Re-screening and preliminary *in vitro* evaluation of resorcinol based Hsp90 inhibitors for potential protocidal activity

Théoneste Umumararungu[1,2]* 🆔, Michelle Isaacs[3] 🆔, Heinrich C. Hoppe[3,4] 🆔,, Eleonora D. Goosen[2] 🆔 and
Setshaba D. Khanye[2,3]* 🆔

[1]Department of Pharmacy, School of Medicine and Pharmacy, College of Medicine and Health Sciences, University of Rwanda, Rwanda
[2]Division of Pharmaceutical Chemistry, Faculty of Pharmacy, Rhodes University, Makhanda, South Africa
[3]Centre for Chemico- and Biomedicinal Research, Rhodes University, Makhanda, South Africa
[4]Department of Biochemistry and Microbiology, Rhodes University, Makhanda, South Africa

ABSTRACT

This study explored a series of resorcinol derivatives originally identified as heat shock protein 90 inhibitors for activity against protozoan parasites. The repurposing of this series revealed that some of these compounds are active against chloroquine-sensitive *Plasmodium falciparum* (3D7) and *Trypanosoma brucei brucei* strains with IC$_{50}$ values below 10 µM without obvious cytotoxic effects against human cervix adenocarcinoma (HeLa) cells at 25 µM.

INTRODUCTION

Malaria is still considered a serious global concern to a large population in tropical regions. In 2020, WHO reported approximately 627 000 malaria deaths, with sub-Saharan Africa accounting for 96% of these.[1] The slight increase in deaths in 2020 from 558 000 in 2019 is attributable to the COVID-19 pandemic, which stalled malaria control programmes. Although a slight upward trend emerged in 2020, global efforts in the last two decades have led to a significant decline in deaths among children under five years in sub-Saharan Africa.[1] Following a period in which antimalarial drugs lost efficacy due to drug resistance, the World Health Organization (WHO) declared artemisinin-based combination therapies (ACTs) the first line of defence against uncomplicated *P. falciparum* malaria.[2] Concerns of emergence of clinical resistance to ACTs — including to both artemisinin derivatives and partner drugs such as the 4-aminoquinolines or aminoalcohols — are on the rise.[3] Thus, fears that this resistance will spread to resource-limited endemic parts of Africa and reverse the gains achieved against malaria eradication are justified.[3-5] Despite numerous on-going efforts to bring a malaria vaccine to the clinic, developing an effective vaccine suitable for a diverse population remains an elusive target.[4,6] More recently, clinical trials for the novel, promising fast-acting anti-malarial drug candidates MMV048, P218 and DSM265 (Figure 1) were discontinued because of clinical toxicity.[7] Such discontinuation of clinical trials for investigational new drugs (INDs) is not restricted to malaria drug discovery and development. Rather, it highlights the high attrition rate of compounds in drug development endeavours prevalent across all drug discovery projects. The limitations of existing antiparasitic drugs in clinical use, coupled with the high attrition rates encountered in drug development phases, thus obviates the need to continue searching for new classes of compounds with novel mechanisms of action.[3,4] On the other hand, human African Trypanosomiasis (HAT) or sleeping sickness, caused by *Trypanosoma brucei* parasites is an important insect-borne protozoan parasitic disease that affects poor populations in remote areas of sub-Saharan Africa.[8,9] Geographically, *T. b. gambiense* is common in central and West Africa, while *T. b. rhodesiense* is widespread in the Eastern part of

Africa.[10-12] These parasites are transmitted to humans following bites by tsetse flies during feeding on human blood. The treatment options for HAT include a handful of FDA-approved drugs: pentamidine, suramin, melarsoprol, eflornithine and nifurtimox, which have been used for several decades.[13,14] These drugs, however, have shortcomings that limit their clinical use, such as reduced efficacy, toxic side effects and the emergence of resistance. Currently, the first-line treatment includes pentamidine for stage 1 of the disease. On other hand, nifurtimox-eflornithine combination therapy (NECT) is used to treat stage 2 of the disease, an important stage of the disease wherein parasites spread and invade the central nervous system (CNS). The recent increased campaigns for alternative drug candidates have yielded two effective compounds, fexinidazole and SCYX-7158 (Figure 1), which are currently undergoing clinical trials for the treatment of HAT.[14-18] Moreover, these drug candidates show positive outcomes for treating stages 1 and 2 of the disease.

Exploiting inhibitors of human enzymes that are homologous to parasitic equivalents is an efficient strategy that researchers have used to successfully identify new antiparasitic agents.[19-22] The heat shock protein 90 (Hsp90) inhibitors, which have drawn considerable attention
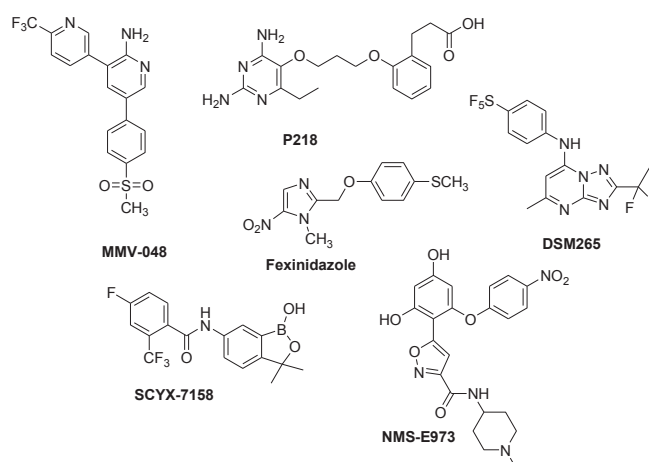

Figure 1: Antiprotozoal drugs in clinical development and the Hsp90 inhibitor NMS-E973

*To whom correspondence should be addressed
Email: s.khanye@ru.ac.za, umumararungut@yahoo.fr

as anticancer agents, are increasingly being explored as potential antiprotozoal agents.[23-25] Hsp90, a molecular chaperone that regulates protein folding and gene expression, is critical for survival of protozoan parasites such as *P. falciparum*, *T. evansi*, *Eimeria tenella*, *Leishmania donovani* and *Toxoplasma gondii* under the severe environmental conditions in which they exist within the hosts.[24] Previous studies on Hsp90 inhibitors showed that treating mice infected with malaria and surra with geldanamycin — an Hsp90 inhibitor — prolonged their survival rate compared with the untreated ones.[26] A recent study by Giannini *et al* explored Hsp90 inhibitors, originally developed for cancer treatment, for their ability to inhibit growth of human protozoan parasites *in vitro*.[27] Data from this this study revealed that *P. falciparum* and *T. b. rhodesiense* parasites showed sensitivity to the Hsp90 inhibitors, while *T. cruzi* and *Giardia lamblia* were less sensitive. Considering the effects of these Hsp90 inhibitors against protozoan parasites, we sought to repurpose the resorcinol derivatives previously reported by Brasca et al[28] for their potential antiplasmodial and antitrypanosomal activities against *P. falciparum* and *T. b. brucei*, respectively. We hypothesized that the hit compound NMS-E973 (Figure 1) — a selective inhibitor of Hsp90 with significant efficacy against human ovarian A2780 cancer cell line — would be active against protozoan parasites that express Hsp90. The aim of this study was, thus, to repurpose novel derivatives of NMS-E973 Hsp90 inhibitors as antiprarasitic agents, and to determine their potential as growth inhibitors of *P. falciparum* and *T.b. brucei*. Herein, we report the synthesis and *in vitro* evaluation of selected resorcinol derivatives for their potential pharmacological activity against strains of the protozoal parasites *P. falciparum* and *T. b. brucei*.

EXPERIMENTAL

Commercially available chemicals and reagents used in this project were purchased from Sigma-Aldrich and Merck and were used without further purification unless stated otherwise. The synthesis and characterisation of compounds **TU-011** to **TU-019** have been reported previously.[28] The full characterisation data is provided in the Supplementary Information file.

In vitro antiplasmodial activity assay

The 3D7 strain *P. falciparum* was cultured in medium consisting of RPMI 1640 containing 25 mM Hepes (Lonza), supplemented with 5% (w/v) Albumax II (ThermoScientific), 22 mM glucose, 0.65 mM hypoxanthine, 0.05 mg/mL gentamicin and 2–4% (v/v) human erythrocytes. Cultures were maintained at 37 °C under an atmosphere of 5% CO_2, 5% O_2, 90% N_2 gas mixture. To measure the antiplasmodial activity, three-fold serial dilutions of each test compound in culture medium were added to parasite culture (adjusted to 2% parasitaemia, 1% haemotocrit) in 96-well plates and incubated at 37 °C for 48 h. Duplicate wells per compound concentration were used. Parasite lactate dehydrogenase (pLDH) enzyme activity in the individual wells was assessed as previously described.[29,30]

In vitro antitrypanosomal activity assay

Trypanosoma brucei brucei 427 trypomastigotes were cultured in Iscove's Modified Dulbecco's medium (IMDM, Lonza, Basel Switzerland) and supplemented with 10% fetal calf serum, HMI-9 supplement,[31,32] hypoxanthine and penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. Serial dilutions of each test compound were incubated with the parasites in 96-well plates at 37 °C for 24 h. The residual parasite viability in the wells was determined by adding 20 µL resazurin toxicology reagent (Sigma-Aldrich) and incubating for an additional 24 h. The reduction of resazurin to resorufin by viable parasites was assessed by fluorescence readings (excitation 560 nm, emission 590 nm) in a Spectramax M3 plate reader (Molecular Devices, San Jose, CA, USA). Fluorescence readings were converted to % parasite viability relative to the average readings obtained from untreated control wells. IC_{50} values were determined by plotting % viability vs. log[compound]

and performing non-linear regression using GraphPad Prism (v. 5.02) software.[29]

In vitro cell cytotoxicity assay

HeLa cells (Cellonex) seeded in 96-well plates were incubated with 25 µM test compounds for 24 h as previously described.[33] The cell viability was assessed using a resazurin fluorescence assay.

Computational methods

Virtual screening of the candidate compounds was done using Autodock vina.[34] Firstly, the energy of each ligand was minimised with Vegazz. Then the ligand and the target protein (*Pf*Hsp90 (PDB code: 3K60) or human Hsp90 (PDB code: 1BYQ)) were prepared with Autodock tools for docking with Autodock vina. Compounds whose estimated maximum length was less than 15 Å, were docked in a 25 Å × 25 Å × 25 Å box, and those with an estimated maximum length equal to or larger than 15 Å, were docked in a 30 Å × 30 Å × 30 Å box. The intention was to increase the docking speed, thus minimising the docking time while preserving the accuracy of predictions of the binding modes of the candidate compounds. Then, the ratio between the inhibition constants of the best binding modes of each ligand for human Hsp90 and *Pf*Hsp90 was determined and used to decide which compounds would selectively bind to *Pf*Hsp90 in the presence of human Hsp90. The approximate dimensions of the ligands were determined using Vegazz. ADMET properties of synthesised compounds were obtained from SwissADME online server[35] using the SMILES inputs of each compound generated by ChemDraw software.

RESULTS and DISCUSSION

Chemistry

Synthesis of this series has been reported previously by Brasca and co-workers.[28] In a slight modification of Brasca's methodology,
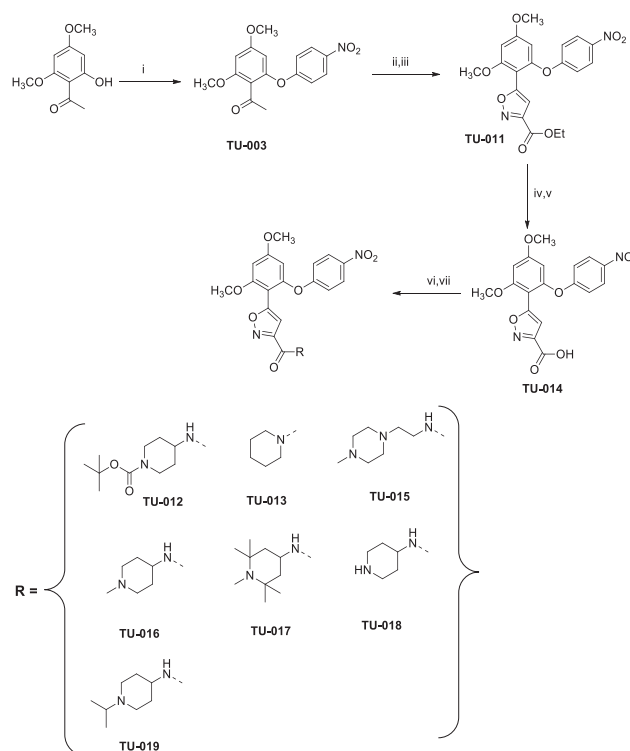
Scheme 1: Reagents conditions: (i) 1-Fluoro-4-nitrobenzene, K_2CO_3, DMSO, 45 °C, 4 h; (ii) THF, lithium bis(trimethylsilyl)amide, diethyl oxalate, −5 °C to r.t., overnight under Ar; (iii) NH_2OH, ethanol, reflux for 5 h; (iv) H_2O, KOH, 80 °C, overnight; (v) 1 M HCl; vi) DMA, TBTU, DIPEA, an amine, r.t., 48 h; vii) Saturated $NaHCO_3$ solution

compounds **TU-011** to **TU-019** were readily obtained in moderate to good yields (Scheme 1). All the synthesised compounds **TU-011** to **TU-019** were fully characterised by ^{1}H and ^{13}C NMR and mass spectrometry (MS) and information is provided as the electronic supplementary file.

In vitro biological assays

The series of resorcinol derivates **TU-014–019** (Scheme 1) were screened *in vitro* for activity against *P. falciparum* (3D7 strain) and *T. brucei* (427 strain). Solutions of compounds **TU-014** to **TU-019** suspended in DMSO were used to determine their cytotoxicity against human cervix adenocarcinoma (HeLa) cells. Chloroquine, pentamidine and emetine were used as positive controls for antiplasmodial, antitrypanosomal activity and cytotoxicity (Figure S2), respectively. The results are summarised in Table 1 (below) and the electronic supplementary file.

Except for compounds **TU-014** (IC_{50} ~ 25 µM), **TU-017** (IC_{50} = 28.5 µM), **TU-018** (IC_{50} = 24.8 µM) and **TU-019** (IC_{50} ~ 25.0 µM), which showed modest cytotoxicity, the rest of compounds showed minimal growth inhibition of HeLa cell line at 100 µM concentration, resulting in >70% HeLa cell viability. Initially, the resorcinol series **TU-011** to **TU-019** was evaluated for *in vitro P. falciparum* inhibitory activity at a single concentration of 25 µM (DMSO) in duplicates using malaria parasite lactate dehydrogenase (pLDH) assays.[36] At this concentration, most of the compounds showed no activity, with parasite percentage viability above 80% for most (Table 1). Compounds **TU-015** and **TU-017** to **TU-019** reduced parasite viabilities below the 50% threshold; accordingly, dose-response analyses were conducted

on these compounds to determine the corresponding IC_{50} values (Figure 2). In general, these compounds were moderately active against the 3D7 strain, with IC_{50} values below 10 µM. Compound **TU-018** (IC_{50} = 1.83 µM) emerged as the most active derivate from this series. Similarly, the solutions containing **TU-011** to **TU-019** were prepared to a single fixed concentration of 25 µg/mL (DMSO). Of the nine compounds, compounds **TU-012** and **TU-017** to **TU-019** suppressed *T. b. brucei* viability below 30% parasite (Table 1) at 25 µg/mL (DMSO). The IC_{50} values of the four active compounds were determined, and the data is depicted in **Fig. 3**. The results show that the compounds were active against *T. b. brucei* within a narrow IC_{50} window of 2 – 6 µM. Once again, the most active compound was **TU-018**, with an IC_{50} value of 2.87 µM. The superior activity of **TU-018** compared to analogous compounds (e.g., **TU-012**, **TU-016** and **TU-019**) could be from the increased basicity of the secondary amine of the piperidine ring, which maybe more favourable for accumulation at the site of action and therefore activity against *P. falciparum* and *T.b. brucei*, respectively. Compared to the reference drugs choroquine and pentamidine, **TU-014** to **TU-019** displayed reduced activity.

In silico drug-likeness prediction

To be effective as a chemotherapeutic agent, a drug must reach a specific target in sufficient concentration. Each year, many promising therapeutic agents that are advanced from early drug discovery stages fail to reach clinical trials because of undesirable drug-likeness and poor pharmacokinetic properties.[37,38] Thus, it is critical to profile the absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of new molecules intended for medicinal applications. In recent years, researchers have applied open access *in silico* tools to assess compound ADME parameters in early-phase drug discovery and development processes.[35,37] In this work, each compound's drug-likeness was assessed using the SwissADME[35] web tool to generate relevant physicochemical parameters with structural inputs derived from the synthesised compounds. The compounds **TU-011** to **TU-019** were profiled by applying Lipinski's rule of five (Ro5), a decision guide that assesses the theoretical oral bioavailability of orally administered drugs. Lipinski's Ro5 proposes that in order to exhibit good oral bioavailability a compound should have a partition coefficient (XLogP) ≤ 5.0, molecular weight ≤ 500, hydrogen bond acceptors ≤ 10, and donors' ≤ 5. A compound that violates more than one of these rules is likely to demonstrate/have unfavourable oral bioavailability.[39] Data from *in silico* screening of our compound set using SwissADME web tool are shown in Table 2. These *in silico* ADME data suggest that the compounds have acceptable ADME properties despite compounds **TU-012**, **TU-015**, **TU-017** and **TU-019** showing molecular weights > 500 Da. Within the context of possessing

Table 1: *In vitro* antiprotozoal activity and cytotoxicity of compounds

Compound	%Viability ± SD		
	Pf (3D7)	*T.b. brucei (427)*	*HeLa cells*
TU-011	95.1 ± 2.8	103.9 ± 0.1	100.3 ± 8.7
TU-012	105.7 ± 0.8	23.8 ± 0.5	108.9 ± 0.7
TU-013	93.5 ± 9.5	111.7 ± 4.5	106.4 ± 12.4
TU-014	113.7 ± 3.4	99.9 ± 4.9	69.3 ± 2.4
TU-015	19.5 ± 2.9	68.1 ± 10.7	78.2 ± 7.7
TU-016	111.1 ± 2.4	101.0 ± 5.4	107.6 ± 6.1
TU-017	32.7 ± 0.4	2.29 ± 0.1	27.1 ± 0.4
TU-018	37.5 ± 1.6	2.60 ± 0.1	−0.44 ± 0.6
TU-019	49.7 ± 9.3	2.70 ± 0.7	6.47 ± 5.9
Control	0[a]	0[b]	0[c]

Positive control drugs: [a]CQ = Chloroquine, [b]PMD = Pentamidine and [c]EMT = Emetine.
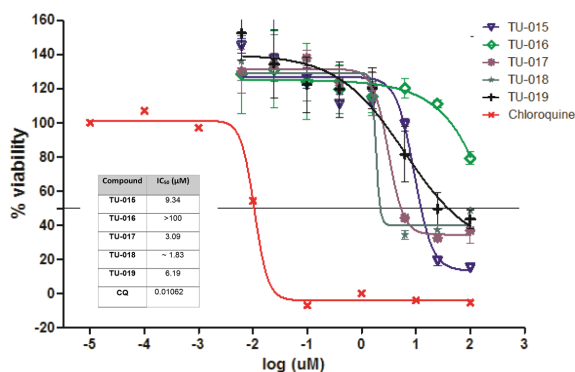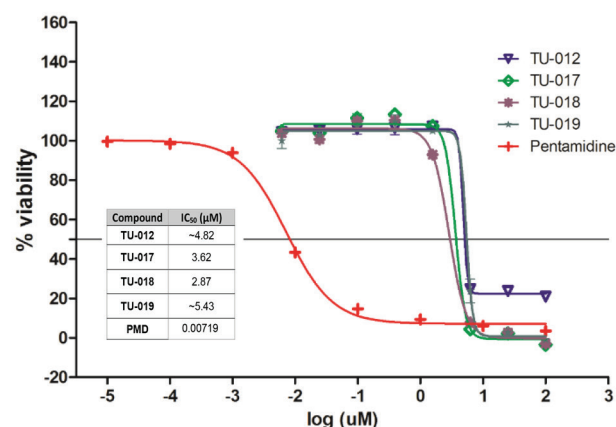

Figure 2: The antiplasmodial activity of selected compounds. Plot of percentage viability against log concentration of selected compounds and control drug (CQ = chloroquine), showing IC_{50} values against CQS 3D7 strain of *P. falciparum*

Figure 3: The antitrypanosomal activity of selected compounds. Plot of percentage viability against log concentration of selected compounds and control drug (PMD = pentamidine), showing IC_{50} values against *T.b. brucei*

Table 2: Physicochemical properties and drug-likeness parameters

Compound	Mw	XlogP3	#HBD	#HBA	#NRB	tPSA	PA
TU-011	414.37	3.88	0	9	9	125.84	0
TU-012	568.58	4.38	1	10	12	158.18	0
TU-013	453.44	3.97	0	8	8	119.85	0
TU-014	386.31	3.19	1	9	7	136.84	0
TU-015	511.53	2.79	1	10	11	135.12	0
TU-016	482.49	3.53	1	9	9	131.88	0
TU-017	538.59	4.77	1	9	9	131.88	0
TU-018	468.46	3.07	2	9	9	140.67	0
TU-019	510.54	4.33	1	9	10	131.88	0

Note: Molecular weight (MW), atomistic octanol-water partition coefficient (XLogP3), number of hydrogen donors (#HBD), number of hydrogen acceptors (#HBA), number of rotatable bonds (#NRB), topological polar surface area (tPSA), PAINS alert (PA)

desirable *in silico* ADME properties, we predict **TU-014** to **TU-019** to exhibit a low risk of acting as pan-assay interference (PAINS), suggesting they are not promiscuous binders or inhibitors.

Molecular docking studies

To investigate the possible molecular mechanism responsible for the antiplasmodial activity of selected compounds **TU-015** and **TU-017** to **TU-019**, we performed molecular docking analyses using AutoDock Vina with *P. falciparum* Hsp90 (*Pf*Hsp90). These compounds showed activity against *P. falciparum* and *T.b. brucei* protozoan parasites with IC$_{50}$ values below 10 µM. We reasoned that such docking analyses would provide the insight into key interactions between ligands **TU-015** and **TU-017** to **TU-019** and *Pf*Hsp90 enzyme. The structure of the *N*-terminal nucleotide, which is the drug binding domain of *Pf*Hsp90 has been reported in literature.[40] Thus, the ADP-bound crystal structures of human Hsp90 (PDB code: 1BYQ) and *Pf*Hsp90 (PDB code: 3K60) were retrieved from the protein data bank to perform the molecular docking studies. Based on the inhibition constants of *h*Hsp90 and *Pf*Hsp90 from the molecular docking studies, compounds **TU-015** and **TU-017** to **TU-019** showed acceptable minimum energies in which all cases these energies were associated with low binding affinity (K$_i$) values (Table 3). More importantly, analysis of conformations and positions of **TU-018** showed that these compounds exhibit a slight variability in terms of conformations inside the *h*Hsp90 and *Pf*Hsp90 binding pockets (Figure 4). Previously, Wang and co-workers identified antimalarial compounds target a unique *Pf*Hsp90 hydrophobic pocket that extend from the lower edge of the conserved Hsp90 ATP binding pocket.[41] It is possible that the compounds showing low binding affinity values in this study could be interacting with specific *Pf*Hsp90 hydrophobic pocket.[42]

CONCLUSIONS

In this work, we synthesized a focused series of resorcinol derivatives **TU-014** to **TU-019**, initially developed as human Hsp90 inhibitors, and evaluated them for their activity against protozoan parasites *P. falciparum* and *T.b. brucei*, respectively. Although most compounds showed modest to weak activities, **TU-018** displayed superior antiplasmodial and antitrypanosomal activity against both *P. falciparum* and *T.b. brucei* (IC$_{50}$ < 3 µM against both) without cytotoxicity against the HeLa cell line. Amongst other potential drug targets, the docking studies suggested that the antiprotozoal activity of these compounds could result from inhibition of Hsp90, an important molecular chaperone for survival of *P. falciparum* and *T.b. brucei* protozoan parasites. Despite the limited small compound set, the preliminary structure-activity relationship (SAR) suggests that the isoxazole amide moiety consisting of non-aromatic heterocyclic ring

Table 3: Binding energies of compounds **TU-015** and **TU-017** to **TU-019** obtained with AutoDock Vina

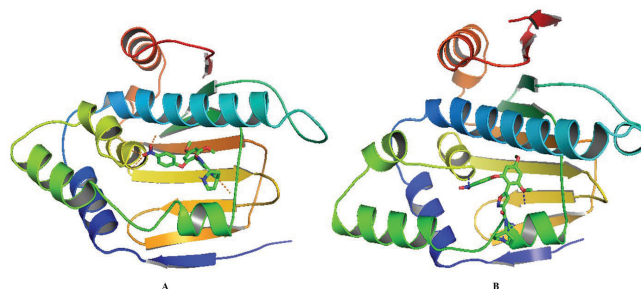| Compound	Parameters associated with binding to hHsp90		Parameters associated with binding to *Pf*Hsp90	
	Binding energy (kcal/mol)	Inhibition constant Ki (µM)	Binding energy (kcal/mol)	Inhibition constant Ki (µM)
TU-015	−8.2	1.0	−8.1	1.2
TU-017	−8.6	0.5	−8.6	0.5
TU-018	−7.8	1.8	−7.8	1.9
TU-019	−8.3	0.8	−8.3	0.8

Figure 4: Molecular docking study of **TU-018** and Hsp90 enzymes. Propose docking pose of **TU-018** bound to human *h*Hsp90 (**A**) and *P. falciparum* *Pf*Hsp90 (**B**) binding pockets

systems provide extra interactions with Hsp90 enzyme and is critical for activity. *In silico* SwissADME prediction of the series indicated that these compounds possess acceptable ADMET and physicochemical properties. We believe that prosecuting these hits further against *P. falciparum* and *T.b. brucei* will increase the pool of quality starting points and spur further synthetic efforts in the quest for new antiprotozoal and antitrypanosomal agents. Finding new agents that act *via* new modes of action will have positive implications on the present treatment regiments that are under threat from emerging drug resistance, or at best, produce suboptimal therapeutic outcomes. Further optimization of these compounds through appropriate structural modifications core structure is currently underway in our labs and will be reported elsewhere.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

Supplementary information for this article is provided as an online supplement. It contains experimental procedure, spectral characterisation data for the title compounds, including copies of the IR, MS, ^{1}H NMR and ^{13}C NMR spectra (Appendix B). The supplementary information file also included pLDH malaria (Figure S1) and cell cytotoxicity (Figure S2) assays.

SUPPLEMENTARY MATERIAL

Supplementary information for this article is provided in the online supplement.

ORCID IDs

Théoneste Umumararungu – https://orcid.org/0000-0002-5684-9607
Michelle Isaacs – https://orcid.org/0000-0002-0694-7487
Heinrich C Hoppe – https://orcid.org/0000-0003-4409-1955
Eleonora D Goosen – https://orcid.org/0000-0002-1470-9956
Setshaba D Khanye – https://orcid.org/0000-0003-0725-5738

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