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In vitro antitrypanosomal activity of synthesized nitrofurantoin-triazole hybrids against *Trypanosoma* species causing animal African trypanosomosis

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ABSTRACT

Animal African trypanosomosis (AAT) is a disease caused by *Trypanosoma brucei brucei*, *T. vivax*, *T. evansi* and *T. congolense* which are mainly transmitted by tsetse flies (maybe the family/genus scientific name for the tsetse flies here?). Synthetic trypanocidal drugs are used to control AAT but have reduced efficacy due to emergence of drug resistant trypanosomes. Therefore, there is a need for the continued development of new safe and effective drugs. The aim of this study was to evaluate the *in vitro* anti-trypanosomal activity of novel nitrofurantoin compounds against trypanosomes (*Trypanosoma brucei brucei*, *T. evansi* and *T. congolense*) causing AAT. This study assessed previously synthesized nineteen nitrofurantoin-triazole (NFT-TZ) hybrids against animal trypanosomes and evaluated their cytotoxicity using Madin–Darby bovine kidney cells. The *n*-alkyl sub-series hybrids, **8** (IC_{50} 0.09 ± 0.02 μM; SI 686.45) and **9** (IC_{50} 0.07 ± 0.04 μM; SI 849.31) had the highest antitrypanosomal activity against *T. b. brucei*. On the contrary, the nonyl **6** (IC_{50} 0.12 ± 0.06 μM; SI 504.57) and nitrobenzyl **18** (IC_{50} 0.11 ± 0.03 μM; SI 211.07) displayed the highest trypanocidal activity against *T. evansi*. The nonyl hybrid **6** (IC_{50} 0.02 ± 0.01 μM**;** SI 6328.76) was also detected alongside the undecyl **8** (IC_{50} 0.02 ± 0.01 μM; SI 3454.36) and 3-bromobenzyl **19** (IC_{50} 0.02 ± 0.01 μM; SI 2360.41) as the most potent hybrids against *T. congolense*. These hybrids had weak toxicity effects on the mammalian cells and highly selective submicromolar antiparasitic action efficacy directed towards the trypanosomes, hence they can be regarded as potential trypanocidal leads for further *in vivo* investigation.

1. Introduction

Animal African trypanosomosis (AAT) which is commonly known as nagana, is a disease caused by tsetse transmistted *Trypanosoma brucei brucei*, *T. congolense* and *T. vivax* (Morrison et al., 2016), whilst *T. evansi* which is mainly transmitted mechanically by haematophagous flies, causes AAT called surra (Aregawi et al., 2019). Both *T b. brucei* and *T. evansi* belong to subgenus *Trypanozoon*, *T. vivax* and *T. congolense* respectively belong to subgenera *Duttonella* and *Nannomonas* (Bengaly et al., 2002; Sánchez et al., 2015). The animal hosts for AAT are domestic livestock including cattle, goats, pigs, horses and camels (Desquesnes et al., 2022), while wild animals serve as reservoirs (Anderson et al., 2011). Clinical symptoms associated with trypanosomosis include fever, weight loss and anemia (Suganuma et al., 2014). Multiple *Trypanosoma* species can cause mixed infections resulting in different clinical presentations (Morrison et al., 2016).

The main pathogenic species causing AAT in livestock are *T. congolense* and *T. vivax*, whilst *T. brucei* displays less pathogenicity (Lalmanach et al., 2002). These trypanosomes are either intravascular (*T. congolense* and *T. vivax*) or both intravascular and extravascular (*T. b. brucei* and *T. evansi* and *T. equiperdum*). Intravascular (haematic) trypanosomes affect and remain in the blood while extravascular trypanosomes invade tissue of the host (Ukpai and Nwabuko, 2014). However, *T. evansi* is distinct from other trypanosomes because it infects

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both the blood and tissue of the host (Mohd Rajdi et al., 2021).

Chemotherapy is still the most commonly used method to control AAT in sub–Saharan Africa as it is easier than controlling the vector, with an estimated 35 million doses of veterinary drugs used annually (Ngumbi and Silayo, 2017). Treatment of AAT depends on chemotherapy and chemoprophylaxis of drugs with diminazene aceturate, isometamidium chloride, suramin, melarsomine, quinapyramine sulphate and homidium salts (Isaac et al., 2017; Suganuma et al., 2022). Studies report that farmers in sub–Saharan Africa use diminazene aceturate (55–79%) and isometamidium (8–21%) as their first choice of treatment against AAT (Liebenehm et al., 2011; Tekle et al., 2018; Odeniran et al., 2019). Most trypanocides, including diminazene aceturate are used as a prophylaxis even though they are therapeutics, whereas only a few like isometamedium have prophylactic capability (Richards et al., 2021). Veterinary trypanocides have been associated with poor quality, extreme side effects, toxicity (Giordani et al., 2016), inaccessibility, unavailability (Legros et al., 2002) and development of drug–resistant parasites (Morrison et al., 2016). Drug uptake mechanisms and cross-resistance between existing drugs also needs to be taken into consideration for the development of new trypanocidal drugs (Tran et al., 2018).

Nitrofuran compounds (furazolidone, nitrofurantoin, nitroimidazone and nitrofurazone) possess antibacterial and antitrypanosomal activity and have been used since the early 1940's (Bot et al., 2013). Nitrofurazone was reported to be effective against *T. brucei* and *T. equiperdum* and over the years there has been modification of nitrofuran derivatives, which led to the discovery of effective and promising drugs against microbial and protozoan parasite diseases (Patterson and Wyllie, 2014). Nitrofurantoin is used to treat bacterial urinary tract infections in humans, cats and dogs (Weese et al., 2011; Leuin et al., 2021; Porreca et al., 2021). However, since 1995 and 2002 nitrofuran drugs have been banned by the European Union and the U.S. Food and Drug Administration due to the drug carcinogenic hazard of consuming animal products (Suganuma et al., 2022).

The efficacy of nitrofurantoin analogs against AAT causing diseases in livestock and humans were reported by Munsimbwe et al. (2021). A study by Suganuma et al. (2022) evaluated the *in vivo* efficacy of nitrofurantoin against *T. congolense*. Species of genera *Trypanosoma* and *Leishmania* are taxonomically related and have similar biochemical and structural features which may lead to effective agents or combinations of drugs against one pathogen being active against the other (Kaufer et al., 2017). The nitrofurantoin-triazole (NFT-TZ) hybrids used in the current study have previously shown to possess leishmanicidal activity (Zuma et al., 2022) and trypanocidal activity against trypanosome species causing human African trypanosomosis which is also known sleeping sickness (Seetsi et al., 2023). Hence, we hypothesized that they may present with potential trypanocidal activity against trypanosome species causing animal trypanosomosis. The current study assessed the *in vitro* antitrypanosomal activity of these synthesized NFT-TZ hybrids against three species of animal trypanosomes, namely, *T. b. brucei, T. evansi* and *T. congolense.*

2. Materials and methods

2.1. Chemistry

The compounds under investigation were previously synthesized in low to moderate yields (30–80%) as described by Zuma et al. (2022) and comprised two sub-series that differed by the substituents on the triazole ring. Sub-series 1 hybrids (**2**–**10**) contained *n*-alkyl groups while sub-series 2 (**11**–**20**) bore benzyl groups (Table 1).

2.2. In vitro trypanosome cultures

Trypanosome species included *T. b. brucei* GuTat3.1 strain originally isolated in Uganda in 1966; *T. evansi* Tansui, akinetosplatic strain isolated in Taiwan (Hirumi et al., 1997); and *T. congolense* IL3000, a savanna subgroup strain isolated near the Kenya/Tanzania border in 1966. Bloodstream forms (BSF) of *T. congolense* were cultivated at 33 °C while *T. b. brucei* and *T. evansi* were cultured at 37 °C in 5% CO_2, using HMI–9 medium (Hirumi and Hirumi, 1991). As reported by Suganuma et al. (2014) and Molefe et al. (2017), *T. congolense* was sub–cultured every other day when the cultures reached high cell densities by replacing the entire culture supernatant with fresh medium, whereas for *T. b. brucei* and *T. evansi*, only part of the culture supernatant was replaced.

2.3. In vitro cell line culture

The Madin–Darby bovine kidney (MDBK) cells were grown in GIT medium Dulbecco's Modified Eagle's Medium (DMEM: Sigma-Aldrich, Tokyo, Japan) supplemented with 20% heat–inactivated fetal bovine serum (HI-FBS) and 1% penicillin-streptomycin (ThermoFisher Scientific, Yokohama, Japan) in a 37 °C incubator with 5% CO_2. Cells were maintained as described by Molefe et al. (2019), where the entire cell suspension was removed from the cell culture flask and approximately 11 mL of fresh medium was added and changed every 3–4 days.

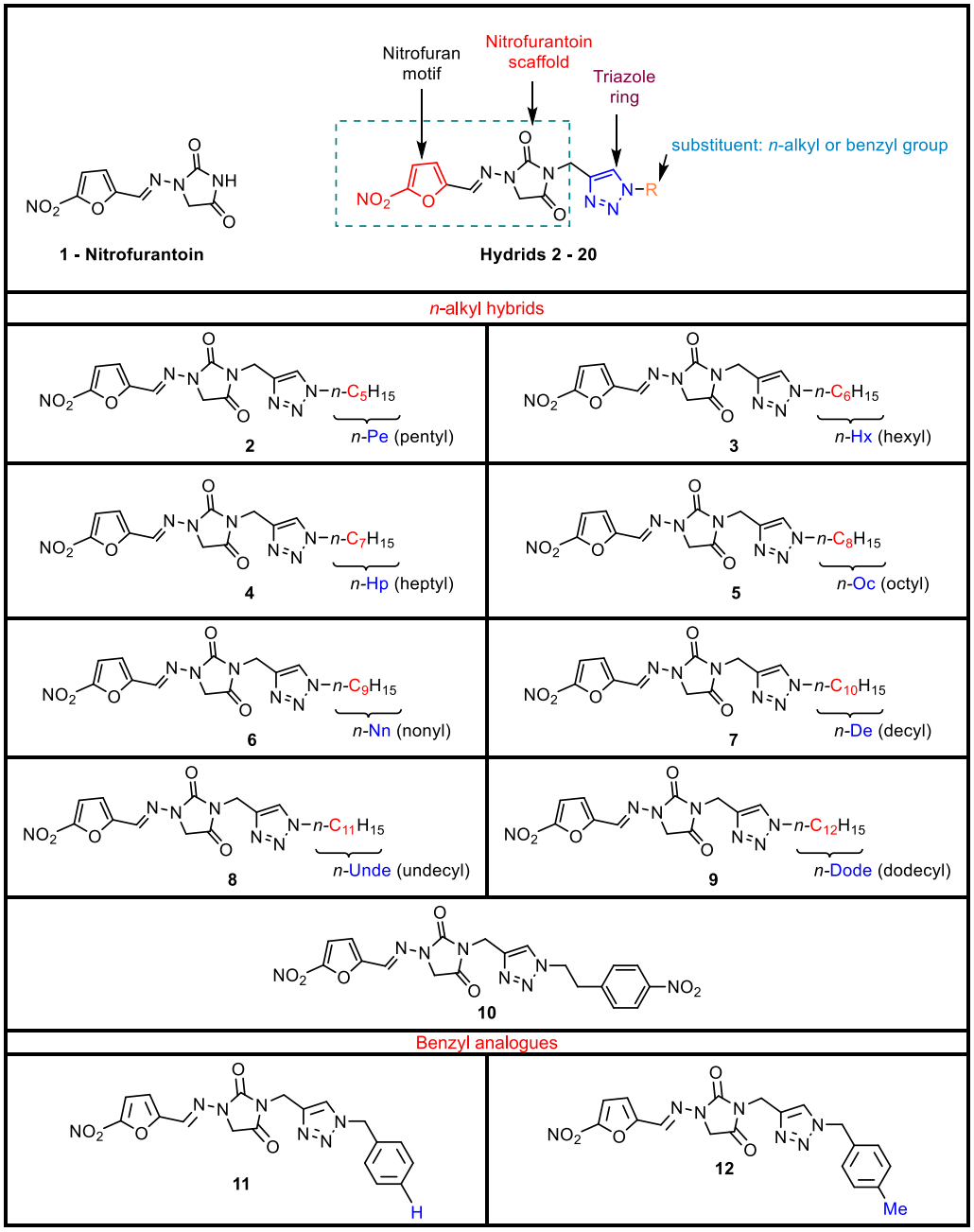
2.4. In vitro evaluation of anti-trypanosomal activity

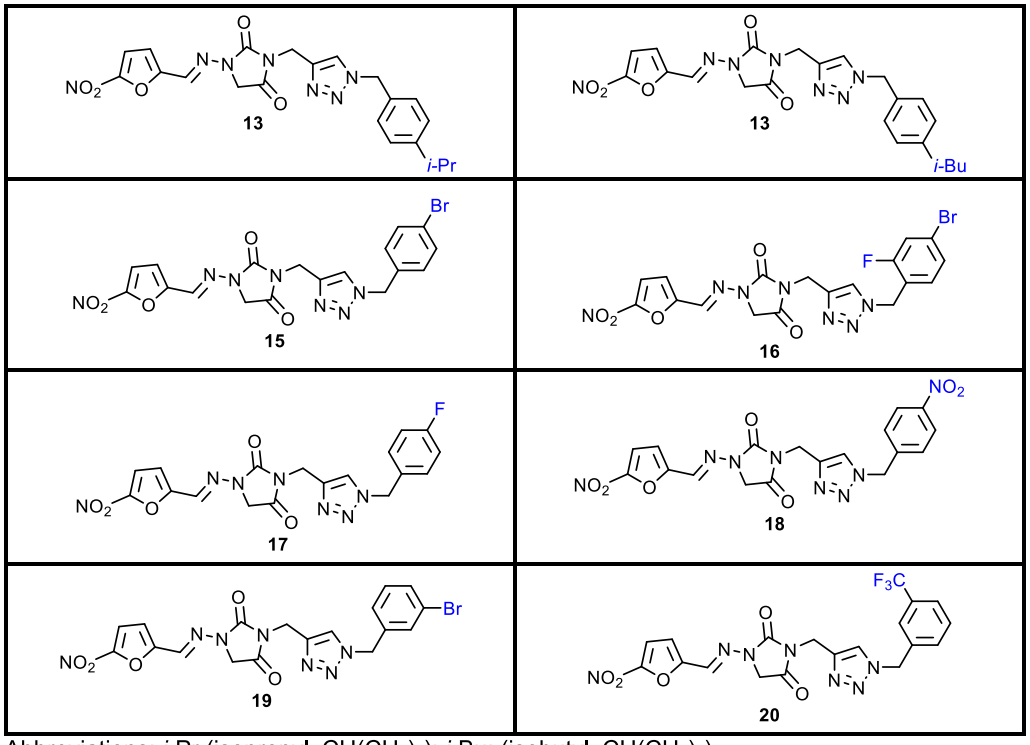
The activity of the nitrofurantoin hybrids against *T. b. brucei*, *T. evansi* and *T. congolense* were assessed as described by Suganuma et al. (2017). Fifty microliters of each nitrofurantoin hybrid together with the commercial reference drugs suramin, pentamidine, nifurtimox, eflornithine and diminazene aceturate were diluted in HMI-9 medium with 2% dimethyl sulfoxide [DMSO] (Wako Pure Chemical Industries, Osaka, Japan). The test compounds were added to Nunc® MicroWell 96 well optical bottom plates (ThermoFisher Scientific, Yokohama, Japan) at various concentrations from 25 μg/mL to 0.003 μg/mL by 5 times serial dilution and incubated in a humid atmosphere at 37 °C. Additional Nunc® MicroWell 96 well optical bottom plates were seeded with 100 μL of 5×10^3 cells/mL (*T. b. brucei*), 1×10^4 cells/mL (*T. evansi*) and 2×10^5 cells/mL (*T. congolense*). The number of living trypanosomes were observed under an optical microscope and counted manually after every 24 h using a counting chamber. Fifty microliters of each *Trypanosoma* strain were added to Nunc® MicroWell 96 well optical bottom plate containing nitrofurantoin hybrids and reference drugs. All plates were then incubated of 24, 48 and 72 h at 37 °C for *T. b. brucei* and *T. evansi*, whilst and at 33 °C *T. congolense*. Subsequently, 23 μL of CellTiter Glo™ Luminescent Cell Viability Assay (Promega, Tokyo, Japan) was added for evaluation of intracellular ATP concentration. The plates were shaken for 2 min at 500 rpm using a MS3 basic plate shaker (IKA® Japan K.K., Osaka, Japan) to enable cell lysis and release of intracellular ATP and the plates were incubated for another 10 min at room temperature. A GloMax®-Multi + Detection System plate reader (Promega Japan) was used to measure the bioluminescence and the experiments were replicated three times as described by Suganuma et al. (2014).

2.5. In vitro cytotoxicity assay

The (MDBK) NBL-1 cells were used for determining the cytotoxicity testing of NFT-TZ hybrids. The cells were seeded in a 96 well microtiter plate (Thermo Fisher Scientific, Yokohama, Japan) at a cell suspension of 1×10^5 cells/mL and 50 μL of the cells were added to nitrofurantoin compounds at various concentrations (100 μg/mL to 0.80 μg/mL), other wells contained culture medium as controls. The plates containing cells were incubated for 72 h in a 5% CO_2 incubator at 37 °C. Viable cells were observed under an optical microscope and were quantified by using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). A 10 μL volume of CCK-8 solution was added to each well and cell viability was determined using a GloMax®-Multi + Detection System plate reader (Promega Japan) at 450 nm. Absorbance readings were

Table 1
Synthesized nitrofurantoin-triazole hybrids.





Abbreviations: *i*-Pr (isopropyl, $CH(CH_3)_2$); *i*-Bu: (isobutyl, $CH(CH_3)_3$).

taken once again after the cells were further incubated for 4 h at 37 °C and the assays were performed in triplicate as described by Lou et al. (2010) and Molefe et al. (2017). The selectivity index was used to determine the relation between 50% cytotoxic concentration (CC_{50}) of the cells and the inhibition concentration (IC_{50}) of the parasites to reveal NFT-TZ hybrids with high efficacy and low cytotoxicity (Nunes et al., 2016).

2.6. Statistical analysis

The IC_{50} values were expressed as the mean ± standard deviation (S.D.) for each of the three experimental trials and the results were analyzed by GraphPad PRISM version 5 software (GraphPad Software, Inc., CA, USA) using nonlinear regression (curve fit).

3. Results

3.1. In vitro evaluation of antitrypanosomal activity

Antitrypanosomal activity of nineteen NFT-based hybrids were screened *in vitro* for efficacy against *T. b. brucei* (GUTat 3.1), *T. evansi* (Tansui) and *T. congolense* (IL3000). Antitrypanosomal activity IC_{50} values are shown in (Fig. 1b).

The data revealed that all hybrids were active with IC_{50} values varying in the range of 0.07–0.63 μM, 0.11–0.77 μM and 0.02–0.42 μM against *T. b. brucei, T. evansi* and *T. congolense,* respectively. The hybrids outperformed nifurtimox, eflornithine and the parent drug nitrofurantoin against all three strains, as they displayed superior activity in comparison to suramin, pentamidine and diminazene against *T. congolense*, but were found to be less potent than diminazene against *T. b. brucei* and *T. evansi*. Thus, the trypanosomes were susceptible to most hybrids with *T. congolense* being the most vulnerable as indicated by the comparatively low activity IC_{50} of the compounds (Table 2).

The was no observed significant difference on the antitrypanosomal activity between *n*-alkyl and benzyl sub-series hybrids. In contrast, the antitrypanosomal activity IC_{50} on individual trypanosome strain revealed that sixteen hybrids (**2, 3**; **6–15**; **17–20**) exhibited submicromolar ($IC_{50} < 1$ μM) and three (4, 5 and 16) possessed moderate micromolar ($IC_{50} > 1$ μM) anti-trypanosomal activity against *T. b. brucei.* In particular, hybrids undecyl **8** (IC_{50} 0.09 ± 0.02 μM) and dodecyl **9** (IC_{50} 0.07 ± 0.03 μM) displayed nanomolar IC_{50} activity hence were identified as the most active. This activity was comparable to that of diminazene aceturate (IC_{50} 0.07 ± 0.02 μM).

The overwhelming majority (15) of hybrids (**2, 3**; **6–15**; **17–19**) showed strong submicromolar activity in the 0.11 ± 0.04–0.78 ± 0.17 μM IC_{50} range while the remaining four (4, 5, 16 and 20) displayed moderate micromolar activity against *T. evansi*. Overall, the best performing hybrids were undecyl **8** (IC_{50} 0.18 ± 0.02 μM), dodecyl **9** (IC_{50} 0.12 ± 0.06 μM), benzyl **11** (IC_{50} 0.17 ± 0.05 μM) and nitrobenzyl **18** (IC_{50} 0.11 ± 0.03 μM), with activity comparable to that of suramin (IC_{50} 0.38 ± 0.06 μM).

Furthermore, all hybrids (**2–20**) exhibited good activity ($IC_{50} < 1$ μM) against *T. congolense* with IC_{50} values ranging from 0.02 ± 0.01 μM to 0.42 ± 0.31 μM. Most hybrids except **3** (0.27 ± 0.17 μM) and **16** (0.42 ± 0.31 μM) showed greater antitrypanosomal potency than pentamidine (0.23 ± 0.02 μM) and diminazene aceturate (0.33 ± 0.05 μM). The hexyl (C6) **3** (IC_{50} 0.02 ± 0.01 μM), octyl (C8) **5** (IC_{50} 0.02 ± 0.01 μM) and the bromobenzyl **19** (IC_{50} 0.02 ± 0.01 μM) stood out as the best performing hybrids against *T. congolense* parasites.

3.2. In vitro cytotoxicity

Cytotoxicity of the hybrids was evaluated on mammalian MDBK cells and the IC_{50} values are presented in Table 1. Of the nineteen hybrids, eight (**2, 4–7, 12, 15** and **20**) showed low cytotoxicity ($IC_{50} > 100$ μM) (Adewusi et al., 2013; Fu et al., 2014), another eight (**3, 8–11, 13, 14** and **19**) displayed weak cytotoxicity ($50 < IC_{50} < 100$ μM) (Liu et al., 2017) and three (**15–17**) were moderately cytotoxic to the mammalian

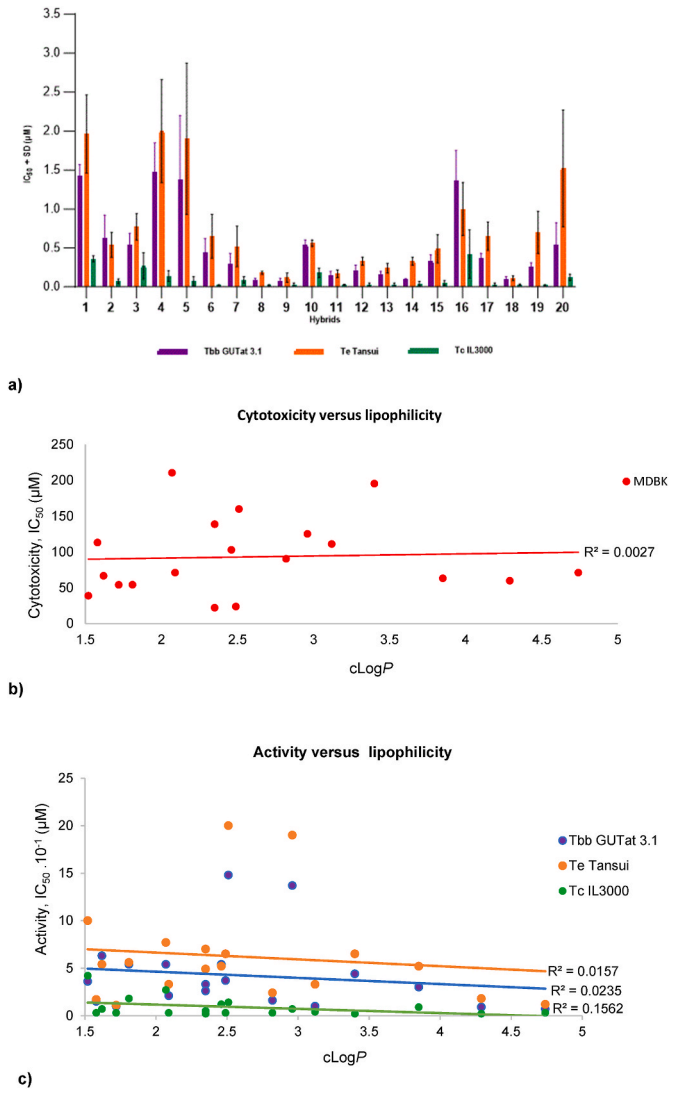


Fig. 1. (a) Antitrypanosomal activity of nitrofurantoin **1*** and hybrids **2**–**20** (IC_{50} ±SD, μM) against *T. b. brucei* GuTat3.1 (Tbb GuTat3.1), *T. evansi* Tansui (Te Tansui) and *T. congolense* IL3000 (Tc IL3000); Correlation plots: **(b)** cytotoxocity on MDBK cells (IC_{50} ±SD, μM) versus lipophilicity (cLog*P*); and **(c)** Antitrypanosomal activity of hybrids (IC_{50} ±SD, μM) against *T. b. brucei* GuTat3.1 (Tbb GuTat3.1), *T. evansi* Tansui (Tev Tansui) and *T. congolense* IL3000 (Tc IL3000) versus with lipophilicity (cLog*P*) of hybrids **2**–**20**.

cells ($10 < IC_{50} < 50$ μM) (Finiuk et al., 2017; Liu et al., 2017). All hybrids with good anti-trypanosomal activity (**3, 8–11, 13, 14, 17–19**) had weak to low cytotoxicity with IC_{50} ranging from 53.96 ± 32.58 μM to 210.51 ± 41.91 μM which translate into selectivity indexes (SI) of >100 of all three trypanosome strains (Table 1). The moderately active hybrids (**4**, **5, 16** and **20**) on the other hand, possessed moderate to low cytotoxicity with $20 < IC_{50s} > 100$ μM which translate into selectivity indexes (SI) of >10. These SI values are indicative of the observed activities of all hybrids being intrinsic, i.e., not attributed to their general toxicity.

The sub-series 1 hybrids appeared slightly less cytotoxic than the sub-series 2 hybrids (Fig. 1a) with the latter containing the three moderately cytotoxic compounds (**15**, **16** and **17**). However, no sensitive correlation could be established between structural properties of hybrids and cytotoxicity data. Similar observation was reported by Zuma et al. (2022).

4. Discussion

4.1. In vitro trypanocidal activity and hit/lead identification

The discovery and development of new drugs is a lengthy and daunting process that often results in failure during clinical trial. This has led experts in the field of drug discovery to establish various strategies to shorten the process and minimize the risk of failed trials. At basic discovery stage, a series of criteria to identify hit and lead compounds intended for the treatment of infectious diseases (e. g., malaria, tuberculosis, leishmaniasis and trypanosomiasis etc.) prolific in developing countries, have been established (Katsuno et al., 2015). With regard to trypanosomiasis, Katsuno et al. (2015) defined an antitrypanosomal "hit" as possessing anti-pathogenic cellular activity $IC_{50} < 10$ μM that is 10-fold selective (SI > 10) than that on mammalian cells. An early lead must possess *inter alia* an antiparasitic cellular activity ($IC_{50} < 1$ μM) and selectivity index (SI > 100). The criteria was established in reference to the bloodstream forms of *Trypanosoma cruzi*, the causative pathogen of Chagas disease (American trypanosomiasis), a human infection that is endemic in South American countries. The application of this criteria to the animal trypanosomes in this study allowed the identification of potential hits and leads among the tested compounds. Hybrids **16** against *T. b. brucei*, and **5, 16** and **20** against *T. evansi*, were uncovered as hits while the remainder stood as early leads. Of these, hybrids **8** and **9** were identified as most promising early leads against *T. b. brucei* based on the combination of their nanomolar activity with more favorable safety profiles (SI > 600–850) while **9** and **18** were unraveled as potential leads against *T. evansi*. Furthermore, all hybrids (except hybrid number **16**) qualified as early leads against *T. congolense*. Of these, the nonyl **6**, undecyl **8**, and the 3-bromobenzyl **19** were identified as most promising early leads on account of their IC_{50} 20 nM activity and highly selective antiparasitic profiles (SI 2400–6300).

4.2. Structure-activity relationship (SAR)

Toxicity is responsible for more than 33% of drug failure in pharmaceutical development, therefore the screening of toxicity in early drug development is critical (Guengerich, 2011). Nitrofurantoin toxicity can also be mediated by redox cycling, which results in oxidative stress and mitochondrial toxicity that is caused by the production of oxygen electron-withdrawing nature of the nitro moiety (Boelsterli et al., 2006). Hall et al. (2010) showed that type I nitroreductases, which reduces the nitro group, is responsible for generating toxic metabolites for antiparasitic activity but may also inhibit mammalian cell growth. Maaland and Guardabassi (2011) studied *in vitro* antimicrobial activity of nitrofurantoin against *Escherichia coli* and *Staphylococcus pseudintermedius* from dogs and cats. The authors concluded that nitrofurantoin was not satisfactory in treating urinary tract infections in small animals due to poor efficacy and high toxicity.

The hybrids of this study had cLog*P* values < 5, thus demonstrating drug-likeness of five according to Lipinski's rule (Lipinski et al., 2001). The *n-alkyl* hybrids **2**–**10** displayed cLog*P* values higher (1.62–4.74) than benzyl hybrids **11**–**12** (1.58–2.82) and better than the parent nitrofurantoin (cLog*P* −0.22) reported by Munsimbwe et al. (2021). The cLog*P* values of *n-alkyl* hybrids increased as the chain length increased, in agreement with the findings of Zuma et al. (2020). Compounds that have high cLog*P* values are often lipophilic and poorly soluble whereas those with low cLog*P* are hydrophilic and associated with poor membrane permeability.

In general, the short alkyl chain hybrids **2**–**5** were more active against *T. congolense* than *T. b. brucei* and *T. evansi*. However, as the chain length increased no significant difference in activity among these hybrids could be observed across all three strains. Hence, for these hybrids the variation in carbon chain length (C5–C8) had marginal influence on the activity. This contradicts an early observation on the

Table 2
Anti-trypanosomal activity of nitrofurantoin-triazole hybrids and cytotoxicity on MDBK cells.

Compd./Drug	Clog*P*[a]	Cytotoxicity, IC_{50} (μM) MDBK[b]) (Mean ± SD)[c]	Anti-trypanosomal activity, IC_{50} (μM) (Mean ± SD)[b]					
			Tbb	SI_1[d]	*Te*	SI_2[e]	*Tc*	SI_3[f]
1*	−0.22	102.75 ± 19.01	1.42 ± 0.15	–	1.96 ± 0.50	–	0.36 ± 0.04	–
2	1.62	66.57 ± 44.62	0.63 ± 0.29	163.65	0.54 ± 0.16	190.54	0.07 ± 0.03	1535.52
3	2.07	210.51 ± 41.91	0.54 ± 0.15	122.34	0.77 ± 0.17	85.49	0.27 ± 0.17	245.37
4	2.51	159.77 ± 78.97	1.48 ± 0.37	141.82	2.00 ± 0.66	105.22	0.14 ± 0.07	1512.12
5	2.96	125.11 ± 2.01	1.37 ± 0.83	116.96	1.90 ± 0.97	84.03	0.07 ± 0.06	2184.71
6	3.40	195.35 ± 43.74	0.44 ± 0.18	285.66	0.65 ± 0.28	193.54	0.02 ± 0.01	6328.76
7	3.85	63.13 ± 30.48	0.30 ± 0.13	661.76	0.52 ± 0.26	374.73	0.09 ± 0.04	2293.50
8	4.29	59.73 ± 12.25	0.09 ± 0.02	686.45	0.18 ± 0.02	349.78	0.02 ± 0.01	3454.36
9	4.74	71.04 ± 9.99	0.07 ± 0.04	849.31	0.12 ± 0.06	504.57	0.03 ± 0.02	2046.63
10	1.81	54.16 ± 5.84	0.54 ± 0.06	131.19	0.56 ± 0.04	127.93	0.18 ± 0.06	385.67
11	1.58	112.95 ± 12.47	0.15 ± 0.05	362.31	0.17 ± 0.05	317.26	0.03 ± 0.01	2127.26
12	2.09	71.04 ± 24.56	0.21 ± 0.07	543.54	0.33 ± 0.05	342.26	0.03 ± 0.02	3569.68
13	2.82	90.31 ± 13.56	0.16 ± 0.04	445.07	0.24 ± 0.06	300.28	0.03 ± 0.02	2423.14
14	3.12	111.00 ± 53.06	0.10 ± 0.04	915.65	0.33 ± 0.05	270.55	0.04 ± 0.03	2487.81
15	2.35	22.02 ± 12.11	0.33 ± 0.08	332.30	0.49 ± 0.18	157.83	0.05 ± 0.03	2112.42
16	1.52	38.73 ± 9.55	1.36 ± 0.39	16.29	1.00 ± 0.34	22.11	0.42 ± 0.31	53.12
17	2.49	23.67 ± 5.26	0.37 ± 0.06	105.28	0.65 ± 0.18	59.72	0.03 ± 0.02	1123.83
18	1.72	53.96 ± 32.58	0.10 ± 0.03	247.04	0.11 ± 0.03	211.07	0.03 ± 0.01	687.78
19	2.35	138.56 ± 44.20	0.26 ± 0.05	208.92	0.70 ± 0.27	109.97	0.02 ± 0.01	2360.41
20	2.46	102.75 ± 19.01	0.54 ± 0.28	256.04	1.52 ± 0.75	91.15	0.12 ± 0.04	1111.77
Suramin		nd	0.07 ± 0.01	–	0.38 ± 0.06	–	7.17 ± 0.87	–
Pentamidine		nd	0.04 ± 0.00	–	0.001 ± 0.00	–	0.33 ± 0.05	–
Nifurtimox		nd	4.66 ± 1.97	–	2.62 ± 1.40	–	1.06 ± 0.22	–
Eflornithine		nd	38.56 ± 9.88	–	57.22 ± 17.56	–	16.13 ± 2.93	–
Diminazene aceturate (DA)		>40[g]	0.07 ± 0.02	>571.43	0.01 ± 0.002	>4000	0.23 ± 0.02	>173.91

Nitrofurantoin 1* reported drug by Munsimbwe et al. (2021).
[a] Calculated from ChemDraw Ultra 12.
[b] MDBK: Madin-Darby bovine kidney.
[c] IC_{50} values (μM) mean ± S.D; Tbb: *Trypanosoma brucei brucei*; Te: *Trypanosoma evansi*; Tc: *Trypanosoma congolense*; SI: Selectivity Index: SI_1.
[d] IC_{50} MDBK/IC_{50} *Tbb*; SI_2.
[e] IC_{50} MDBK/IC_{50} *Te*; SI_3.
[f] IC_{50} MDBK/IC_{50} Tc.
[g] Literature value (Guswanto et al., 2018); blue = hit and black = early lead according to Katsuno et al. (2015) (hit compound: $IC_{50} < 10$ μM and SI > 10; early lead: $IC_{50} < 1$ μM and SI > 100).

antiparasitic action of phosphonium lipocations where short chain compounds (C4–C6) demonstrated reduced activity compared to longer chain (C10) compounds (Long et al., 2012). As the lipophilicity increased so did the activity of *n*-alkyl hybrids **6** (C9) –**9** (C12) against *T. b. brucei* and *T. evansi* parasites (Table 1, Fig. 1c). This may be attributed to better cellular uptake of these hybrids due to favorable lipophilicity enhancing cell permeability as previously reported by Branco Santos et al. (2020) and Munsimbwe et al. (2021). Hence, the lipophilicity influenced the activity of these hybrids as reflected by previous reports (Suganuma et al., 2016; Zuma et al., 2020; El-Wakil et al., 2021; Munsimbwe et al., 2021).

Another physical factor that influences the biological activity of a drug is the electronic effect of the chemical groups in its structure. In this study, the sub-series 2, benzyl hybrids (**11**–**20**) differ by the substituents on the phenyl (Phe) ring that range from electron donating groups (EDG: Me (**12**), *i*-Pr (**13**) and *i*-Bu (**14**) by order of increasing strength), neutral (H (**11**)) to electron withdrawing groups (EWG: 4-Br (**15**), 3-Br (**19**), 4-F (**17**), 2-F 4-Br (**16**), 3-CF_3 (**20**), 4-NO_2 (**18**) by order of increasing strength). These hybrids had variations in cLog*P* values. As the electronic effect increased so did the lipophilicity and activity of EDG-containing hybrids against *T. b. brucei* strain unlike against *T. evansi* and *T. congolense,* where comparable potencies were observed (Table 1, Fig. 1b). No correlation could be found between electronic effect and lipophilicity among EWG-bearing hybrids, which contradicted observations of El-Wakil et al. (2021) who reported derivatives with EWG (F, Cl, Br) in *para*-position having the highest lipophilicity with cLog*P* in the 2.1–2.73 range.

These results corroborated previous findings which showed that an electronic effect influences activity (Munsimbwe et al., 2021). However, hybrid **18** that bears NO_2, the strongest EWG showed nanomolar activity against *T. b. brucei* (0.10 ± 0.03 μM) and *T. evansi* (0.11 ± 0.03 μM) was identified as the most active of all sub-series 2 hybrids against these strains. Hybrid **16** containing two EWGs (3-F and 4-Br) stood out as the least performer, displaying reduced activity against *T. congolense* (0.42 ± 0.31 μM) and micromolar potency ($IC_{50} > 1$ μM) against *T. b. brucei* and *T. evansi*. While conclusive correlation could be found between electronic effect and trypanocidal activity against *T. b. brucei* and *T. congolense* strains among EWG benzyl hybrids, an increase in electronic strength resulted in decreased activity against *T. evansi* with hybrids **17** and **18** being outliers.

Moreover, the hybrids had comparable activity against *T. b. brucei* and *T. evansi* which could be ascribed to both trypanosome species belonging to the subgenus *Trypanozoon* making them very genetically similar (Radwanska et al., 2018). Furthermore, *T. evansi* originates from multiple strains of *T. b. brucei*, hence the hybrids showed similar anti-trypanosomal activity to both species (Carnes et al., 2015; Kamidi et al., 2017). The hybrids displayed higher activity against *T. congolense* than against *T. b. brucei* and *T. evansi*. Similarly, Molefe et al. (2017) noted that azithromycin showed higher *in vitro* efficacy against *T. congolense* than *T. b. brucei* and *T. evansi*. Suganuma et al. (2016) also indicated that three mycophelonic acid derivatives had lower inhibitory activity against *T. b. brucei* and *T. evansi* than against *T. congolense*. *Trypanosoma brucei* leave blood vessels and invade extravascular tissues in the central nervous system (Mulenga et al., 2001). *Trypanosoma congolense* is strictly intravascular, it only invades the blood and is exposed to the host's immune system and this exposure has enabled these African trypanosomes to develop mechanisms for immune evasion (Coustou et al., 2010; Kuriakose et al., 2012).

Zuma et al. (2022) reported that the same hybrids had moderate anti-leishmanial activity. Anti-leishmanial drugs may have trypanocidal

activity because *Leishmania* and *Trypanosoma* spp. share taxonomic similarities (Morrison, 2009). Suramin, pentamidine and diminazene aceturate displayed activity against *T. b. brucei* and *T. evansi*. Similarly, pentamidine and diminazene aceturate displayed activity against *T. congolense*, however, suramin showed weak antitrypanosomal activity against *T. congolense*, which may be associated with pathogen altered specific transport proteins; oxygen consumption, electron transport chain (ETC) and nucleoside transporters (Kasozi et al., 2022). Invariant surface glycoproteins (ISG65) or (ISG75) are conserved in *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi* and *T. equiperdum* (Tran et al., 2018). A study by Makarov et al. (2023) showed that ISG75 directly binds to suramin compounds and mediates the uptake of suramin in *T. brucei* bloodstream form. However, the role of ISGs has not been established in *T. congolense* and this can be associated with resistance to suramin (Awuoche et al., 2018).

The antitrypanosomal activities of pentamidine and diminazene aceturate were better against *T. b. brucei* and *T. evansi* with IC_{50} values lower than against *T. congolense*. Previous studies reported that IC_{50} values of pentamidine and diminazene against *T. b. brucei* and *T. evansi* were 2–100 times lower than against *T. congolense* (Gillingwater et al., 2007; Sykes and Avery, 2009; Suganuma et al., 2014). These studies reported that different transporters of *T. congolense* and *T. b. brucei* are responsible for the different results in pentaminide and diminazene. The P1-type purine transporter (*TbNT10*) was responsible for uptake of diminazene and pentamidine in *T. congolense*. The P2 aminopurine encoded by *T. brucei* adenosine transporter 1 (*TbAT1*) was identified as a transporter in *T. brucei* (Munday et al., 2013) and this gene exists in *T. brucei* subspecies, *T. evansi* and *T. equiperdum* (Matovu et al., 2003; de Koning et al., 2004).

5. Conclusion

In this study, *in vitro* anti-trypanosomal activity of nineteen NFT-triazole hybrids was evaluated against African animal trypanosomes. Hybrids **8** and **9** containing *n*-alkyl substituents and **11**, **13** and **18** bearing benzyl groups exhibited strong antitrypanosomal activity against *T. b. brucei*, *T. congolense* and *T. evansi* with low cytotoxicity. They were identified as trypanocidal early lead compounds and potential candidates for future *in vivo* studies for antitrypanosomal activity and toxicity evaluation.

Ethics approval statement

This study was approved by the scientific committee of the Integrated Pest Management, of the Unit for Environmental Sciences and Management and the Faculty of Natural and Agricultural Sciences Ethics Committee of North–West University (Reference number: NWU–01310–20–A9).

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CRediT authorship contribution statement

Anna Seetsi: Data curation, Formal analysis, Writing – original draft, Investigation, Methodology. **David D. N'Da:** Conceptualization, Data curation, Formal analysis, Writing – review & editing, Supervision. **Nthatisi Nyembe:** Formal analysis, Methodology, Writing – review & editing, Supervision. **Keisuke Suganuma:** Conceptualization, Methodology, Writing – review & editing. **Tsepo Ramatla:** Writing – review & editing, Validation. **Oriel Thekisoe:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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References

Adewusi, E.A., Steenkamp, P., Fouche, G., Steenkamp, V., 2013. Isolation of cycloeucalenol from Boophone disticha and evaluation of its cytotoxicity. Nat. Prod. Commun. 8 (9), 1213–1216. https://doi.org/10.1177/1934578X130080.

Anderson, N.E., Mubanga, J., Fevre, E.M., Picozzi, K., Eisler, M.C., Thomas, R., Welburn, S.C., 2011. Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. PLoS Neglected Trop. Dis. 5 (6), e1211. https://doi.org/10.1371/journal.pntd.0001211.

Aregawi, W.G., Agga, G.E., Abdi, R.D., Büscher, P., 2019. Systematic review and meta–analysis on the global distribution, host range, and prevalence of *Trypanosoma evansi*. Parasites Vectors 12 (67), 1–25. https://doi.org/10.1186/s13071–019–3311–4.

Awuoche, E.O., Weiss, B.L., Mireji, P.O., Vigneron, A., Nyambega, B., Murilla, G., Aksoy, S., 2018. Expression profiling of *Trypanosoma congolense* genes during development in the tsetse fly vector *Glossina morsitans morsitans*. Parasites Vectors 11 (380), 1–18. https://doi.org/10.1186/s13071-018-2964-8.

Bengaly, Z., Sidibé, I., Ganaba, R., Desquesnes, M., Boly, H., Sawadogo, L., 2002. Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. Vet. Parasitol. 108 (1), 1–19. https://doi.org/10.1016/s0304–4017(02)00164–4.

Boelsterli, U.A., Ho, H.K., Zhou, S., Yeow Leow, K., 2006. Bioactivation and hepatotoxicity of nitroaromatic drugs. Curr. Drug Metabol. 7 (7), 715–727. https://doi.org/10.2174/138920006778520606.

Bot, C., Hall, B.S., Álvarez, G., Di Maio, R., González, M., Cerecetto, H., Wilkinson, S.R., 2013. Evaluating 5–nitrofurans as trypanocidal agents. Antimicrob. Agents Chemother. 57 (4), 1638–1647. https://doi.org/10.1128/AAC.02046–12.

Branco Santos, J.C., De Melo, J.A., Maheshwari, S., De Medeiros, W.M.T.Q., de Freitas Oliveira, J.W., Moreno, C.J., Mario Amzel, L., Gabelli, S.B., Sousa Silva, M., 2020. Bisphosphonate–based molecules as potential new antiparasitic drugs. Molecules 25 (11), 2602. https://doi.org/10.3390/molecules25112602.

Carnes, J., Anupama, A., Balmer, O., Jackson, A., Lewis, M., Brown, R., Cestari, I., Desquesnes, M., Gendrin, C., Hertz–Fowler, C., Imamura, H., 2015. Genome and phylogenetic analyses of *Trypanosoma evansi* reveal extensive similarity to *T. brucei* and multiple independent origins for dyskinetoplasty. PLoS Neglected Trop. Dis. 9 (1), e3404 https://doi.org/10.1371/journal.pntd.0003404.

Coustou, V., Guegan, F., Plazolles, N., Baltz, T., 2010. Complete in vitro life cycle of *Trypanosoma congolense*: development of genetic tools. PLoS Neglected Trop. Dis. 4 (3), e618. https://doi.org/10.1371/journal.pntd.0000618.

de Koning, H.P., Anderson, L.F., Stewart, M., Burchmore, R.J., Wallace, L.J., Barrett, M. P., 2004. The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in African trypanosomes. Antimicrob. Agents Chemother. 48 (5), 1515–1519. https://doi.org/10.1128/AAC.48.5.1515–1519.2004.

Desquesnes, M., Gonzatti, M., Sazmand, A., Thévenon, S., Bossard, G., Boulangé, A., Gimonneau, G., Truc, P., Herder, S., Ravel, S., Sereno, D., 2022. A review on the diagnosis of animal trypanosomoses. Parasites Vectors 15 (1), 1–24. https://doi.org/10.1186/s13071–022–05190–1.

El-Wakil, M.H., Meheissen, M.A., Abu-Serie, M.M., 2021. Nitrofurazone repurposing towards design and synthesis of novel apoptotic–dependent anticancer and antimicrobial agents: biological evaluation, kinetic studies and molecular modeling. Bioorg. Chem. 113 (104971), 1–17. https://doi.org/10.1016/j.bioorg.2021.104971.

Finiuk, N.S., Hreniuh, V.P., Ostapiuk, Y.V., Matiychuk, V.S., Frolov, D.A., Obushak, M.D., Stoika, R.S., Babsky, A.M., 2017. Antineoplastic activity of novel thiazole derivatives. Biopolym. Cell 33 (2), 135–146. https://doi.org/10.7124/bc.00094B.

Fu, J., Chen, H., Soroka, D.N., Warin, R.F., Sang, S., 2014. Cysteine–conjugated metabolites of ginger components, shogaols, induce apoptosis through oxidative stress–mediated p53 pathway in human colon cancer cells. J. Agric. Food Chem. 62 (20), 4632–4642. https://doi.org/10.1021/jf501351r.

Gillingwater, K., Büscher, P., Brun, R., 2007. Establishment of a panel of reference *Trypanosoma evansi* and *Trypanosoma equiperdum* strains for drug screening. Vet. Parasitol. 148 (2), 114–121. https://doi.org/10.1016/j.vetpar.2007.05.020.

Giordani, F., Morrison, L.J., Rowan, T.G., De Koning, H.P., Barrett, M.P., 2016. The animal trypanosomiases and their chemotherapy: a review. Parasitology 143 (14), 1862–1889. https://doi.org/10.1017/S0031182016001268.

Guengerich, F.P., 2011. Mechanisms of drug toxicity and relevance to pharmaceutical development. Drug Metabol. Pharmacokinet. 26 (1), 3–14. https://doi.org/10.2133/dmpk.dmpk–10–rv–062.

Guswanto, A., Nugrah, a A.B., Tuvshintulga, B., Tayebwa, D.S., Rizk, M.A., Batiha, G.E., Gantuya, S., Sivakumar, T., Yokoyama, N., Igarashi, I., 2018. 17-DMAG inhibits the multiplication of several *Babesia* species and *Theileria equi* on *in vitro* cultures, and *Babesia microti* in mice. Int J Parasitol Drugs Drug Resist 8 (1), 104–111. https://doi.org/10.1016/j.ijpddr.2018.02.005.

Hall, B.S., Wu, X., Hu, L., Wilkinson, S.R., 2010. Exploiting the drug–activating properties of a novel trypanosomal nitroreductase. Antimicrob. Agents Chemother. 54 (3), 1193–1199. https://doi.org/10.1128/AAC.01213–09.

Hirumi, H., Hirumi, K., 1991. In vitro cultivation of *Trypanosoma congolense* bloodstream forms in the absence of feeder cell layers. Parasitology 102 (2), 225–236. https://doi.org/10.1017/s0031182000062533.

Hirumi, H., Martin, S., Hirumi, K., Inoue, N., Kanbara, H., Saito, A., Suzuki, N., 1997. Cultivation of bloodstream forms of *Trypanosoma brucei* and *T. evansi* in a serum-free medium. Trop. Med. Int. Health 2 (3), 240–244. https://doi.org/10.1046/j.1365–3156.1997.d01–268.x.

Isaac, C., Ohiolei, J.A., Ebhodaghe, F., Igbinosa, I.B., Eze, A.A., 2017. Animal African Trypanosomiasis in Nigeria: a long way from elimination/eradication. Acta Trop. 176, 323–331. https://doi.org/10.1016/j.actatropica.2017.08.032.

Kamidi, C.M., Saarman, N.P., Dion, K., Mireji, P.O., Ouma, C., Murilla, G., Aksoy, S., Schnaufer, A., Caccone, A., 2017. Multiple evolutionary origins of *Trypanosoma evansi* in Kenya. PLoS Neglected Trop. Dis. 11 (9), e0005895 https://doi.org/10.1371/journal.pntd.0005895.

Kasozi, K.I., MacLeod, E.T., Ntulume, I., Welburn, S.C., 2022. An update on African trypanocide pharmaceutics and resistance. Front. Vet. Sci. 9 (828111), 1–23. https://doi.org/10.3389/fvets.2022.828111.

Katsuno, K., Burrows, J.N., Duncan, K., Van Huijsduijnen, R.H., Kaneko, T., Kita, K., Mowbray, C.E., Schmatz, D., Warner, P., Slingsby, B.T., 2015. Hit and lead criteria in drug discovery for infectious diseases of the developing world. Nat. Rev. Drug Discov. 14 (11), 751–758. https://doi.org/10.1038/nrd4683.

Kaufer, A., Ellis, J., Stark, D., Barratt, J., 2017. The evolution of trypanosomatid taxonomy. Parasites Vectors 10 (287), 1–17. https://doi.org/10.1186/s13071-017-2204-7.

Kuriakose, S., Muleme, H.M., Onyilagha, C., Singh, R., Jia, P., Uzonna, J.E., 2012. Diminazene aceturate (Berenil) modulates the host cellular and inflammatory responses to *Trypanosoma congolense* infection. PLoS One 7 (11), e48696. https://doi.org/10.1371/journal.pone.0048696.

Lalmanach, G., Boulangé, A., Serveau, C., Lecaille, F., Scharfstein, J., Gauthier, F., Authié, E., 2002. Congopain from *Trypanosoma congolense*: drug target and vaccine candidate. Bio Chem 383 (5), 739–749. https://doi.org/10.1515/BC.2002.077.

Legros, D., Ollivier, G., Gastellu–Etchegorry, M., Paquet, C., Burri, C., Jannin, J., Büscher, P., 2002. Treatment of human African trypanosomiasis—present situation and needs for research and development. Lancet Infect. Dis. 2 (7), 437–440. https://doi.org/10.1016/s1473–3099(02)00321–3.

Leuin, A.S., Hartmann, F., Viviano, K., 2021. Administration of nitrofurantoin in dogs with lower urinary tract infections: 14 cases (2013-2019). J. Small Anim. Pract. 62 (1), 42–48. https://doi.org/10.1111/jsap.13252.

Liebenehm, S., Affognon, H., Waibel, H., 2011. Collective livestock research for sustainable disease management in Mali and Burkina Faso. Int. J. Agric. Sustain. 9 (1), 212–221. https://doi.org/10.3763/ijas.2010.0572.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 46 (1–3), 3–26. https://doi.org/10.1016/s0169–409x(00)00129–0.

Liu, S., Su, M., Song, S.J., Jung, J.H., 2017. Marine–derived Penicillium species as producers of cytotoxic metabolites. Mar. Drugs 15 (10), 329. https://doi.org/10.3390/md15100329.

Long, T.E., Lu, X., Galizzi, M., Docampo, R., Gut, J., Rosenthal, P.J., 2012. Phosphonium lipocations as antiparasitic agents. Bioorg. Med. Chem. Lett 22 (8), 2976–2979. https://doi.org/10.1016/j.bmcl.2012.02.045.

Lou, J., Chu, G., Zhou, G., Jiang, J., Huang, F., Xu, J., Zheng, S., Jiang, W., Lu, Y., Li, X., Chen, Z., 2010. Comparison between two kinds of cigarette smoke condensates (CSCs) of the cytogenotoxicity and protein expression in a human B–cell lymphoblastoid cell line using CCK–8 assay, comet assay and protein microarray. Mutat Res Genet 697 (1–2), 55–59. https://doi.org/10.1016/j.mrgentox.2010.01.014.

Maaland, M., Guardabassi, L., 2011. In vitro antimicrobial activity of nitrofurantoin against *Escherichia coli* and *Staphylococcus pseudintermedius* isolated from dogs and cats. Vet. Microbiol. 151 (3–4), 396–399. https://doi.org/10.1016/j.vetmic.2011.03.009.

Makarov, A., Began, J., Mautone, I.C., Pinto, E., Ferguson, L., Zoltner, M., Zoll, S., Field, M.C., 2023. The role of invariant surface glycoprotein 75 in xenobiotic acquisition by African trypanosomes. Microb Cell 10 (2), 18–35. https://doi.org/10.15698/mic2023.02.790.

Matovu, E., Stewart, M.L., Geiser, F., Brun, R., Mäser, P., Wallace, L.J., Burchmore, R.J., Enyaru, J.C., Barrett, M.P., Kaminsky, R., Seebeck, T., 2003. Mechanisms of arsenical and diamidine uptake and resistance in *Trypanosoma brucei*. Eukaryot. Cell 2 (5), 1003–1008. https://doi.org/10.1128/EC.2.5.1003–1008.2003.

Mohd Rajdi, N.Z.I., Mohamad, M.A., Tan, L.P., Choong, S.S., Reduan, M.F.H., Hamdan, R.H., Cw Zalati, C.S., 2021. First case report on the occurrence of *Trypanosoma evansi* in a siam B mare in kelantan, Malaysia. Vet. Med. Sci. 7 (2), 303–309. https://doi.org/10.1002/vms3.379.

Molefe, N.I., Musinguzi, P.S., Kondoh, D., Watanabe, K., Thekisoe, O.M., Xuan, X., Inoue, N., Suganuma, K., 2019. Short–and long–term effects of orally administered azithromycin on *Trypanosoma brucei brucei*–infected mice. Exp. Parasitol. 199, 40–46. https://doi.org/10.1016/j.exppara.2019.02.018.

Molefe, N.I., Yamasaki, S., Macalanda, A.M.C., Suganuma, K., Watanabe, K., Xuan, X., Inoue, N., 2017. Oral administration of azithromycin ameliorates trypanosomosis in *Trypanosoma congolense*–infected mice. Parasitol. Res. 116 (9), 2407–2415. https://doi.org/10.1007/s00436–017–5542–7.

Morrison, D.A., 2009. Evolution of the Apicomplexa: where are we now? Trends Parasitol. 25 (8), 375–382. https://doi.org/10.1016/j.pt.2009.05.010.

Morrison, L.J., Vezza, L., Rowan, T., Hope, J.C., 2016. Animal African trypanosomiasis: time to increase focus on clinically relevant parasite and host species. Trends Parasitol. 32 (8), 599–607. https://doi.org/10.1016/j.pt.2016.04.012.

Mulenga, C., Mhlanga, J.D.M., Kristensson, K., Robertson, B., 2001. *Trypanosoma brucei brucei* crosses the blood–brain barrier while tight junction proteins are preserved in a rat chronic disease model. Neuropathol. Appl. Neurobiol. 27 (1), 77–85. https://doi.org/10.1046/j.0305–1846.2001.00306.x.

Munday, J.C., López, K.E.R., Eze, A.A., Delespaux, V., Van Den Abbeele, J., Rowan, T., Barrett, M.P., Morrison, L.J., de Koning, H.P., 2013. Functional expression of TcoAT1 reveals it to be a P1–type nucleoside transporter with no capacity for diminazene uptake. Int J Parasitol Drugs Drug Resist 3, 69–76. https://doi.org/10.1016/j.ijpddr.2013.01.004.

Munsimbwe, L., Seetsi, A., Namangala, B., N'Da, D.D., Inoue, N., Suganuma, K., 2021. *In vitro* and *in vivo* trypanocidal efficacy of synthesized nitrofurantoin analogs. Molecules 26 (11), 3372. https://doi.org/10.3390/molecules26113372.

Ngumbi, A.F., Silayo, R.S., 2017. A cross–sectional study on the use and misuse of trypanocides in selected pastoral and agropastoral areas of eastern and northeastern Tanzania. Parasites Vectors 10 (607), 1–9. https://doi.org/10.1186/s13071–017–2544–3.

Nunes, B.C., Martins, M.M., Chang, R., Morais, S.A., Nascimento, E.A., de Oliveira, A., Cunha, L.C., da Silva, C.V., Teixeira, T.L., Ambrósio, M.A., Martins, C.H., 2016. Antimicrobial activity, cytotoxicity and selectivity index of *Banisteriopsis laevifolia* (A. Juss.) B. Gates leaves. Ind. Crops Prod. 92, 277–289. https://doi.org/10.1016/j.indcrop.2016.08.016.

Odeniran, P.O., Macleod, E.T., Ademola, I.O., Welburn, S.C., 2019. Practices of cattle keepers of southwest Nigeria in relation to bovine trypanosomosis. Trop. Anim. Health Prod. 51 (8), 2117–2126. https://doi.org/10.1007/s11250–018–1694–4.

Patterson, S., Wyllie, S., 2014. Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. Trends Parasitol. 30 (6), 289–298. https://doi.org/10.1016/j.pt.2014.04.003.

Porreca, A., D'Agostino, D., Romagnoli, D., Del Giudice, F., Maggi, M., Palmer, K., Falabella, R., De Berardinis, E., Sciarra, A., Ferro, M., Artibani, W., 2021. The clinical efficacy of nitrofurantoin for treating uncomplicated urinary tract infection in adults: a systematic review of randomized control trials. Urol. Int. 105 (7–8), 531–540. https://doi.org/10.1159/000512582.

Radwanska, M., Vereecke, N., Deleeuw, V., Pinto, J., Magez, S., 2018. Salivarian trypanosomosis: a review of parasites involved, their global distribution and their interaction with the innate and adaptive mammalian host immune system. Front. Immunol. 9 (2253), 1–20. https://doi.org/10.3389/fimmu.2018.02253.

Richards, S., Morrison, L.J., Torr, S.J., Barrett, M.P., Manangwa, O., Mramba, F., Auty, H., 2021. Pharma to farmer: field challenges of optimizing trypanocide use in African animal trypanosomiasis. Trends Parasitol. 37 (9), 831–843. https://doi.org/10.1016/j.pt.2021.04.007.

Sánchez, E., Perrone, T., Recchimuzzi, G., Cardozo, I., Biteau, N., Aso, P.M., Mijares, A., Baltz, T., Berthier, D., Balzano–Nogueira, L., Gonzatti, M.I., 2015. Erratum to: molecular characterization and classification of *Trypanosoma* spp. Venezuelan isolates based on microsatellite markers and kinetoplast maxicircle genes. Parasites Vectors 8 (536), 1–11. https://doi.org/10.1186/s13071–015–1177–7.

Seetsi, A., N'Da, D.D., Molefe-Nyembe, N., Suganuma, K., Ramatla, T., Thekisoe, O., 2023. *In vitro* anti-trypanosomal activity of synthetic nitrofurantoin-triazole hybrids against Trypanosoma species causing human African trypanosomosis. Fundam. Clin. Pharmacol. 1–12 https://doi.org/10.1111/fcp.12940.

Suganuma, K., Allamanda, P., Hakimi, H., Zhou, M., Angeles, J.M., Kawazu, S.I., Inoue, N., 2014. Establishment of ATP–based luciferase viability assay in 96–well plate for *Trypanosoma congolense*. J. Vet. Med. Sci. 76 (11), 1437–1441. https://doi.org/10.1292/jvms.14–0273.

Suganuma, K., Molefe, N.I., Inoue, N., 2017. An ATP–based luciferase viability assay for animal African trypanosomes using a 96–well plate. Methods Mol. Biol. 1601, 89–95. https://doi.org/10.1007/978–1–4939–6960–9_8.

Suganuma, K., N'Da, D.D., Watanabe, K.I., Tanaka, Y., Mossaad, E., Elata, A., Inoue, N., Kawazu, S.I., 2022. Therapeutic efficacy of orally administered nitrofurantoin against animal African trypanosomosis caused by *Trypanosoma congolense* Infection. Pathogens 11 (3), 331. https://doi.org/10.3390/pathogens11030331.

Suganuma, K., Sarwono, A.E.Y., Mitsuhashi, S., Jąkalski, M., Okada, T., Nthatisi, M., Yamagishi, J., Ubukata, M., Inoue, N., 2016. Mycophenolic acid and its derivatives as potential chemotherapeutic agents targeting inosine monophosphate dehydrogenase in *Trypanosoma congolense*. Antimicrob. Agents Chemother. 60 (7), 4391–4393. https://doi.org/10.1128/AAC.02816–15.

Sykes, M.L., Avery, V.M., 2009. Development of an Alamar Blue™ viability assay in 384–well format for high throughput whole cell screening of *Trypanosoma brucei brucei* bloodstream form strain 427. Am. J. Trop. Med. Hyg. 81 (4), 665–674. https://doi.org/10.4269/ajtmh.2009.09–0015.

Tekle, T., Terefe, G., Cherenet, T., Ashenafi, H., Akoda, K.G., Teko–Agbo, A., Van Den Abbeele, J., Gari, G., Clausen, P.H., Hoppenheit, A., Mattioli, R.C., 2018. Aberrant

use and poor quality of trypanocides: a risk for drug resistance in southwestern Ethiopia. BMC Vet. Res. 14 (4), 1–8. https://doi.org/10.1186/s12917–017–1327–6.

Tran, T., Büscher, P., Vandenbussche, G., Wyns, L., Messens, J., De Greve, H., 2018. Heterologous expression, purification and characterisation of the extracellular domain of trypanosome invariant surface glycoprotein ISG75. J. Biotechnol. 135 (3), 247–254. https://doi.org/10.1016/j.jbiotec.2008.04.012.

Ukpai, O.M., Nwabuko, O.P., 2014. Effects on haematological parameters and pathology of internal organs of *Trypanosoma brucei brucei* infected albino rats. Niger. J. Biotechnol. 27, 8–13.

Weese, J.S., Blondeau, J.M., Boothe, D., Breitschwerdt, E.B., Guardabassi, L., Hillier, A., Lloyd, D.H., Papich, M.G., Rankin, S.C., Turnidge, J.D., Sykes, J.E., 2011. Antimicrobial use guidelines for treatment of urinary tract disease in dogs and cats: antimicrobial guidelines working group of the international society for companion animal infectious diseases. Vet. Med. Int. 2011 (263768), 1–9. https://doi.org/10.4061/2011/263768.

Zuma, N.H., Aucamp, J., Viljoen, M., N'Da, D.D., 2022. Synthesis, *in vitro* antileishmanial efficacy and hit/lead identification of nitrofurantoin-triazole hybrids. ChemMedChem 17 (10), e202200023. https://doi.org/10.1002/cmdc.202200023.

Zuma, N.H., Smit, F.J., Seldon, R., Aucamp, J., Jordaan, A., Warner, D.F., David, D.D., 2020. Single–step synthesis and *in vitro* anti–mycobacterial activity of novel nitrofurantoin analogs. Bioorg. Chem. 96 (103587), 1–11. https://doi.org/10.1016/j.bioorg.2020.103587.