhodesiense*.
[a]Calculated from ChemDraw Ultra 12.
[b]$SI_2 = IC_{50}$ MDBK/IC_{50} Tbr; nd.

Thirteen NFT hybrids, **2**, **3**, **6–15**, and **17–20**, displayed good activity against *T. b. gambiense* with IC_{50} < 1 μM. Of these hybrids, **8**, **9**, and **18** displayed nanomolar activity with IC_{50} values of 0.09 ± 0.03 μM, 0.07 ± 0.03 μM, and 0.08 ± 0.04 μM, respectively. On the contrary, **1**, **4**, **5**, and **16** demonstrated micromolar activity with IC_{50} > 1 μM: 1.62 ± 0.28, 1.04 ± 0.26 μM, 1.14 ± 045 μM, and 1.03 ± 0.41 μM, respectively (Table 2). The nitrofurantoin hybrids bearing *n*-alkyl substituents **8** and **9** and benzyl substituent **18** showed excellent activity than their respective hybrids against *T. b. gambiense*. Sub-series 2 hybrids **11–19** bearing benzyl substituents displayed increased activity than hybrids bearing *n*-alkyl substituents **2–7** and **10**. The benzyl nitrofurantoin hybrids bearing methyl electron-donating groups **11–14** showed increased activity than hybrids bearing electron-withdrawing groups **15–20** against *T. b. gambiense* (Table 2).

Nitrofurantoin hybrids **9**, **11**, and **18** showed nanomolar activity against *T. b. rhodesiense* with IC_{50} values ranging 0.11–0.17 μM in (Table 3). The majority (70%) of the hybrids **2**, **6–15**, **17**, **18**, and **19** exhibited potent sub-micromolar activity against *T. b. rhodesiense* with IC_{50} < 1 μM, while hybrids **1**, **3**, **4**, **5**, **16**, and **20** showed moderate micromolar activity $IC_{50} \approx 1$ (Table 3). Overall, the best performers were hybrids **11**, **13**, and **16** against *T. b. gambiense* and hybrids **9**, **11**, and **18** against *T. b. rhodesiense*, and they displayed dual potent nanomolar activities as shown in Table 3, respectively. Similar good potency was observed against *T. b. rhodesiense* parasites, the majority of hybrids (70%), including **2**, **6–15**, **17**, **18**, and **19**, exhibited potent sub-micromolar activity with IC_{50} < 1 μM, while hybrids **1**, **3**, **4**, **5**, **16**, and **20** showed moderate micromolar activity $IC_{50} \approx 1$ (Table 3). Overall, the best performers were hybrids **11**, **13**, and **16** against *T. b. gambiense* and hybrids **9**, **11**, and **18** against *T. b. rhodesiense*, and they displayed dual potent nanomolar activities as shown in Table 3, respectively.

3.2 | In vitro cytotoxicity evaluation

Hybrids **2**, **4**, **5**, **6**, **7**, **12**, **15**, and **20** had no cytotoxicity to mammalian cells IC_{50} > 100 μM; eight hybrids, **3**, **8**, **9**, **10**, **11**, **13**, **14**, and **19**, showed weak cytotoxicity to mammalian cells IC_{50} < 100 μM; and lastly, hybrids **16**, **17**, and **18** were moderately toxic to mammalian cells IC_{50} < 50 μM, respectively. All the NFT hybrids had SIs >10 on both *T. b. gambiense* and *T. b. rhodesiense*. The NFT hybrids that displayed anti-trypanosomal activity with no toxicity on the MDBK cells **2**, **4**, **5**, **6**, **7**, **12**, **15**, and **20** had SIs of >10 toward *T. b. gambiense* and *T. b. rhodesiense* in Tables 2 and 3. Interestingly, there are other NFT hybrids that displayed good anti-trypanosomal activity but showed cytotoxicity on the MDBK cells **3**, **8**, **9**, **10**, **11**, **13**, **14**, **16**, **17**, **18**, and **19**; they also had selectivity >10 toward *T. b. gambiense* and *T. b. rhodesiense*. Furthermore, hybrids **8**, **9**, and **17** displayed the strongest activity against *T. b. gambiense* and *T. b. rhodesiense*, but they were more toxic to MDBK cells, and their selectivity was 706.71, 832.53, and 298.47 toward *T. b. gambiense* and 231.84, 410.11, and 220.54 toward *T. b. rhodesiense* (Tables 2 and 3), respectively.

4 | DISCUSSION

4.1 | In vitro anti-trypanosomal activity

In the current study, 19 synthesized NFT hybrids were screened against *T. gambiense* and *T. rhodesiense*. The findings in this study showed that an increase in the chain length and the number of carbon atoms in the *n*-alkyl hybrids **5–9** influenced the increase in anti-trypanosomal activity against *T. b. gambiense* and hybrids **4–9** against *T. b. rhodesiense*. Experts implemented criteria for screening potential compounds for infectious diseases (leishmaniasis, malaria, TB, and trypanosomosis) and the in vitro activity criteria for a "hit" compound against *T. b. rhodesiense* is IC_{50} < 0.2 μg/mL and for *T. cruzi* is IC_{50} < 1.0 μg/mL [23–25].

The short-chain *n*-alkyl hybrids **2**, **3**, **4**, **5**, and **10** showed decreased activity compared to long-chain *n*-alkyl hybrids **6**, **7**, **8**, and **9** with increased activity against both *T. b. gambiense* and *T. b. rhodesiense* (Tables 2 and 3). The results of this study agree with previous studies regarding the structure–activity relationship, chain length, and the number of carbon atoms do influence trypanocidal [26, 27]. According to Zuma et al. [28], the addition of carbons to extend the alkyl chain allows for more flexibility and structural diversity. Electrophilic aromatic substitution with electron withdrawing groups; Br, F, CF_3, NO_2 and electron donating groups, methyl, isopropyl and *tert*-butyl on the phenyl ring had an influence on trypanocidal activity. Incorporation of additional electron-donating substituents in NFT hybrids **11–14** showed increased anti-trypanosomal activity compared to electron-withdrawing substituents in NFT hybrids **15**, **16**, and **18–20**, as shown in Tables 1–3. Complementary to the results, electronegativity has been reported to correlate with trypanocidal activity [29, 30]. The position of the substituents on the phenyl ring appeared to influence anti-trypanocidal activity. Hybrid **15** incorporating a bromine substituent in the *para*-position was marginally more active than hybrid **19** with a bromine substituent in the *meta*-position against both *T. b. gambiense* and *T. b. rhodesiense* (Tables 1–3). However, hybrids with the fluorine substituent **17** were more active in the *para*-position than **18** in the *ortho*-position or **20** in the *meta*-position against both *T. b. gambiense* and *T. b. rhodesiense* (Tables 1–3). Zhou et al. [31] reported that the nitro group position of synthesized 5-nitrofurans containing nitroaromatic rings influenced activity as it was in *meta*-position and more potent than those in *ortho*- or *para*-position. The effects of *ortho*-, *meta*-, and *para*-substitution on the biological activity of (*E*)-*N*,-1-diphenylmethanimine derivatives were investigated by Mkpenie et al. [32], and the results displayed that *meta*- and *para*-substituents were more active against some fungal and bacterial microorganisms.

Hu et al. [33] reported that 4-nitrobenzyl phosphoramide mustard displayed high potency against *T. brucei*, *T. cruzi*, and *Leishmania donovani* with IC_{50} values of 0.007, 0., and 1.29 μM, respectively. The activity can be explained by the type I nitroreductase mechanism and the benzylic C–O bond necessary for prodrug activation. Kaiser et al. [34] reported the findings of 5-nitrofuran repurposed drugs and nitrofurantoin as being exceptionally potent with good selectivity against *T. b. rhodesiense* with IC_{50} of 0.5 μM; SI:181 but inactive against other antiprotozoal parasites *L. donovani* amastigotes IC_{50} 2.12 μM; SI: 59 and *T. cruzi* IC_{50} 4.35 μM SI: 21, respectively. Reduced anti-trypanosomal activity was observed on hybrids **1**, **3**, **4**, **5**, and against both *T. b. gambiense* and *T. b. rhodesiense* with IC_{50} > 1 μM and hybrid **20** showed no activity against *T. b. rhodesiense* with IC_{50} of 17.46 ± 1.54, respectively. All of the tested hybrids **2–20** displayed better anti-trypanosomal activity against *T. b. gambiense* than *T. b. rhodesiense*. However, the parent drug, nitrofurantoin, displayed slightly better anti-trypanosomal activity against *T. b. rhodesiense*; IC_{50} 1.42 ± 0.25 μM than *T. b. gambiense* IC_{50} 1.62 ± 0.28 μM, respectively (Tables 2 and 3). This poor activity in comparison to hybrids **2**, **3**, **6–16**, **18**, and **19** may be due to poor parasitic cell permeability and the hybrids being unstable, and this could also have influenced the lack of activity in Bains et al. [35]. Poor anti-trypanocidal activity against *T. b. rhodesiense* was also observed compared to *T. b. gambiense* in a study by Kibona et al [36] stated that drug resistance was associated with reduced susceptibility of *T. b. rhodesiense*.

A compound containing dinitrophynyl and its analogs were screened against T. b. rhodesiense, and most were potent with IC_{50} ranging from 0.0075 to 0.05 μg/mL in vitro [24].

Pharmacokinetic properties such as absorption, distribution, excretion, metabolism, permeability, potency, selectivity, and toxicity are very important when developing new drugs [37]. It is crucial for a potential drug to have good solubility, which enables the compound's bioavailability and absorption and its ability to permeate biological membranes [38]. Nitrofurantoin has been reported to have low lipophilicity, which makes it poorly soluble in oils, and it influences its ability for intestinal permeability by passive diffusion for oral administration [39]. There is a need for the development of drugs that display optimal physicochemical properties for oral administration, as there are a limited number of orally administered anti-trypanocidal drugs [18].

The measure between lipophilicity and hydrophilicity of a compound is calculated by partition coefficient cLog*P* value [40]. Lipinski's rule of five for drug-likeness indicates that the logarithm of the partition coefficient log*P* value requirement is ≤5 with < being the ideal target [41]. Most NFT hybrids in this study showed good drug-like properties; the cLog*P* values were within the target range of cLog*P* <5 (1.52–4.74). Hybrids with a large log*P* value indicate high lipophilicity and poor water solubility, whereas hybrids with a low log*P* value showed high hydrophilicity associated with poor permeability and low [42, 43]. Sub-series 1 *n*-alkyl hybrids **2**, **4–9** chain length had an influence on lipophilicity; as the chain length increased, there was an increase in cLog*P* and anti-trypanosomal activity (Table 2). The two most active *n*-alkyl hybrids, **8** (C11) and **9** (C12), had high cLog*P* values of 4.29 and 4.74, respectively. An increase in the lipophilicity of compounds tends to increase potency; however, due to high binding affinity, highly lipophilic compounds can bind to off-target hydrophobic environments, increasing the risk of toxicity [44]. In a study conducted by Cao et al. [45], it was observed that the antiviral activity of highly potent compounds was partially affected by their configuration and lipophilicity.

There was no effect on lipophilicity observed on the NFT benzyl hybrids, as the electronic effect of the substituents varied. However, hybrids **11** and **17** that showed the strongest activity were among the least lipophilic, with cLog*P* values of 1.58 and 1.62, respectively. Zuma et al. [28] reported the same outcomes with regards to the effect of lipophilicity on the antimycobacterial activity of *n*-alkyl analogs with increasing side chain length. The benzyl sub-series, however, displayed no relation between the electronic effect of the substituents and lipophilicity. There were NFT hybrids within the cLog*P* target range, but they were found to be inactive, confirming that biological activity is dependent on many parameters apart from physicochemical properties [46]. The difference in the anti-trypanosomal activity of the NFT hybrids observed in this study may be because of many factors, such as the bioassay, parasite species, and taking into consideration the different bio-activation enzymes between *Trypanosoma* species [47].

Hybrid **16**, which was among the best performing hybrids against both *T. b. gambiense*; 0.08 ± 0.04 μM and *T. b. rhodesiense* 0.11 ± 0.06 μM, the activity might be influenced by the introduction of a fluorine in the *para*-position on the benzyl ring. Fluorination of aromatic rings by the replacement of hydrogen with fluorine has been reported to have an effect on stability, solubility, lipophilicity in terms of distribution, and absorption, as well as biological activity [48]. A study by Cuevas-Herna'ndez et al. [49] showed a trifluoromethyl compound to have better trypanocidal activity than benznidazole, which is a commercially used drug for the treatment of Chagas disease caused by *T. cruzi*.

The trypanocidal drugs suramin, pentamidine, and diminazine aceturate used in this study showed more potency than all the NFT hybrids. However, nifurtimox and eflornithine showed no anti-trypanosomal activity at IC_{50} values of 4.59 ± 2.38; 16.13 ± 2.93 μM against *T. b. gambiense* and 4.36 ± 1.59; 45.99 ± 17.07 μM against *T. b. rhodesiense*, respectively. Similar results were reported by Vincent et al. [50]; both nifurtimox and eflornithine failed to produce antitrypanosomal activity in vitro with IC_{50} values of 4 and 35 μM against *T. brucei*. Stewart et al. [51] investigated the trypanocidal activity of nitroheterocyclic compounds containing melamine groups. Only one melamine nitrofuran compound showed the strongest in vitro activity, with IC_{50} value of 0.025 M against *T. b. rhodesiense*, and the compound was compared to nifurtimox, which had an in vitro IC_{50} value of 1.5 M against *T. b. rhodesiense*. Strains of *T. b. gambiense* (STIB754/130R and STIB930), and *T. b. rhodesiense* (TBI900) have been reported to be sensitive to melarsoprol, pentamidine, and suramin [24]. Diminazene aceturate is used for the treatment of animal trypanosomosis; however, it has also been used for the first-stage treatment of *T. b. gambiense* and *T. b. rhodesiense* [52].

4.2 | In vitro cytotoxicity

The cytotoxicity effects of NFT hybrids were determined on MDBK cells using CCK-8 assay and results were expressed as IC_{50}. Five sub-series 1 *n*-alkyl hybrids, **2**, **4**, **5**, **6**, and **7**, and four sub-series 2 benzyl hybrids, **12**, **13**, **15**, and **20**, were non-toxic to mammalian cells LC_{50} > 100 μM. The n-alkyl and benzyl hybrids **3**, **8**, **9**, **10**, **11**, **13**, **14**, and **19** had weak cytotoxicity 50 μM < IC_{50} < 100 μM. Only sub-series 2 benzyl hybrids **16**, **17**, and **18** displayed moderate cytotoxicity 10 μM < IC_{50} < 50 μM, and interestingly, none of the NFT hybrids in this study displayed high cytotoxicity <10 μM. These results are in accordance with the criteria for toxicity classification, whereby a compound is

considered to be of low toxicity when the IC_{50} > 100 μM [53, 54], weak toxicity when 50 μM < IC_{50} < 100 μM [55], moderate toxicity with 10 μM < IC_{50} < 50 μM [55, 56], and high cytotoxicity with IC_{50} < 10 μM [55–57].

Hybrid **18** containing a nitro group substituent displayed moderate cytotoxicity with IC_{50} of 22.20 ± 12.11 μM, and a study conducted by Li et al. [58] showed that the nitrobenzene-containing group on the furan ring of nitrofurantoin played a role in toxicity. There was no correlation observed between chain length and cytotoxicity of the *n*-alkyl hybrids, as well as the chain length of the benzyl hybrids bearing electron-donating methyl substituents. However, the benzyl hybrids with electron-withdrawing substituents suggested that the position of the substituent on the phenyl ring played a role in cytotoxicity. The bromine substituent at *para*-position on hybrid **15** was nontoxic with IC_{50} of 111.00 ± 53.06 μM, whereas when the bromine substituent (hybrid **19)** was at *meta*-position, it displayed weak cytotoxicity with LC_{50} of 53.96 ± 32.58 μM, respectively. Similar results were observed with hybrid **20** with a fluorine substituent in the *ortho*-position, which showed no toxicity with IC_{50} of 138.56 ± 44.20 μM, but the fluorine substituent (hybrid **17**) in the *para*-position displayed moderate cytotoxicity with LC_{50} of 23.67 ± 5.26 μM, respectively.

As reported by Luethi et al. [59], *para*-substituents to the nitro moiety may increase toxicity influenced by mitochondrial impairment. Nitrofurantoin toxicity can also be mediated by redox cycling, which results in oxidative stress, and mitochondrial toxicity is caused by the production of oxygen electron-withdrawing nature of the nitro moiety [60]. Hall et al. [61] showed findings of type I nitroreductases, which reduce the nitro group and are responsible for generating toxic metabolites for anti-parasitic activity that may also inhibit mammalian cell growth. Interference of the substituents on the benzyl ring is considered to influence toxicity, and substituents in the *meta*-position decrease toxicity, whereas *para*-position substituents decrease toxicity [62].

Toxicity and poor pharmacokinetics account for 20%–40% of drug failure in drug development, especially for candidate drugs, and compounds with high lipophilicity are associated with toxicity [43, 63]. The use of nitrofurantoin has been associated with hepatotoxicity and pulmonary toxicity; however, the mechanism remains undetermined for reasons that are so far unexplained [64]. Mechanisms in in vitro studies indicated that an increase in the production of oxidants results in toxic metabolic products of nitrofurantoin in the presence of oxygen with long-term use [65].

Lack of suitable cell lines for cytotoxicity tests may hinder reliable results for cytotoxicity; MDBK cells may be susceptible, but they have been used for their bovine origin, epithelial traits, and adherent growth characteristics [66]. Cytotoxic assay can also influence the optical quantitative measurement of electrochemical properties, and in this study, cell viability was evaluated by the CCK-8 assay. According to Cai et al. [67], the CCK-8 assay shows detection sensitivity using a highly water-soluble tetrazolium salt measured by the dye of WST-8 reduced by dehydrogenase in cells to form a water-soluble orange-colored product called formazan. The amount of formazan dye produced by cellular dehydrogenases is correlated with the number of living cells. Therefore, cell viability can be simply estimated by recording the optical density (OD) of formazan at 450 nm using a microplate reader.

All of the nitrofurantoin hybrids in this study had SI values ranging from 18 to greater than 915, and these results were in agreement with previous studies by Katsuno et al. [25] and Finiuk et al. [56] that the hybrids were up to 10–100 times fold selective in their anti-trypanosomal activity. The synthesized NFT hybrids showed strong selectivity >10 to *T. b. gambiense* and *T. b. rhodesiense*, which indicates the initial criteria for selecting potential hit drugs [68].

5 | CONCLUSION

The anti-trypanosomal activity of 19 NFT-triazole hybrids was evaluated against *T. b. gambiense* and *T. b. rhodesiense*. Hybrids **9**, **11**, **13**, **16**, and **19** exhibited high anti-trypanosomal activity; even so, these hybrids showed weak to moderate cytotoxicity on the MDBK cells. The findings in the current study add to the library potential compounds for the design and development of trypanocidal drugs. These NFT-triazole hybrids must be further evaluated in vivo for trypanocidal activity using experimental animals.

AUTHOR CONTRIBUTIONS

Conceptualization: Anna Seetsi, Nthatisi Molefe-Nyembe, David D. N'da, and Oriel Thekisoe. *Methodology*: Anna Seetsi, Nthatisi Molefe-Nyembe, David D. N'da, and Oriel Thekisoe. *Formal analysis*: Keisuke Suganuma, Anna Seetsi, Nthatisi Molefe-Nyembe, and David D. N'da. *Writing original draft preparation*: Keisuke Suganuma. *Writing review and editing*: Keisuke Suganuma, Anna Seetsi, Nthatisi Molefe-Nyembe, David D. N'da, Tsepo Ramatla, and Oriel Thekisoe. *Supervision*: Nthatisi Molefe-Nyembe, David D. N'da, and Oriel Thekisoe. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

ETHICS APPROVAL STATEMENT

This study was approved by the scientific committee of the Integrated Pest Management of the Unit for Environmental Sciences and Management, North-West University, reference number: NWU-01310-20-A9.

ORCID

Tsepo Ramatla https://orcid.org/0000-0002-0473-8075

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