

Investigation of Novel Isatinylyhydantoin Derivatives as Potential Anti-Kinetoplastid Agents

Keamogetswe Sechoaro,^[a] Janine Aucamp,^[a] Christina Kannigadu,^[a]
Helena D. Janse van Rensburg,^[a] Keisuke Suganuma^{+,*[b]} and David D. N'Da^{+,*[a]}

Neglected tropical diseases are a group of infectious diseases with a high endemicity in developing countries of Africa, Asia, and the Americas. Treatment for these diseases depends solely on chemotherapy, which is associated with severe side effects, toxicity, and the development of parasitic resistance. This highlights a critical need to develop new and effective drugs to curb these diseases. As a result, a series of novel isatinylyhydantoin derivatives were synthesized and evaluated for *in vitro* anti-kinetoplastid activity against seven human- or animal-infective *Trypanosoma* and two human-infective *Leishmania* species. The synthesized derivatives were tested for potential cytotoxicity against human, animal, and parasite host-related cell lines. The

isatinylyhydantoin hybrid **4b** bearing 5-chloroisatin and *p*-bromobenzyl moieties, showed strong trypanocidal activity against blood-stage *T. congolense* parasites; however, the promising *in vitro* trypanocidal potency of **4b** could not be translated to *in vivo* treatment efficacy in a preliminary animal study. Compounds **5**, **2b**, and **5b**, were the most active against amastigotes of *L. donovani*, showing higher leishmanicidal activity than the reference drug, amphotericin B. These compounds were identified as early antileishmanicidal leads, and future investigations will focus on confirming their antileishmanial potential through *in vivo* efficacy evaluation as well as their exact mechanism of action.

1. Introduction

Neglected tropical diseases (NTDs) are widespread in subtropical and tropical countries and affect more than one billion people in poor areas.^[1] Trypanosomiasis and leishmaniasis are the most common NTDs that have a significant impact on human and animal health globally.^[2] These diseases are caused by taxonomically related protozoan parasites, *Trypanosoma* and *Leishmania*, which belong to the Trypanosomatidae family and have similar biochemical and structural features.^[3]

Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by two sub-species of unicellular flagellate parasites, *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, and is spread through the bite of an infected tsetse fly.^[4] *T. b. gambiense* is responsible for causing anthroponotic disease, whereas *T. b. rhodesiense* leads to zoonotic disease.^[5] The number

of HAT cases recorded in 2022 has decreased worldwide to < 1000 yearly.^[6] However, over fifty million people remain at risk of contracting this disease, particularly in sub-Saharan Africa.^[6]

HAT treatment depends on the form and stage of the disease.^[6] Pentamidine and suramin are used to treat the first or haemolymphatic stage of *T. b. gambiense* and *T. b. rhodesiense*, respectively, and they have been reported to have poor efficacy.^[2] Eflornithine and nifurtimox (only as a component of NECT) are active against the second or meningo-encephalitic stage of *T. b. gambiense*, whereas melarsoprol is used for the treatment of the second stage of both *T. b. gambiense* and *T. b. rhodesiense* infection.^[2] However, all of these drugs have been shown to have severe problems such as toxic side effects, high cost, time-consuming parenteral administration, and the development of parasitic resistance.^[4] In 2021, US FDA (Food and Drug Administration) approved fexinidazole as the first all-oral treatment for both stages of HAT.^[7]

American trypanosomiasis, also known as Chagas disease (CD), is a deadly disease caused by the flagellated protozoan parasite *Trypanosoma cruzi*.^[8] It is transmitted to humans through contact with the infected feces of triatomine bugs.^[9] More than sixty million people are infected worldwide, particularly in impoverished areas in Latin America.^[8]

The current curative options for Chagas disease are limited, as benznidazole and nifurtimox are the only approved drugs developed more than fifty years ago.^[9] These drugs can potentially prevent or reduce acute infections if given early, but they are ineffective in treating chronic infections.^[10] They are also associated with significant adverse effects, which prompt therapy termination and restrict their long-term usage.^[11]

Animal trypanosomiasis (AT) is caused by several species and strains of the genus *Trypanosoma* and affects both domestic and wild animals.^[12] *T. b. brucei* and *T. congolense*

[a] K. Sechoaro, J. Aucamp, C. Kannigadu, H. D. Janse van Rensburg, D. D. N'Da⁺
Centre of Excellence for Pharmaceutical Sciences (Pharmacem), Faculty of Health Sciences, North-West University, 11 Hoffmann Street, Potchefstroom 2520, South Africa
E-mail: David.nda@nwu.ac.za

[b] K. Suganuma⁺
National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan
E-mail: k.suganuma@obihiro.ac.jp

[†] Equal contribution

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cmdc.202400533>

© 2024 The Authors. ChemMedChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

mainly infect cattle, causing a complex disease called nagana, and are transmitted cyclically by tsetse flies in sub-Saharan Africa.^[13] It is estimated that more than 50 million cattle and approximately 100 million small ruminants are at risk of contracting this disease.^[14]

Other pathogenic trypanosomes affecting livestock include *T. evansi*, the causative agent of surra, a major disease in camels and horses. It is transmitted mechanically by biting insects such as tabanids (horseflies) and *Stomoxys* spp..^[15] About 12 million camels worldwide are at risk of developing this dangerous disease, which is predicted to cost a total of 1.3 billion US dollars annually as compared to the price of both milk and meat.^[16] *T. equiperdum*, the causative agent of dourine, is a fatal disease sexually transmitted among equines.^[15,17]

Currently, control drugs for AT include homidium bromide, isometamidium chloride, quinapyramine sulphate, diminazene aceturate, and melarsomine, which have been reported to have poor effectiveness, toxicity, drug resistance, and lower standards of quality control.^[18]

Leishmaniasis is the second most common vector-borne parasitic disease after malaria, which affects about 700,000 to 1 million people every year, resulting in approximately 20,000 to 30,000 deaths yearly.^[19] This disease is transmitted to humans by the bite of infected female phlebotomine sandflies and can develop into three distinct forms, namely visceral (VL), cutaneous (CL), and mucocutaneous leishmaniasis (MCL).^[19b] CL is the most common, causing skin sores and significant scarring, while VL is the most severe, affecting several internal organs and being typically fatal if not treated.^[19b] The main drugs used to treat leishmaniasis include pentavalent antimonials, miltefosine, pentamidine, paromomycin, and liposomal amphotericin B.^[20] However, these drugs are toxic, and their excessive usage and patient compliance issues have led to the development of drug resistance.^[20]

These limitations, along with the absence of an effective vaccine, emphasize the need to develop novel drugs for these diseases.^[21] In this study, the focus was on isatin and hydantoin heterocyclic scaffolds, which have been of great interest in drug discovery. Their derivatives have been shown to possess diverse biological activities, including antitrypanosomal and antileishmanial properties.

Isatin derivatives demonstrate inhibitory activity against cysteine proteases.^[22] Cysteine proteases (CPs) are secreted proteins widely present in various parasites. CPs play key roles in the pathogenesis of protozoan parasites, including cell/tissue penetration, hydrolysis of host or parasite proteins, autophagy, and evasion or modulation of the host immune response. CPs also adhere to host cells, tissue invasion, cytotoxicity and nutrient uptake,^[23] making them attractive chemotherapeutic and vaccine targets. In *Leishmania*, CPs are putative virulence factors affecting growth, viability, and pathogenicity.^[24] Cruzipains are the main papain-like cysteine proteases of *T. cruzi*, the protozoan parasite that causes Chagas disease.^[25]

Chiyanzu et al.^[26] previously investigated a series of isatin-3-thiosemicarbazone and *N*-substituted isatin derivatives against *T. cruzi* and *T. b. rhodesiense*. Among these, the derivative bearing the thiosemicarbazone moiety and that containing the

biphenyl substructural motif, were the most active against *T. cruzi* and *T. b. rhodesiense*, respectively, as shown in Figure 1.

In another study conducted by Sabt et al.,^[27] 20 novel quinoline-isatin hybrids were tested for their *in vitro* antileishmanial activity against *L. major* strains. The compounds bearing 5-bromoindoline and 5-fluoroindoline moieties, respectively, showed the highest antileishmanial activity against both promastigote and amastigote forms, with no cytotoxicity in Vero cells (Figure 1).

Furthermore, hydantoin is considered a valuable and privileged scaffold in medicinal chemistry, the importance of which in drug discovery has been supported by several drugs in clinical use, including phenytoin, nitrofurantoin, and enzalutamide.^[28] While the exact mechanism of action of hydantoin has yet to be elucidated, investigation of the anthelmintic activity of several aryl-hydantoins revealed the presence of the hydantoin core as a prerequisite for enhanced antiparasitic activity despite their antiandrogenic side effects in the host, which has been attributed to the structural similarity to the antiandrogenic drug nilutamide.^[29]

On these grounds, Buchynskyy et al.^[30] investigated a series of 2-thiohydantoin derivatives' *in vitro* antitrypanosomal activity against *T. brucei*. The derivative bearing 4-fluoro and 3-chloro benzyl, and the compound that containing 2-chloro and 3-methoxy on the phenyl group, demonstrated good activity against *T. brucei* parasites (Figure 2). These compounds were further evaluated *in vivo* in the acute mouse model of HAT, and it was found that they cured all the treated mice, with no parasitemia observed for sixty days after infection.

In another study by Zuma et al.,^[20] a series of nitrofurantoin-triazole hybrids were synthesized and evaluated for *in vitro* antileishmanial activity and cytotoxicity. The propargyl bearing nitrofurantoin analogue, and *n*-heptyl substituted 1,2,3-triazole

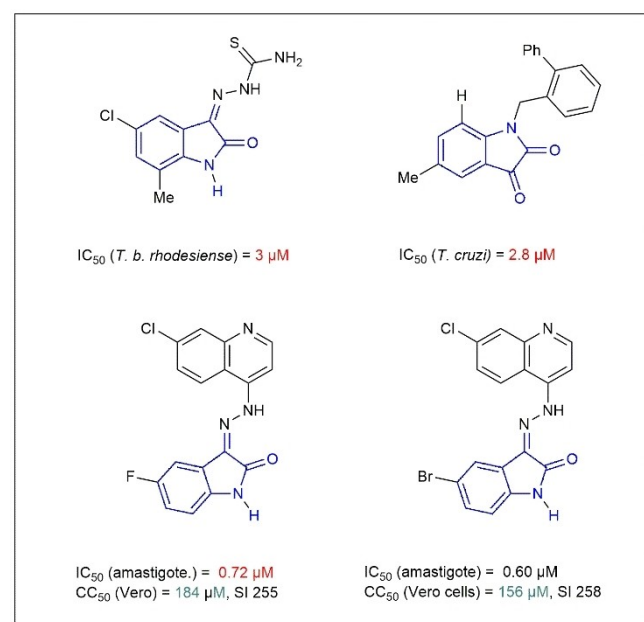


Figure 1. Structures of antitrypanosomal^[26] and antileishmanial^[27] active isatin derivatives.

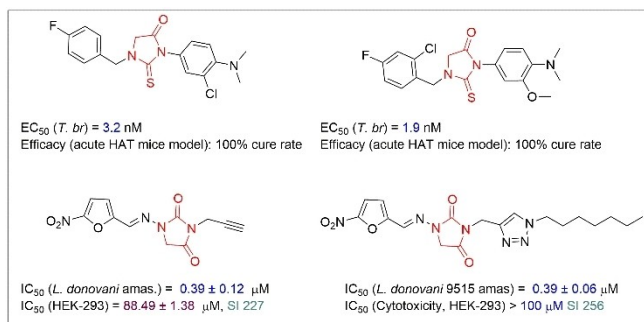


Figure 2. Structures of hydantoin-related compounds active against *T. brucei* and *Leishmania* major strains.^[20,30]

hybrid, were identified as early antileishmanial leads with no cytotoxicity to human cells (Figure 2).

Furthermore, aromatic rings are considered versatile pharmacophores that enhance the individual non-bonding and electrostatic interactions between the ligand and macromolecule.^[31] Thus, we hypothesized that the addition of these aromatic rings to isatin and hydantoin pharmacological synthons would afford derivatives with increased binding affinity, stability, biological activity, and specificity.^[32]

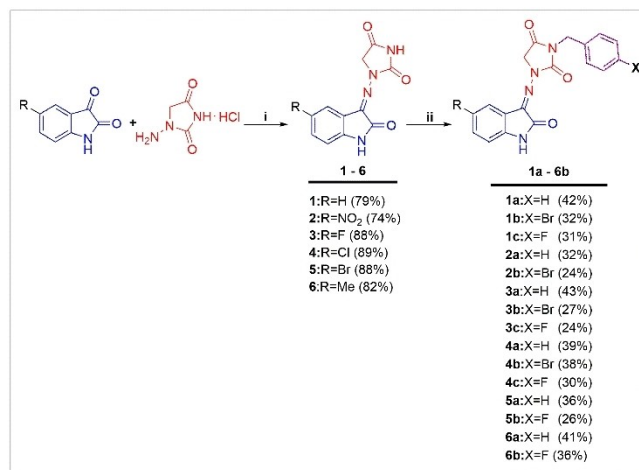
Based on this evidence, novel isatinhydantoin derivatives were synthesized in this study, and their anti-kinetoplastid activities were examined both *in vitro* and *in vivo*. We herein report the synthesis and the biological activities of these derivatives.

2. Results and Discussion

2.1. Chemistry

A series of isatinhydantoin derivatives (**1–6**, **1a–6b**) were successfully synthesized through a two-step process involving one Schiff base condensation and one nucleophilic substitution (SN), as illustrated in Scheme 1. Firstly, 1-aminohydantoin hydrochloride was deprotected with a mild base, potassium carbonate, and reacted with commercially available isatins (Schiff condensation) to afford the isatinhydantoin intermediates (**1–6**). Secondly, the intermediates were treated with various substituted benzyl bromides in SN reaction to provide the isatinhydantoin benzyl derivatives (**1a–6b**) in poor to moderate yields (24–43%) as powder or crystals after recrystallization from ethanol and water (1:1, v/v).

The structures of all the synthesized compounds were confirmed using routine molecular analysis techniques, such as ¹H NMR, ¹³C NMR, HRMS, and IR (Supplementary Information). The formation of the isatinhydantoin intermediates (**1–6**) was confirmed by ¹H NMR spectra through the disappearance of the singlet assigned to NH₂ ca. δ 5.00 ppm of the starting 1-aminohydantoin, supporting the successful Schiff condensation. This was further confirmed by the presence of the singlet ca. δ 4.17 ppm, which was attributed to H-5' of the methylene group (CH₂) of the hydantoin ring.



Scheme 1. Two-step synthesis of isatinhydantoin derivatives. Reagents and conditions: i. Dry EtOH (10 mL), K₂CO₃, rt, 12 h; ii. DMF (10 mL), benzyl bromide (1.5 equiv.), TEA (1.5 equiv.), rt, 24 h.

The successful confirmation of compounds **1a–6b** was observed by the presence of the singlet ca. 4.89 ppm that was attributed to methylene proton H-6' of the benzyl moiety, which supports the successful nucleophilic substitution at the nitrogen N-3. The phenyl ring of isatin moiety showed three aromatic resonances that appeared as two pairs of coupled doublets and a triplet at δ 7.49, δ 6.90, and δ 7.05 ppm with a *J* value of 8.1 Hz, 8.0 Hz, and 7.3 Hz, respectively. Furthermore, the aromatic protons H-2", -3" and -4" of benzyl moiety gave a multiplet (m) in the range δ 7.36–7.29 ppm region. All derivatives possessed a singlet around δ 11.03 ppm assigned to the acidic proton H-1 of the isatin moiety. The fluorine-containing compounds (**3**, **1c**, **3a**, **3b**, **3c**, **4c**, **5b** & **6b**) presented with the aromatic resonance due to the fluorine-proton coupling and consisting of a doublet of doublets (dd) ca. δ 7.17 ppm with *J*_{H-F} = 8.7, 5.5 Hz assigned to resonance of protons H-3" and -2". In summary, all the protons of each derivative were accounted for.

In the ¹³C NMR spectra, the carbonyl carbons appeared as singlets at δ 168.5 (C-4'), 158.6 (C-2), and 155.7 (C-2') present in all synthesized compounds. The hydantoin ring methylene carbon C-5' was a singlet at δ 55.6 ppm region, and the methylene carbon C-6' of the substituted benzyl showed as a singlet at δ 42.5 ppm. The aromatic carbons of the isatin moiety appeared as singlets at δ 140.8 (C-7a), 136.3 (C-3), 134.6 (C-5), 132.6 (C-6), 122.2 (C-4), 122.0 (C-3a), 110.7 (C-7) while the phenyl ring carbons appeared as singlets at 139.8 (C-1"), 131.6 (C-4"), 129.0 (C-3") and 128.1 (C-2").

For the fluorine-containing compounds (**3**, **1c**, **3a**, **3b**, **3c**, **4c**, **5b** & **6b**), the carbon C-4" resonated as a doublet at δ 162.0 ppm resulting from the coupling with neighboring fluorine with ¹*J*_{C-F} = 243.1 Hz. The carbon C-3" exhibited lower coupling with fluorine (2 bonds away), resulting in a doublet at δ 115.7 ppm with ²*J*_{C-F} = 21.1 Hz, and C-2" showed even weaker coupling with fluorine (3 bonds away), as indicated by a doublet at δ 130.38 ppm with ³*J*_{C-F} = 7.5 Hz. The carbon C-1"

presented even weaker coupling to fluorine (4 bonds away), as demonstrated by the doublet at δ 132.5 ppm with $^4J_{\text{C-F}} = 3.0$ Hz.

The IR spectra confirmed the formation of compounds **1–6** and **1a–6b** by the appearance of strong bands in the 1625–1795 cm^{-1} , which are the indicative stretching of the three carbonyl groups (C=O). This was further verified by the appearance of two NH bands stretching in 2997–3305 cm^{-1} region for **1–6**, and one sharp NH band stretching in 3310–3662 cm^{-1} for **1a–6b**. The compounds were also confirmed by the presence of stretching bands in the ranges of 605–640 cm^{-1} (C–Br), 802–826 cm^{-1} (C–Cl), 1334–1355 cm^{-1} (N–O), 1343–1410 cm^{-1} (C–F). Furthermore, HRMS verified the expected molecular ion of each synthesized compound (except for compounds **3**, **2a**, and **2b**).

2.2. Predicted Pharmacokinetic Properties

A summary of the predicted physicochemical and pharmacokinetic properties, drug-likeness, and medicinal chemistry friendliness of the isatinyldihydroantoin intermediates **1–6** and derivatives **1a–6b** can be found in the Supplementary Information, Tables S1–S3 and Figures S1 and S2.

Overall, according to Lipinski's rule-based filter, the test compounds were considered drug-like, i.e., a molecule with the potential to be an oral drug regarding bioavailability ($\text{MW} < 500$, $\log P_{\text{ow}} < 5$, N or O < 10 , NH or OH < 5).^[33] Nonetheless, adhering to these rules does not guarantee that a molecule will be orally bioavailable. The WLOGP-versus-TPSA referential, also known as the BOILED-Egg model, was also used to predict passive human gastrointestinal absorption (HIA) and blood-brain barrier (BBB) permeation.^[34] All test compounds, except intermediate **2**, were expected to have high gastrointestinal absorption (TPSA lower than 142 $\text{\AA}^2$ and $\log P_{\text{ow}}$ between -2.3 and $+6.8$). Unfortunately, none of the compounds were predicted to cross the BBB, since moderately polar (TPSA $< 79 \text{\AA}^2$) and relatively lipophilic ($\log P_{\text{ow}}$ between $+0.4$ and $+6.0$) molecules have a higher probability of entering the central nervous system (CNS) and, thus, crossing the BBB.

2.3. Pharmacology

2.3.1. Antitrypanosomal Activity

The synthesized isatinyldihydroantoin derivatives were initially screened at 10 μM for their *in vitro* antitrypanosomal activities against the *T. cruzi* strain CL epimastigotes and trypomastigotes with benznidazole used as reference antitrypanosomal drug. The selection cut-offs for further screening against axenic epimastigotes and trypomastigotes were set to greater than 70%, as was the case with axenic *Leishmania* promastigotes.^[35] None of the compounds, however, qualified for antitrypanosomal IC_{50} determination against *T. cruzi* epimastigotes and trypomastigotes due to growth inhibitions $< 6\%$ as reported in Table 1.

The synthesized isatinyldihydroantoin intermediates **1–6** and derivatives **1a–6b** were also evaluated for antitrypanosomal

Table 1. Growth inhibition (%) of compounds at 10 μM against *T. cruzi* strain CL epimastigotes and trypomastigotes.

Compd.	Antitrypanosomal growth inhibition (%)	
	<i>T. cruzi</i> strain CL epimastigotes	<i>T. cruzi</i> strain CL trypomastigotes
1	0	0
1a	0	0
1b	0	0
1c	0	0
2	0	2
2a	0	4
2b	0	4
3	0	0
3a	0	0
3b	0	0
3c	0	0
4	0	0
4a	0	0
4b	0	6
4c	0	0
5	0	0
5a	0	0
5b	0	1
6	0	0
6a	0	0
6b	0	0
Benznidazole	100	100

activity via *in vitro* trypomastigote cultures of tsetse transmitted human infective trypanosomes *T. b. gambiense* strain IL1922 and *T. b. rhodesiense* strain IL1501, tsetse transmitted animal infective trypanosomes *T. b. brucei* strain GuTat3.1 and *T. congolense* strain IL3000, and non-tsetse transmitted animal infective trypanosomes *Trypanosoma evansi* strain Tansui, and *Trypanosoma equiperdum* strain IVM-t1. The trypanocidal activities of the test compounds are summarized in Table 2. All test compounds were initially screened for potential antitrypomastigote activity at a maximum concentration of 25 $\mu\text{g/mL}$ and a minimum concentration of 0.25 $\mu\text{g/mL}$, and compounds with growth inhibition $\geq 50\%$ at the maximum tested concentration qualified for IC_{50} determination. Notably, intermediates **3–5** and derivative **2b** presented solubility issues when 10 mg/mL stock solutions were prepared in 100% dimethyl sulfoxide (DMSO) and then serially diluted with growth medium to 0.5% DMSO, forming quickly separating suspensions, making uniform sampling challenging.

Compounds **1–3**, **1a–c**, **3a–b**, and **6a–b** showed low basal toxicity towards mammalian cells ($\text{CC}_{50} \geq 100 \mu\text{M}$),^[38] while **2b**, **4a**, **4c** and **5b** were weakly toxic ($50 \mu\text{M} \leq \text{CC}_{50} \leq 100 \mu\text{M}$)^[39] and **4–6**, **2a**, and **4b** were mild to moderately toxic ($10 \mu\text{M} \leq \text{CC}_{50} \leq 50 \mu\text{M}$).^[39,40]

The synthesized compounds generally showed poor trypanocidal activity against the investigated human and animal infective trypanosome species, and only derivatives **1b**, **3a**, **4a–**

Table 2. *In vitro* trypanocidal potency of test compounds against *Trypanosoma* species.

Compound	Cytotoxicity CC ₅₀ ± SD (μM) ^[a]	Cidal antitrypomastigote activity IC ₅₀ ± SD (μM) ^[b] (SI) ^[c]					
		MDBK	Tbg IL1922	Tbr IL1501	Tbb GuTat3.1	Tc IL3000	Tev Tansui
1	> 100	> 10	> 10	> 10	> 10	> 10	> 10
2	> 100	> 10	> 10	> 10	> 10	> 10	> 10
3	> 100	> 10	> 10	> 10	> 10	> 10	> 10
4	43.67 ± 5.88	> 10	> 10	> 10	> 10	> 10	> 10
5	27.04 ± 16.86	> 10	> 10	> 10	> 10	> 10	> 10
6	44.97 ± 13.60	> 10	> 10	> 10	> 10	> 10	> 10
1a	> 100	> 10	> 10	> 10	> 10	> 10	> 10
1b	> 100	> 10	> 10	> 10	8.43 ± 3.12 (> 12)	> 10	> 10
1c	> 100	> 10	> 10	> 10	> 10	> 10	> 10
2a	13.13 ± 9.23	> 10	> 10	> 10	> 10	> 10	> 10
2b	88.99 ± 15.67	> 10	> 10	> 10	> 10	> 10	> 10
3a	> 100	> 10	> 10	> 10	3.89 ± 1.05 (> 26)	> 10	> 10
3b	> 100	> 10	> 10	> 10	> 10	> 10	> 10
3c	> 100	> 10	> 10	> 10	> 10	> 10	> 10
4a	65.67 ± 7.02	> 10	> 10	> 10	3.85 ± 1.75 (17)	> 10	> 10
4b	49.35 ± 26.68	> 10	> 10	> 10	1.78 ± 0.50 (28)	> 10	> 10
4c	89.93 ± 58.64	> 10	> 10	> 10	> 10	> 10	> 10
5a	22.65 ± 5.93	> 10	> 10	> 10	6.77 ± 4.75 (3)	> 10	> 10
5b	60.63 ± 28.87	> 10	> 10	> 10	> 10	> 10	> 10
6a	> 100	> 10	> 10	> 10	8.49 ± 4.97 (> 12)	> 10	> 10
6b	> 100	> 10	> 10	> 10	> 10	> 10	> 10
Pentamidine	-	0.014 ^[d]	0.029 ^[d]	0.041 ^[d]	0.33 ^[d]	0.00097 ^[d]	0.0013 ^[d]
Eflornithine	-	36.66 ^[d]	45.99 ^[d]	38.56 ^[d]	16.13 ^[d]	57.21 ^[d]	-
Nifurtimox	-	4.58 ^[d]	4.35 ^[d]	4.66 ^[d]	1.06 ^[d]	2.62 ^[d]	-
Suramin	-	0.064 ^[d]	0.076 ^[d]	0.066 ^[d]	7.17 ^[d]	0.38 ^[d]	0.038 ^[d]
Diminazene aceturate	-	-	-	-	0.11 ^[d]	-	0.011 ^[d]

[a] Half maximal cytotoxic concentration (CC₅₀, μM) represented as the mean ± standard deviation (SD), three biological replicates. [b] Half maximal inhibitory concentration (IC₅₀, μM) represented as the mean ± standard deviation (SD), three biological replicates. [c] Selectivity index (SI): CC₅₀ of MDBK/IC₅₀ of trypanosome. [d] IC₅₀ values obtained from the literature.^[36] MDBK: Madin-Darby bovine kidney cells; Tbg IL1922: *T. brucei gambiense* strain IL1922; Tbr IL1501: *T. brucei rhodesiense* strain; Tbb GUTat3.1: *T. brucei brucei* strain GUTat3.1; Tc IL3000: *T. congolense* strain IL3000; Tev Tansui: *T. evansi* strain Tansui; Teq IVM-t1: *T. equiperdum* strain IVM-t1; Green: qualifies as hit compound if IC₅₀ ≤ 10 μM and SI ≥ 10.^[37]

b, and **6a** showed hit antitrypanosomal activity against *Trypanosoma congolense* strain IL3000 trypomastigotes (IC₅₀ ≤ 10 μM, SI ≥ 10).^[37] Derivative **4b** containing 5-chloroisatin and *p*-bromobenzyl moieties had the best *in vitro* trypanocidal activity against the tsetse-transmitted animal trypanosome *T. congolense* strain IL3000 (IC₅₀ 1.78 μM), as well as relatively good selectivity towards the said trypanosome (SI 28) and was subsequently evaluated to determine its preliminary *in vivo* treatment efficacy in *T. congolense* strain IL3000 infected BALB/c mice. Derivative **4b** showed four-fold greater *in vitro* activity against *T. congolense* strain IL3000 trypomastigotes than the standard clinical drug suramin but was sixteen times less active than diminazene aceturate.

2.3.2. Preliminary Treatment Efficacy

This animal study evaluated the preliminary treatment efficacy of the isatinylhydantoin derivative **4b** (which showed the best *in vitro* trypanocidal potency of the investigated series) administered to *T. congolense* strain IL3000-infected BALB/C mice either orally (p.o.) at a dose of 100 mg/kg or intraperitoneally (i.p.) at a dose of 10 mg/kg from five days post-infection (d.p.i.) for seven consecutive days. Based on the *in silico* predicted pharmacokinetic properties (Supplementary Information, Tables S2–S3 and Figure S2), derivative **4b** had high GI absorption and > 10% bioavailability in rat or measurable Caco-2 permeability.^[14] However, none of the pharmacokinetic properties were investigated experimentally. A smaller dosage was used for intraperitoneal injection than oral gavage since it has been found that the absolute bioavailability of a drug may be

higher after i.p. vs. p.o. administration.^[16] Although both administration routes are subject to first-pass metabolism by the liver, intraperitoneal injection avoids the gastrointestinal tract and the drug's potential biopharmaceutical degradation/modification.^[17]

The median survival of the infected but untreated group I mice was nine d.p.i., while the median survival of infected group II mice treated with either 100 mg/kg p.o. of **4b** or 10 mg/kg i.p. of **4b** was 10 d.p.i. (Figure 3A). The survival rates of the treated mice were not significantly different from those of the untreated mice (P value 0.3173, log-rank test). All mice died within 10 d.p.i. due to high parasitemia (Figure 3B). No mentionable *in vivo* treatment efficacy was observed against *T. congolense* strain IL3000-infected BALB/c mice after oral or intraperitoneal administration of the isatinylhydantoin derivative **4b** (group II) since no cure (defined as no parasites present in the blood and survival after 30 days) was achieved. Plausible reasons that may justify the poor treatment efficacy include inter alia (a) the relatively lower *in vitro* potency of **4b** compared to the standard drug diminazene aceturate and (b) an insufficient dosing regimen as 10 mg/kg i.p. might not be sufficient while with 100 mg/kg p.o., the lack of bioavailability data gave no indication of the quantity of the compound which

entered the systemic circulation. These preliminary results did not warrant further animal studies using more mice per group.

2.3.3. Antileishmanial Activity

The antileishmanial activity and cytotoxicities of the isatinylhydantoin derivatives are summarized in Table 3. All synthesized derivatives were initially screened for potential antileishmanial activity at 10 μ M, and compounds with growth inhibition $\geq 60\%$ were considered for further IC_{50} determination. Only compounds **2b**, **5**, and **5b** qualified for IC_{50} determination against the clinically relevant amastigotes species of antimony-resistant *L. donovani* strain 9515, but none was so against *L. major*. Cytotoxicity profiles of the derivatives were determined using Vero cells with emetine as a positive control. Amphotericin B was used as the reference antileishmanial drug. These results are reported in Table 3.

All synthesized compounds (except **5**, **2b**, and **5b**) exhibited no activity. Hence, they were not reported in Table 3. The cytotoxicity data demonstrated that the three active derivatives showed no toxicity against Vero and THP1 cells ($IC_{50} > 100 \mu$ M); hence, their experimental antileishmanial activities were

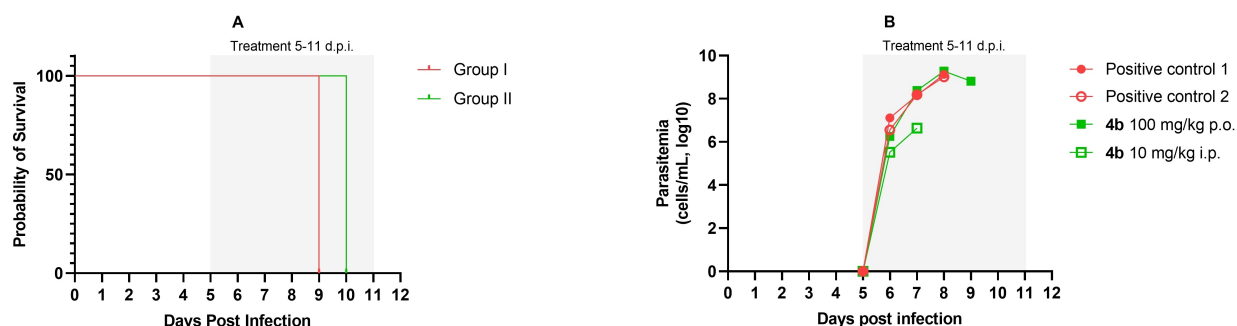


Figure 3. *In vivo* evaluation of preliminary treatment efficacy of the isatinylhydantoin derivative **4b**. **A** Survival (%) of female 6-week-old BALB/c mice infected with *T. congolense* strain IL3000 parasites and left untreated (Group I: positive control) or treated with **4b** (Group II). The survival rates of the treated mice were not significantly different from those of the untreated mice (P value 0.3173, log-rank test). P -values < 0.05 were considered significantly different. **B** Parasitemia (cells/mL, log10) in female 6-week-old BALB/c mice infected with *T. congolense* strain IL3000 parasites and left untreated (positive controls) or treated with either 100 mg/kg of **4b** orally or 10 mg/kg of **4b** intraperitoneally from 5 d.p.i. Group II mice died before treatment could be completed. d.p.i.: days post-infection; p.o.: per os; i.p. intraperitoneal.

Table 3. Antileishmanial activity (μ M $\pm$ Standard deviation) of compounds against *L. donovani* strain 9515.

Compd.	General cytotoxicity Vero IC_{50} (μ M)	Host cell cytotoxicity THP1 IC_{50} (μ M)	Antipromastigote activity		Static anti-amastigote activity			Cidal anti-amastigote activity		
			IC_{50} (μ M)	$SI_1^{[a]}$	IC_{50} (μ M)	$SI_2^{[b]}$	$Spl_1^{[c]}$	IC_{50} (μ M)	$SI_3^{[d]}$	$Spl_2^{[e]}$
2b	> 100	> 100	2.88 ± 0.84	35	0.55 ± 0.7	182	5	0.44 ± 0.08	227	7
5	> 100	> 100	> 10		0.40 ± 0.2	250		0.43 ± 0.02	233	
5b	> 100	> 100	12.39 ± 3.67	8	0.72 ± 0.7	139	17	0.73 ± 0.01	137	17
Emetine	0.08 ± 0.009	-			-	-		-	-	
AMB	57.80 ± 3.20	14.86 ± 0.09			0.05 ± 0.0	116		0.45 ± 0.05	128	

[a] Selectivity Index of *L. donovani*: $SI_1 = IC_{50}$ Vero/ IC_{50} promastigote. [b] Selectivity Index of *L. donovani*: $SI_2 = IC_{50}$ Vero/static IC_{50} amastigote. [c] Selectivity Index of *L. donovani*: $SI_3 = IC_{50}$ Vero/cidal IC_{50} amastigote. Specificity index (Spl) < 0.4 indicates more antipromastigote activity, $0.4 < Spl < 2.0$ indicates activity against both forms, $Spl > 2.0$ indicates more anti-amastigote activity.^[41] [d] Specificity index of *L. donovani*: $Spl_1 = IC_{50}$ promastigote/static IC_{50} amastigote. [e] Specificity index of *L. donovani*: $Spl_2 = IC_{50}$ promastigote/cidal IC_{50} amastigote. Vero: African green monkey kidney epithelial cells; THP-1: human acute monocytic leukemia; Red = compounds qualifying as early antileishmanial leads; Blue = compounds qualifying as antileishmanial hits.^[37a] All data reported in the tables were significant at $p < 0.05$.

deemed intrinsic. The low cytotoxicity of these 3 derivatives in Vero and THP1 cells contrasts with their moderate to weak toxicity in MDBK cells, which may indicate species-dependent toxicity for these compounds.

The antileishmanial activity was characterized as either static (inhibition of proliferation) or cidal (parasite death) by determining the IC_{50} values at 24 h and 72 h post host cell lysis. Cidal compounds retain significant antileishmanial activity up to 72 hours post lysis, indicating that the parasites could not recover from treatment. Cidal drugs are often associated with the development of fast-acting antiprotozoals.^[42] Static compounds, on the other hand, present with significant anti-amastigote activity 24 hours post lysis, but lose their activity due to parasite recovery and subsequent promastigote proliferation during further incubation. Static drugs may require higher concentrations and/or longer treatment periods as observed in antibacterial assays,^[43] which could pose a resistance development risk.

When the static (24 h) and cidal (72 h) IC_{50} values of the three compounds were compared, it was observed that antileishmanial activity was retained after 72 h and not lost due to parasite recovery. Accordingly, their antileishmanial activity was deemed cidal in nature. Furthermore, these hybrids showed anti-amastigote activity comparable to that of the reference drug (amphotericin B, IC_{50} 0.45 ± 0.05 μ M), especially **5** with unsubstituted benzyl and **2b** featuring 5-nitroisatin and *p*-bromobenzyl groups with IC_{50} values of 0.43 ± 0.02 and 0.44 ± 0.08 μ M, respectively. Their selectivity towards parasites (SI 137–227) was also significantly improved compared to amphotericin B (SI 128). These compounds therefore qualify as potential early antileishmanicidal leads according to the guidelines of Katsuno et al., (2015) ($IC_{50} \leq 1$ μ M; $SI \geq 100$). However, of these three compounds, intermediate **5** synthesized in a single-step process and tested as suspension, demonstrated exclusive anti-amastigote activity, and displayed the most selective leishmanicidal action and hence stood out as the promising candidate for further investigation. Derivatives, **2b** and **5b**, on the other hand, were preferentially active against the amastigotes of *L. donovani* as can be seen from their high specificity indexes, **5** and **17**, respectively.^[41]

2.3.4. *T. cruzi* Anti-Amastigote Activity

Leishmania and *Trypanosoma* are taxonomically related and have similar biochemical and structural features. This may particularly be the case for their clinically relevant amastigote forms, as both species are obligate intracellular parasites in this form. Thus, the potential for activity against intracellular *T. cruzi* amastigotes was also assessed by screening compounds **2b**, **5** and **5b** with confirmed antileishmanial amastigote activity against *T. cruzi* strain Tulahuen clone C4 expressing β -galactosidase enzyme. However, these compounds showed no notable activity against this strain ($IC_{50} > 10$ μ M) in comparison to benznidazole, used as reference, which had IC_{50} 2.70 ± 0.32 μ M.

2.3.5. Structure-Activity Relationships

When analyzing the electronic effect and strength across the isatinylhydantoin derivatives **1a–6b**, the R substituents can be categorized into three distinct groups, namely electron-withdrawing group (EWG, $NO_2 > F > Cl > Br$), neutral group (H), and electron donating group (EDG, Me). The limited number of active compounds hindered the deduction of further meaningful structure-activity relationships. However, it was noted that in the benzyl sub-series, hybrid **4c** bearing the strongest EWG (F) exhibited an IC_{50} value > 10 μ M, compared to 1.78 μ M of **4b** containing the weakest EWG (Br). Furthermore, the unsubstituted benzyl compounds, **3a**, **4a**, and **6a**, were found to exhibit hit activities against *T. congolense* strain IL3000 trypomastigotes with the EWG-containing analogues, **3a** and **4a** being equipotent while displaying a two-fold higher activity than their EDG-containing counterpart **6a**. Among the chlorinated isatin analogues, **4a**, **4b** and **4c**, the brominated (weaker EWG) **4b** was identified as the most active followed by the neutral **4a**, which in turn was more antitrypanosomal potent than the inactive fluorinated **4c** (stronger EWG).

Comparison of *L. donovani* antileishmanial efficacy among the hits revealed two 5-brominated isatin analogues, the intermediate **5** and the fluorobenzylated **5b** with submicromolar equipotency while the unsubstituted benzyl **5a** was found to be inactive.

3. Conclusions

A series of novel isatinylhydantoin derivatives were synthesized in a two-step process with poor to moderate yields. Most of the compounds were non-cytotoxic against three mammalian cell lines, apart from compounds **2b**, **4a**, **4c**, and **5b** in MDBK cells, which exhibited moderate toxicity.

The compounds showed no growth inhibition against axenic *T. cruzi* epimastigotes and trypomastigotes. The derivatives also exhibited weak axenic trypanocidal activity against species/strains of animal and human trypanosomes, except for compounds **1b**, **3a**, **3c**, **4a**, **4b**, and **6a**, which showed hit trypanocidal activity against *T. congolense* IL3000 ($IC_{50} \leq 10$ μ M, $SI \geq 10$). Hybrid **4b** was identified as the most promising trypanocidal hit based on its *in vitro* trypanocidal potency and further evaluated *in vivo*; however, no mentionable preliminary treatment efficacy was observed against *T. congolense* strain IL3000-infected BALB/c mice after oral or intraperitoneal administration of the isatinylhydantoin derivative.

Against *Leishmania* parasites, however, hybrids **2b**, **5**, and **5b** demonstrated very good activity against the intracellular amastigotes of antimony-resistant *L. donovani* strain 9515. These compounds were identified as potential early antileishmanicidal leads. Thus, future work on isatinylhydantoin derivatives will focus on the reported *in vitro* hit/lead activity against *L. donovani* for further investigations into their *in vivo* antiparasitic potential.

Experimental Sections

Materials and Methods

Materials

Isatin ($C_8H_5NO_2$), 5-nitroisatin ($C_8H_4N_2O_4$), 5-fluoroisatin ($C_8H_4FNO_2$), 5-chloroisatin ($C_8H_4ClNO_2$), 5-bromoisatin ($C_8H_4BrNO_2$), 5-methylisatin ($C_9H_7NO_2$), 1-aminohydantoin hydrochloride ($C_3H_6ClN_3O_2$), Triethylamine ($C_6H_{15}N$), benzyl bromide (C_7H_7Br), 4-bromobenzyl bromide ($C_7H_6Br_2$), 4-fluorobenzyl bromide (C_7H_6BrF) were obtained from Sigma-Aldrich (Johannesburg, South Africa). *N,N*-dimethylformamide (DMF) were purchased from Merck (Johannesburg, South Africa). All other solvents, including ethanol (EtOH), dichloromethane (DCM), potassium carbonate (K_2CO_3) and magnesium sulphate ($MgSO_4$), were purchased from Associated Chemical Enterprises (ACE, South Africa). All chemicals and reagents were of analytical grade, and no further purification was needed.

General Procedures

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance™ III 600 spectrometer at a frequency of 600 and 150 MHz, respectively, in deuterated dimethyl sulfoxide ($DMSO-d_6$). The chemical shifts were reported in parts per million δ (ppm) with the residual protons of the solvent as a reference. The abbreviations of the splitting patterns were as follows: singlet (s), doublet (d), doublet of doublet (dd), doublet of triplets (dt), triplet (t), triplet of doublets (td), doublet of doublet of doublets (ddd) and multiplet (m).

High-resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source set at 200 °C or 180 °C and analysed using a Bruker Compass Data Analysis 4.0 software. A full scan ranged from 50–1500 m/z at a capillary voltage of 4500 V, an end plate offset voltage of –500 V, with the nebulizer set at 1.6 and 0.4 Bar, respectively, and a collision cell RF voltage of 100 Vpp.

Infrared (FTIR) spectra were recorded on a Bruker Alpha-P FTIR instrument, whereas a BÜCHI melting point B-545 instrument was used to determine melting points (mp), which were uncorrected. Thin-layer chromatography (TLC) was performed on silica gel plates (60F254) acquired from Merck (South Africa). The plates were visualized under ultraviolet (UV254 nm and 366 nm) light or stained with iodine vapor.

Syntheses

Synthesis of Isatinylhydantoin Intermediates (1–6)

The starting material, 1-aminohydantoin hydrochloride (10.1 mmol, 1.5 equiv.), was dissolved in dry EtOH (50 ml), and then K_2CO_3 (1.5 equiv.) was added. The solution was stirred at room temperature for 1 h; thereafter, the isatins (6.7 mmol, 1 equiv.) dissolved in dry EtOH (50 ml) were added dropwise. The resulting mixture was stirred at room temperature for 12 h and monitored by thin layer chromatography (TLC). Upon completion, the ensuing precipitate was filtered and dried in an oven to afford the isatinylhydantoin intermediates 1–6, which were subsequently used without further purification.

1-[(2-Oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (1)

Yellow powder; yield: 79%; mp: 226–228 °C; IR ν_{max} (cm^{-1}): 3284 (N–H), 2997 (N–H), 2963 (C–H), 1792 (C=O), 1764 (C=O), 1692 (C=O), 1592 (C=N). ^1H NMR (600 MHz, $DMSO-d_6$) δ (ppm): 10.94 (s, 1H, H-1), 7.46 (d, $J = 7.1$ Hz, 1H, H-4), 7.31 (t, $J = 7.6$ Hz, 1H, H-5), 7.03 (t, $J = 7.4$ Hz, 1H, H-6), 6.89 (d, $J = 8.0$ Hz, 1H, H-7), 4.58 (s, 2H, H-5'). ^{13}C NMR (151 MHz, $DMSO-d_6$) δ (ppm): 170.8 (C-4'), 158.6 (C-2), 158.1 (C-2'), 142.4 (C-7a), 136.5 (C-3), 131.2 (C-5), 122.8 (C-3a), 122.3 (C-4), 121.0 (C-6), 110.6 (C-7), 56.8 (C-5'). HRMS-APCI m/z : 245.0690 [$M + H$]⁺ (calculated for $C_{11}H_9N_4O_3^+$, 245.0669).

(Z)-1-[(5-Nitro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (2)

Light orange powder; Yield: 74%; mp: 210–213 °C; IR ν_{max} (cm^{-1}): 3265 (N–H), 3192 (N–H), 2987 (C–H), 1707 (C=O), 1625 (C=O), 1602 (C=O), 1583 (C=N), 1338 (N–O). ^1H NMR (600 MHz, $DMSO-d_6$) δ (ppm): 8.16–8.11 (m, 2H, H-6, H-4), 7.02 (d, $J = 8.3$ Hz, 1H, H-7), 4.30 (s, 2H, H-5'). ^{13}C NMR (151 MHz, $DMSO-d_6$) δ (ppm): 170.8 (C-4'), 167.3 (C-2), 159.4 (C-2'), 146.7 (C-7a), 142.4 (C-5), 125.2 (C-3), 125.1 (C-6), 124.6 (C-3a), 114.6 (C-4), 110.3 (C-7), 57.5 (C-5'). HRMS-APCI m/z : 290.0520 [$M + H$]⁺ (calculated for $C_{11}H_8N_5O_5^+$, 290.0529).

(Z)-1-[(5-Fluoro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (3)

Orange powder; yield: 88%; mp: 319–322 °C; IR ν_{max} (cm^{-1}): 3262 (N–H), 3158 (N–H), 2988 (C–H), 1773 (C=O), 1715 (C=O), 1617 (C=O), 1583 (C=N), 1408 (C–F). ^1H NMR (600 MHz, $DMSO-d_6$) δ (ppm): 10.79 (s, 1H, H-1), 7.34 (d, $J_{H-F} = 8.3$ Hz, 1H, H-4), 7.22 (d, $J = 8.4$ Hz, 1H, H-7), 6.86 (dd, $J_{H-F} = 8.4$, 2.7 Hz, 1H, H-6), 4.18 (s, 2H, H-5'). ^{13}C NMR (151 MHz, $DMSO-d_6$) δ (ppm): 170.8 (C-4'), 158.8 (C-2), 158.4 (d, $^1J_{C-F} = 237.0$ Hz, C-5), 158.1 (C-2'), 138.4 (C-7a), 134.5 (C-3), 124.4 (d, $^3J_{C-F} = 7.5$ Hz, C-3a), 117.1 (d, $^2J_{C-F} = 24.1$ Hz, C-6), 111.6 (d, $^3J_{C-F} = 7.5$ Hz, C-7), 107.6 (d, $^2J_{C-F} = 25.6$ Hz, C-4), 57.0 (C-5'). HRMS: The molecular ion peak was not found and is likely unstable with the ionization method.

(Z)-1-[(5-Chloro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (4)

Orange powder; yield 89%; mp: 272–274 °C; IR ν_{max} (cm^{-1}): 3271 (N–H), 3167 (N–H), 2967 (C–H), 1767 (C=O), 1711 (C=O), 1651 (C=O), 1585 (C=N), 826 (C–Cl). ^1H NMR (600 MHz, $DMSO-d_6$) δ (ppm): 10.58 (s, 1H, H-1), 7.12 (s, 1H, H-4), 7.01 (*d, $J = 8.0$ Hz, 1H, H-6), 6.81 (*d, $J = 8.0$ Hz, 1H, H-7), 4.17 (s, 2H, H-5'). ^{13}C NMR (151 MHz, $DMSO-d_6$) δ (ppm): ^{13}C NMR (151 MHz, $DMSO$) δ (ppm): 170.8 (C-4'), 158.5 (C-2), 158.1 (C-2'), 140.6 (C-7a), 129.9 (C-3), 126.3 (C-6), 126.1 (C-5), 125.1 (C-3a), 120.0 (C-4), 112.0 (C-7), 57.1 (C-5'). HRMS-APCI m/z : 279.0279 [$M + H$]⁺ (calculated for $C_{11}H_8ClN_4O_3^+$, 279.0286). *d is a coalesced doublet

(Z)-1-[(5-Bromo-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (5)

Orange powder; yield: 88%; mp: 262–264 °C; IR ν_{max} (cm^{-1}): 3262 (N–H), 3158 (N–H), 2988 (C–H), 1773 (C=O), 1715 (C=O), 1617 (C=O), 1583 (C=N), 605 (C–Br). ^1H NMR (600 MHz, $DMSO-d_6$) δ (ppm): 10.81 (s, 1H, H-1), 7.45 (s, 1H, H-4), 7.35 (*d, $J = 8.0$ Hz, 1H, H-6), 6.80 (*d, $J = 8.0$ Hz, 1H, H-7), 4.19 (s, 2H, H-5'). ^{13}C NMR (151 MHz, $DMSO-d_6$) δ (ppm): 183.0 (C-4'), 168.7 (C-2), 158.6 (C-2'), 140.0 (C-7a), 131.1 (C-3), 127.9 (C-6), 126.6 (C-4), 122.0 (C-3a), 113.5 (C-5),

112.0 (C-7), 57.6 (C-5'). HRMS-APCI m/z : 322.9774 $[M+H]^+$ (calculated for $C_{11}H_8 BrN_4O_3^+$, 322.9776). *d is a coalesced doublet

(Z)-1-[(5-Methyl-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (6)

Orange powder; yield: 82% mp: 228–230 °C; IR ν_{max} (cm^{-1}): 3305 (N–H), 3191 (N–H), 2988 (C–H), 1767 (C=O), 1711 (C=O), 1625 (C=O) 1592 (C=N). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 10.74 (s, 1H, H-1), 7.27 (s, 1H, H-4), 7.09 (d, J = 7.9 Hz, 1H, H-6), 6.75 (d, J = 7.9 Hz, 1H, H-7), 4.46 (s, 2H, H-5'), 2.28 (s, 3H, H-8). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm): 170.8 (C-4'), 158.7 (C-2), 158.1 (C-2'), 140.2 (C-7a), 131.6 (C-3), 131.3 (C-5), 122.8 (C-4/6), 121.4 (C-3a), 110.4 (C-7), 56.8 (C-5'), 21.0 (C-8). HRMS-APCI m/z : 259.0826 $[M+H]^+$ (calculated for $C_{12}H_{11}N_4O_3^+$, 259.0823).

Synthesis of Isatinylyhydantoin Derivatives (1 a–6 b).

The previously synthesized isatinylyhydantoin intermediates (1–6) were dissolved in anhydrous DMF (10 mL) upon stirring at room temperature, and then TEA (1.5 equiv.) was added to the solution and stirred for 15 min. Afterwards, benzyl bromides (6.13 mmol, 1.5 equiv.) were added, and the stirring was continued for 24 h whilst the progress of the reaction was monitored by TLC. Upon completion, DMF was removed in *vacuo*, and the residue was suspended in H_2O (20 mL) and extracted with DCM (3×20 mL). The organic layer was washed once with saturated NH_4Cl (20 mL) and then with water (20 mL). Thereafter, the organic phase was dried over anhydrous magnesium sulphate, filtered, dried under vacuum, and then recrystallized from EtOH/ H_2O (1:1, v/v) to obtain the target isatinylyhydantoin derivatives (1 a–6 b).

3-Benzyl-1-[(2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (1 a)

Yellow powder; yield: 42%; mp: 218–221 °C; IR ν_{max} (cm^{-1}): 3320 (N–H), 2963 (C–H), 1772 (C=O), 1712 (C=O), 1619 (C=O), 1581 (C=N). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 11.03 (s, 1H, H-1), 7.49 (d, J = 8.0 Hz, 1H, H-4), 7.36–7.29 (m, 6H, H-2'', –3'', –4'', –5'', –6'', –5), 7.05 (t, J = 7.3 Hz, 1H, H-6), 6.90 (d, J = 8.0 Hz, 1H, H-7), 4.87 (s, 2H, H-6'), 4.69 (s, 2H, H-5'). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm): 168.5 (C-4'), 158.5 (C-2), 155.7 (C-2'), 143.0 (C-7a), 139.7 (C-3), 136.2 (C-1''), 132.2 (C-5), 129.0 (C-3''), 128.1 (C-2''), 128.0 (C-4''), 122.6 (C-4), 122.1 (C-3a), 121.6 (C-6), 110.9 (C-7), 55.6 (C-5'), 42.5 (C-6'). HRMS-APCI m/z : 335.1139 $[M+H]^+$ (calculated for $C_{18}H_{15} N_4O_3^+$, 335.1129).

3-(4-Bromobenzyl)-1-[(2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (1 b)

Yellow powder; yield: 32%; mp: 202–204 °C; IR ν_{max} (cm^{-1}): 3289 (N–H), 2987 (C–H), 1781 (C=O), 1713 (C=O), 1621 (C=O), 1488 (C=N), 609 (C–Br). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 10.99 (s, 1H, H-1), 7.55 (d, J = 8.5 Hz, 2H, H-3'', –5''), 7.49 (d, J = 8.3 Hz, 1H, H-4), 7.36–7.32 (m, 3H, H-5, –2''), 7.04 (t, J = 7.8 Hz, 1H, H-6), 6.89 (d, J = 8.3 Hz, 1H, H-7), 4.86 (s, 2H, H-6'), 4.66 (s, 2H, H-5'). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm): 168.5 (C-4'), 158.5 (C-2), 155.6 (C-2'), 143.0 (C-7a), 139.7 (C-3), 135.7 (C-1''), 132.2 (C-5), 131.8 (C-3''), 130.4 (C-2''), 122.6 (C-4), 122.1 (C-3a), 121.6 (C-6), 121.2 (C-4''), 110.9 (C-7), 55.7 (C-5'), 41.9 (C-6'). HRMS-APCI m/z : 413.0244 $[M+H]^+$ (calculated for $C_{18}H_{14} BrN_4O_3^+$, 413.0228).

3-(4-Fluorobenzyl)-1-[(2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (1 c)

Yellow powder; yield: 31%; mp: 226–228 °C; IR ν_{max} (cm^{-1}): 3319 (N–H), 2967 (C–H), 1773 (C=O), 1713 (C=O), 1620 (C=O), 1508 (C=N), 1410 (C–F). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 10.98 (s, 1H, H-1), 7.49 (d, J = 7.7 Hz, 1H, H-4), 7.42–7.40 (m, 2H, H-2''), 7.35 (t, J = 7.7 Hz, 1H, H-5), 7.20–7.18 (m, 2H, H-3''), 7.04 (t, J = 7.9 Hz, 1H, H-6), 6.89 (d, J = 8.3 Hz, 1H, H-7), 4.86 (s, 2H, H-6'), 4.67 (s, 2H, H-5'). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm): 168.5 (C-4'), 162.0 (d, $^1J_{C-F}$ = 243.1 Hz, C-4''), 158.5 (C-2), 155.7 (C-2'), 143.0 (C-7a), 139.7 (C-3), 132.2 (C-1''), 130.4 (d, $^3J_{C-F}$ = 7.5 Hz, C-3'', C-5''), 122.6 (C-5), 122.4 (C-4), 122.1 (C-3a), 121.6 (C-6), 115.7 (d, $^2J_{C-F}$ = 21.1 Hz, C-2'', C-6''), 110.9 (C-7), 55.7 (C-5'), 41.8 (C-6'). HRMS-APCI m/z : 353.1044 $[M+H]^+$ (calculated for $C_{18}H_{14} FN_4O_3^+$, 353.1039).

3-Benzyl-1-[(5-nitro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (2 a)

Dark orange powder; yield: 32%; mp: 209–212 °C; IR ν_{max} (cm^{-1}): 3662 (N–H), 2971 (C–H), 1790 (C=O), 1719 (C=O), 1616 (C=O), 1521 (C=N), 1334 (N–O). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 8.28 (d, J = 8.0 Hz, 1H, H-6), 8.22 (d, J = 8.0 Hz, 1H, H-4), 7.38–7.36 (m, 5H, H-2'', –3'' and –4''), 7.25 (d, J = 8.7 Hz, 1H, H-7), 5.07 (s, 2H, H-6'), 5.04 (s, 2H, H-5'). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm): 168.1 (C-4'), 157.5 (C-2), 155.3 (C-2'), 147.6 (C-7a), 143.5 (C-5), 136.0 (C-1''), 135.8 (C-3), 134.1 (C-4''), 129.2 (C-3''), 128.1 (C-2''), 127.5 (C-6), 122.6 (C-3a), 115.8 (C-4), 110.5 (C-7), 56.0 (C-5'), 42.7 (C-6'). HRMS: The molecular ion peak was not found and is likely unstable with the ionisation method.

3-(4-Bromobenzyl)-1-[(5-nitro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (2 b)

Light green crystals; yield: 24%; mp: 221–223 °C; IR ν_{max} (cm^{-1}): 3080 (N–H), 2944 (C–H), 1795 (C=O), 1723 (C=O), 1614 (C=O), 1519 (C=N), 1355 (N–O), 642 (C–Br). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 8.29 (d, J = 8.7 Hz, 1H, H-6), 8.21 (s, 1H, H-4), 7.55 (t, J = 7.6 Hz, 2H, H-3''), 7.34 (*d, J = 6.1 Hz, 2H, H-2''), 7.24 (d, J = 6.1 Hz, 1H, H-7), 5.01 (s, 2H, H-6'), 4.71 (s, 2H, H-5'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): ^{13}C NMR (151 MHz, DMSO- d_6) δ 168.0 (C-4'), 157.5 (C-2), 155.2 (C-2'), 147.5 (C-7a), 143.5 (C-5), 135.4 (C-1''), 135.2 (C-3), 132.1 (C-3''), 130.4 (C-2''), 127.5 (C-6), 122.7 (C-3a), 121.3 (C-4''), 115.8 (C-4), 110.54 (C-7), 56.09 (C-5'), 42.15 (C-6'). HRMS: The molecular ion peak was not found and is likely unstable with the ionisation method. *d is a coalesced doublet

3-Benzyl-1-[(5-fluoro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (3 a)

Yellow powder; yield: 43%; mp: 235–238 °C; IR ν_{max} (cm^{-1}): 3306 (N–H), 2967 (C–H), 1769 (C=O), 1714 (C=O), 1579 (C=O), 1478 (C=N), 1343 (C–F). ^1H NMR (600 MHz, DMSO- d_6) δ (ppm): 10.94 (s, 1H, H-1), 7.37–7.34 (m, 5H, H-3'', –4'' and –2''), 7.24 (dd, J_{H-F} = 8.0, 2.7 Hz, 1H, H-4), 7.20 (td, J_{H-F} = 8.3, 3.2 Hz, 1H, H-6), 6.89 (dd, J_{H-F} = 8.5, 4.2 Hz, 1H, H-7), 4.90 (s, 2H, H-6'), 4.69 (s, 2H, H-5'). ^{13}C NMR (151 MHz, DMSO- d_6) δ (ppm): 168.4 (C-4'), 158.6 (C-2), 158.5 (d, $^1J_{C-F}$ = 237.0 Hz, C-5), 155.6 (C-2'), 139.2 (C-7a), 138.4 (C-1''), 136.2 (C-3), 129.0 (C-3''), 128.1 (C-2''), 125.1 (C-4''), 123.5 (d, $^3J_{C-F}$ = 9.0 Hz, C-3a), 118.4 (d, $^2J_{C-F}$ = 25.6 Hz, C-6), 112.0 (d, $^3J_{C-F}$ = 7.5 Hz, C-7), 108.4 (d, $^2J_{C-F}$ = 25.6 Hz, C-4), 55.8 (C-5'), 42.5 (C-6'). HRMS-APCI m/z : 353.1044 $[M+H]^+$ (calculated for $C_{18}H_{14} FN_4O_3^+$, 353.1055).

3-(4-Bromobenzyl)-1-[(5-fluoro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (3 b)

Orange powder; yield: 27%; mp: 262–265 °C; IR ν_{max} (cm⁻¹): 3293 (N–H), 2987 (C–H), 1719 (C=O), 1629 (C=O), 1596 (C=O), 1481 (C=N), 1381 (C–F), 640 (C–Br). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 11.04 (s, 1H, H-1), 7.55 (d, *J* = 8.3 Hz, 2H, H-3''), 7.33 (d, *J* = 8.6 Hz, 2H, H-2''), 7.24–7.19 (m, 2H, H-4, –6), 6.89 (dd, *J*_{H-F} = 8.5, 4.1 Hz, 1H, H-7), 4.88 (s, 2H, H-6'), 4.66 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.3 (C-4'), 159.6 (C-2), 158.5 (d, ¹*J*_{C-F} = 238.5 Hz, C-5), 155.5 (C-2'), 139.2 (C-7a), 138.4 (C-1''), 135.6 (C-3), 131.8 (C-3''), 130.4 (C-2''), 123.5 (d, ³*J*_{C-F} = 9.0 Hz, C-3a), 121.2 (C-4''), 118.4 (d, ²*J*_{C-F} = 22.6 Hz, C-6), 112.0 (d, ³*J*_{C-F} = 7.5 Hz, C-7), 108.4 (d, ²*J*_{C-F} = 25.6 Hz, C-4), 55.8 (C-5'), 42.00 (C-6'). HRMS-APCI *m/z*: 431.0150 [M + H]⁺ (calculated for C₁₈H₁₃BrFN₄O₃⁺, 431.0125).

1-[(5-Fluoro-2-oxoindolin-3-ylidene)amino]-3-(4-fluorobenzyl)imidazolidine-2,4-dione (3 c)

Orange powder; yield: 24%; mp: 204–207 °C; IR ν_{max} (cm⁻¹): 3310 (N–H), 2971 (C–H), 1771 (C=O), 1715 (C=O), 1634 (C=O), 1509 (C=N), 1408 (C–F), 1359 (C–F). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 11.02 (s, 1H, H-1), 7.41 (dd, *J*_{H-F} = 8.0, 2.8 Hz, 2H, H-3''), 7.24 (d, *J*_{H-F} = 8.0 Hz, 1H, H-4), 7.18 (ddd, *J*_{H-F} = 24.0, 12.0, 6.0 Hz, 3H, H-2'' and –6), 6.89 (dd, *J*_{H-F} = 8.2, 3.9 Hz, 1H, H-7), 4.88 (s, 2H, H-6'), 4.68 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.3 (C-4'), 163.7 (d, ¹*J*_{C-F} = 247.6 Hz, C-4''), 162.1 (d, ¹*J*_{C-F} = 244.6 Hz, C-5), 158.6 (C-2), 155.5 (C-2'), 139.2 (C-7a), 138.4 (C-3), 132.4 (d, ⁴*J*_{C-F} = 3.0 Hz, C-1''), 130.4 (d, ³*J*_{C-F} = 9.0 Hz, C-2''), 123.5 (d, ³*J*_{C-F} = 7.5 Hz, C-3a), 118.4 (d, ²*J*_{C-F} = 24.1 Hz, C-6), 115.8 (d, ³*J*_{C-F} = 9.0 Hz, C-3''), 112.0 (d, ³*J*_{C-F} = 9.0 Hz, C-7), 108.4 (d, ²*J*_{C-F} = 24.1 Hz, C-4), 55.8 (C-5'), 41.91 (C-6'). HRMS-APCI *m/z*: 371.0950 [M + H]⁺ (calculated for C₁₈H₁₃F₂N₄O₃⁺, 371.0969).

3-Benzyl-1-[(5-chloro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (4 a)

Orange powder; yield: 39%; mp: 237–239 °C; IR ν_{max} (cm⁻¹): 3229 (N–H), 2987 (C–H), 1777 (C=O), 1713 (C=O), 1616 (C=O), 1576 (C=N), 805 (C–Cl). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 9.75 (s, 1H, H-1), 7.39 (dd, *J* = 8.3, 2.2 Hz, 1H, H-4, H-6), 7.37–7.34 (m, 5H, H-3'', –2'' and –4''), 6.91 (d, *J* = 8.3 Hz, 1H, H-7), 4.90 (s, 2H, H-6'), 4.69 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.3 (C-4'), 158.3 (C-2), 155.5 (C-2'), 141.5 (C-7a), 137.5 (C-1''), 136.2 (C-3), 131.4 (C-6), 129.0 (C-3''), 128.1 (C-2''), 128.1 (C-5), 126.7 (C-4''), 124.0 (C-4), 120.9 (C-3a), 112.5 (C-7), 55.8 (C-5'), 42.6 (C-6'). HRMS-APCI *m/z*: 369.0749 [M + H]⁺ (calculated for C₁₈H₁₄ClN₄O₃⁺, 369.0766).

3-(4-Bromobenzyl)-1-[(5-chloro-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (4 b)

Yellow powder; yield: 38%; mp: 257–259 °C; IR ν_{max} (cm⁻¹): 3239 (N–H), 2987 (C–H), 1778 (C=O), 1714 (C=O), 1619 (C=O), 1577 (C=N), 802 (C–Cl), 622 (C–Br). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 11.11 (s, 1H, H-1), 7.55 (d, *J* = 8.4 Hz, 2H, H-3''), 7.42–7.37 (m, 2H, H-4, –6), 7.34 (d, *J* = 8.3 Hz, 2H, H-2''), 6.91 (d, *J* = 8.3 Hz, 1H, H-7), 4.89 (s, 2H, H-6'), 4.67 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 168.3 (C-4'), 158.3 (C-2), 155.4 (C-2'), 141.5 (C-7a), 137.5 (C-1''), 135.6 (C-3), 131.8 (C-3''), 130.4 (C-2''), 130.4 (C-6'), 126.7 (C-5), 124.0 (C-3a), 121.2 (C-4), 120.9 (C-4''), 112.4 (C-7), 55.9 (C-5'), 42.0 (C-6'). HRMS-APCI *m/z*: 446.9854 [M + H]⁺ (calculated for C₁₈H₁₃BrClN₄O₃⁺, 446.9871).

1-[(5-Chloro-2-oxoindolin-3-ylidene)amino]-3-(4-fluorobenzyl)imidazolidine-2,4-dione (4 c)

Red powder; yield: 30% mp: 213–215 °C; IR ν_{max} (cm⁻¹): 3254 (N–H), 2987 (C–H), 1785 (C=O), 1716 (C=O), 1618 (C=O), 1547 (C=N), 1406 (C–F), 819 (C–Cl). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 11.13 (s, 1H, H-1), 7.43–7.38 (m, 4H, H-2'' and –4''), 7.18 (t, *J*_{H-F} = 8.8 Hz, 2H, H-3''), 6.91 (d, *J* = 8.3 Hz, 1H, H-7), 4.88 (s, 2H, H-6'), 4.68 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.3 (C-4'), 162.1 (d, ¹*J*_{C-F} = 243.1 Hz, C-4''), 158.3 (C-2), 155.5 (C-2'), 141.5 (C-7a), 137.5 (C-1''), 132.4 (C-3), 130.4 (d, ³*J*_{C-F} = 9.0 Hz, C-2''), 126.7 (C-5), 124.0 (C-4), 120.89 (C-3a), 115.7 (d, ²*J*_{C-F} = 21.1 Hz, C-3''), 112.5 (C-7), 55.8 (C-5'), 41.9 (C-6'). HRMS-APCI *m/z*: 387.0655 [M + H]⁺ (calculated for C₁₈H₁₃ClFN₄O₃⁺, 387.0670).

3-Benzyl-1-[(5-bromo-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (5 a)

Orange powder; yield: 36%; mp: 235–238 °C; IR ν_{max} (cm⁻¹): 3245 (N–H), 2987 (C–H), 1774 (C=O), 1713 (C=O), 1616 (C=O), 1575 (C=N), 610 (C–Br). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 11.15 (s, 1H, H-1), 7.53–7.50 (m, 2H, H-4, H-6), 7.38–7.34 (m, 5H, H-2'', –3'' and –4''), 6.86 (d, *J* = 8.2 Hz, 1H, H-7), 4.90 (s, 2H, H-6'), 4.69 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.3 (C-4'), 158.2 (C-2), 155.5 (C-2'), 141.9 (C-7a), 137.3 (C-1''), 136.2 (C-3), 134.2 (C-6), 129.0 (C-3''), 128.1 (C-2''), 128.1 (C-4''), 124.4 (C-4), 123.6 (C-3a), 114.2 (C-5), 112.9 (C-7), 55.8 (C-5'), 42.5 (C-6'). HRMS-APCI *m/z*: 413.0244 [M + H]⁺ (calculated for C₁₈H₁₄BrN₄O₃⁺, 413.0257).

1-[(5-Bromo-2-oxoindolin-3-ylidene)amino]-3-(4-fluorobenzyl)imidazolidine-2,4-dione (5 b)

Red-orange powder; yield: 26%; mp: 211–214 °C; IR ν_{max} (cm⁻¹): 3246 (N–H), 2971 (C–H), 1786 (C=O), 1713 (C=O), 1607 (C=O), 1510 (C=N), 1406 (C–F), 636 (C–Br). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 11.13 (s, 1H, H-1), 7.54–7.51 (m, 2H, H-4, H-6), 7.41 (dd, *J*_{H-F} = 8.7, 5.7 Hz, 2H, H-2''), 7.18 (ddd, *J*_{H-F} = 30.0, 18.0, 6.0 Hz, 2H, H-3''), 6.86 (d, *J* = 8.1 Hz, 1H, H-7), 4.88 (s, 2H, H-6'), 4.68 (s, 2H, H-5'). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.3 (C-4'), 162.1 (d, ¹*J*_{C-F} = 243.1 Hz, C-4''), 158.2 (C-2), 155.5 (C-2'), 141.9 (C-7a), 137.3 (C-3), 134.2 (C-6), 132.4 (d, ⁴*J*_{C-F} = 3.0 Hz, C-1''), 130.4 (d, ³*J*_{C-F} = 7.5 Hz, C-2''), 124.4 (C-4), 123.6 (C-3a), 115.7 (d, ²*J*_{C-F} = 21.1 Hz, C-3''), 14.2 (C-5), 112.9 (C-7), 55.8 (C-5'), 41.9 (C-6'). HRMS-APCI *m/z*: 431.0150 [M + H]⁺ (calculated for C₁₈H₁₃BrFN₄O₃⁺, 431.0149).

3-Benzyl-1-[(5-methyl-2-oxoindolin-3-ylidene)amino]imidazolidine-2,4-dione (6 a)

Orange powder; yield: 41%; mp: 231–234 °C; IR ν_{max} (cm⁻¹): 3264 (N–H), 2987 (C–H), 1792 (C=O), 1706 (C=O), 1625 (C=O), 1578 (C=N). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 10.87 (s, 1H, H-1), 7.38–7.31 (m, 5H, H-3'', –2'' and –4''), 7.18 (dd, *J* = 8.4, 5.5 Hz, 2H, H-4, –6), 6.78 (d, *J* = 7.9 Hz, 1H, H-7), 4.87 (s, 2H, H-6'), 4.69 (s, 2H, H-5'), 2.29 (s, 3H, H-8). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.5 (C-4'), 158.6 (C-2), 155.7 (C-2'), 140.8 (C-7a), 139.8 (C-1''), 136.3 (C-3), 134.6 (C-5), 132.6 (C-6), 131.6 (C-4''), 129.0 (C-3''), 128.1 (C-2''), 122.2 (C-4), 122.0 (C-3a), 110.7 (C-7), 55.6 (C-5'), 42.5 (C-6'), 20.9 (C-8). HRMS-APCI *m/z*: 349.1295 [M + H]⁺ (calculated for C₁₉H₁₇N₄O₃⁺, 349.1312).

3-(4-Fluorobenzyl)-1-[(5-methyl-2-oxindolin-3-ylidene)amino]imidazolidine-2,4-dione (6b)

Orange powder; yield: 36%, mp: 225–228 °C; IR ν_{max} (cm⁻¹): 3280 (N–H), 2987 (C–H), 1781 (C=O), 1712 (C=O), 1628 (C=O), 1510 (C=N), 1407 (C–F). ¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm): 10.89 (s, 1H, H-1), 7.41 (dd, *J* = 8.7, 5.5 Hz, 1H, H-6), 7.31 (s, 1H, H-4), 7.17 (dd, *J*_{H-F} = 8.7, 5.5 Hz, 4H, H-2' and -3'), 6.78 (d, *J* = 7.9 Hz, 1H, H-7), 4.85 (s, 2H, H-6'), 4.67 (s, 2H, H-5'), 2.28 (s, 3H, H-8). ¹³C NMR (151 MHz, DMSO-*d*₆) δ (ppm): 168.5 (C-4'), 162.0 (d, ¹*J*_{C-F} = 243.1 Hz, C-4''), 158.6 (C-2), 155.7 (C-2'), 140.7 (C-7a), 139.8 (C-3), 132.6 (C-5), 132.5 (d, ⁴*J*_{C-F} = 3.0 Hz, C-1''), 131.6 (C-6), 130.3 (d, ³*J*_{C-F} = 7.5 Hz, C-2''), 122.1 (C-4), 122.0 (C-3a), 115.7 (d, ²*J*_{C-F} = 21.1 Hz, C-3''), 110.7 (C-7), 55.7 (C-5'), 41.8 (C-6'), 20.9 (C-8). HRMS-APCI *m/z*: 367.1201 [M + H]⁺ (calculated for C₁₉H₁₆FN₄O₃⁺, 367.1208).

4. Biological Evaluation

4.1. Cytotoxicity Assays

4.1.1. General (Human) Cytotoxicity

The resazurin assay assessed the cytotoxicity of the synthesized isatinylyhydantoin derivatives. The assay includes the irreversible enzymatic reduction of oxidized blue resazurin dye to pink, highly fluorescent resorufin by viable cells.^[44] This non-toxic reagent is useful for evaluating cell proliferation and drug toxicity.

The assay was performed using Vero cells as reported in Zuma et al.^[45] Briefly, 96 well plates containing cell suspension (60 000 cells/mL, 100 μ L/well) were incubated for 24 h and subsequently treated with a two-fold dilution range of 100 μ M compound solution. After incubating the treated plates for 48 h, resazurin was added to all wells and incubated for 2 h. The absorbances, data analysis, and IC₅₀ determinations were carried out as described in Zuma et al.^[45]

4.1.2. Animal Cytotoxicity Assay

The potential animal cytotoxicity of the test compounds was evaluated using *in vitro* cultures of Madin-Darby Bovine Kidney (MDBK) cells (Japanese Collection of Research Bioresources (JCRB) cell bank, National Institute of Biomedical Innovation, Health and Nutrition) and published methods^[36c,46] were followed.

The MDBK cells were cultured at 37 °C (5% CO₂) in minimum essential medium Eagle (MEM) with Earle's salts, L-glutamine, and sodium bicarbonate (Thermo Fisher Scientific), with added penicillin-streptomycin (1%) and heat-inactivated FBS (10%).

The test compounds were evaluated at three concentrations (100 μ g/mL, 10 μ g/mL, and 1 μ g/mL) for their potential cytotoxicity against MDBK cells. Then, prepared 96-well plates (Thermo Fisher Scientific) containing cell suspension (1 $\times$ 10⁴ cells/mL, 50 μ L/well) along with test compound (50 μ L/well) were incubated for 72 h at 37 °C (5% CO₂). Thereafter, Cell Counting Kit-8 solution (10 μ L/well; Dojindo, Kumamoto, Japan) was added. A GloMax-Multi + Detection System plate reader (Prom-

ega) was used to determine the optical density (OD₄₅₀) before and after 4 h of incubation at 450 nm.^[36c,46]

The compounds that obtained cell growth inhibition greater than 50% at 100 μ g/mL, 10 μ g/mL or 1 μ g/mL qualified for CC₅₀ determination, while compounds with cell growth inhibition below 50% at 100 μ g/mL were classified as non-toxic. Then, prepared 96-well plates with cell suspension (1 $\times$ 10⁴ cells/mL, 50 μ L/well) and qualifying test compounds at seven concentrations of a two-fold serial dilution (100–1.56 μ g/mL, 50 μ L/well) were incubated at 37 °C (5% CO₂) for 72 h. The Cell Counting Kit-8 method was then used, as described previously, for the initial screening above.

4.1.3. Host Cytotoxicity Assay

The compounds' toxicity was also assessed in the host macrophage cell line because of the *Leishmania* intramacrophage assay, as detailed in Zuma et al.^[45] Briefly, infected phorbol 12-myristate 13-acetate (PMA)-differentiated THP1 cells (500 000 cells/mL; MOI 30:1) were treated with seven two-fold dilution concentrations of 100 μ M compound and incubated for 72 h before adding resazurin and further incubation for 2 h. The absorbances, data analysis, and IC₅₀ determinations were carried out as described in Zuma et al.^[45]

4.2. Antitrypanosomal Assays Against *Trypanosoma* Species

4.2.1. *T. cruzi* Anti-Epimastigote Assay

The resazurin assay assessed the anti-epimastigote activity of synthesized isatinylyhydantoin derivatives towards the CL strain of *T. cruzi*. A modified technique of Kulshrestha et al.^[47] and Siqueira-Neto et al.^[35] was followed, as detailed in Zuma et al.^[45] Briefly, *T. cruzi* strain CL epimastigotes (1 $\times$ 10⁶ cells/mL) were treated with either 20 μ M test compound for activity screening or 7 two-fold dilution concentrations of compounds for IC₅₀ determination for 48 h. After incubation, resazurin solution was added and the plates further incubated at 25 °C for 24 h. Absorbances and data analysis were carried out as described in Zuma et al.^[45]

4.2.2. *T. cruzi* Antitrypomastigote Assay

The antitrypanosomal activity of the synthesized isatinylyhydantoin derivatives was evaluated using the human-infective extracellular bloodstream forms (trypomastigotes) of *T. cruzi* strain CL, applying a modified version of the resazurin assay^[11] as detailed in Zuma et al.^[45] Briefly, trypomastigote suspension (100 μ L/well) were treated with either 20 μ M test compound for activity screening or a three-fold dilution concentration range of compounds for IC₅₀ determination for 48 h. For the assay, resazurin and the plates incubated for 24 h at 37 °C. The absorbances, data analysis, and IC₅₀ determinations were carried out as described in Zuma et al.^[45]

4.2.3. *T. cruzi* Anti-Amastigote Assay

The antitrypanosomal activity of the synthesized isatinylhydantoin derivatives was evaluated using the obligate intracellular amastigote form of *T. cruzi* strain Tulahuen (clone C4, +*lacZ*), applying a modified version of the β -galactosidase assay of Martinez-Peinado et al.^[48]

T. cruzi trypomastigotes were maintained in 75 cm² flasks of Vero cells with RPMI 1640 medium supplemented with 2% heat-inactivated FBS and 1% penicillin-streptomycin solution (100 U/mL). For the assay, Vero cells and trypomastigotes are seeded in 96 well plates (4×10⁵ cells/mL; MOI 1:1) in phenol red-free medium containing 10% heat-inactivated FBS and incubated overnight at 37 °C and 5% CO₂. Wells without any cells and wells containing only Vero cells served as blanks. The plates are then gently washed with PBS to remove remaining extracellular parasites, treated with a two-fold dilution concentration range of compounds in phenol red-free medium for IC₅₀ determination and incubated for a further 72 h. Wells without treatment served as negative controls and benznidazole was used as reference drug.

For the β -galactosidase assay, a mixture of ortho-nitrophenyl beta-D-galactopyranoside (ONPG, 400 μ M) and Triton X-100 (0.3%) in PBS is freshly prepared and added to all wells (50 μ L/well). The plates were then incubated at room temperature for 24 h. A Thermofisher Scientific GO Multiscan plate reader was used to determine absorbance at 420 nm. SkanIt 4.0 Research Edition software was used to perform the data analysis. The mean absorbance was computed, and the % cell viability calculated using the following equation.

$$\text{Cell viability \%} = \frac{(\Delta \text{ Abs sample} - \Delta \text{ Abs blank})}{(\Delta \text{ Abs neg control} - \Delta \text{ Abs blank})} \times 100$$

GraphPad Prism 5 was used to compute the IC₅₀ and Z-score for each compound's three biological replicates. The final IC₅₀ of each compound was calculated as the mean IC₅₀ of the biological replicates with standard deviation (SD).

4.2.3.1. Trypanocidal Assay Against Axenically Proliferating Blood-Stage Trypomastigotes

The *in vitro* trypomastigote cultures of *T. congolense* IL3000, *T. b. brucei* GUTat3.1, *T. b. gambiense* IL1922, *T. b. rhodesiense* IL1501, *T. evansi* Tansui, and *T. equiperdum* IVM-t1 were used to evaluate the trypanocidal activity of the test compounds, according to established methods.^[36a,b] Dr. Hirumi provided *T. congolense* IL3000, *T. b. brucei* GUTat3.1, *T. b. gambiense* IL1922, *T. b. rhodesiense* IL1501, whereas Dr. Suganuma established *T. equiperdum* IVM-t1.^[36b,49]

The bloodstream form trypanosomes were cultured at 37 °C (33 °C for *T. congolense* IL3000) and 5% CO₂ in Hirumi's modified Iscove's medium-9 (HMI-9)^[50] composed of Iscove's modified Dulbecco's medium (Sigma-Aldrich) with penicillin-streptomycin (1%), L-glutamine (1%), bathocuproine (0.1 mM; Sigma-Aldrich), pyruvic acid sodium salt (1 mM; Sigma-Aldrich),

hypoxanthine (1 mM) and thymidine (16 μ M; HT supplement: Thermo Fisher Scientific), insulin (10 μ g/L), transferrin (5.5 μ g/L), sodium selenite (6.7 ng/L; ITS-X: Thermo Fisher Scientific), 2- β -mercaptoethanol (0.0001%; Sigma-Aldrich), L-cysteine (2 mM; Sigma-Aldrich), HEPES (60 mM; Sigma-Aldrich), bovine serum albumin (0.4 g/L; Sigma-Aldrich), and heat-inactivated FBS (20%).

To determine potential trypanocidal activity, all test compounds were first tested at a high concentration of 25 μ g/mL and a low concentration of 0.25 μ g/mL. After that, Nunc MicroWell 96-well optical bottom plates (Thermo Fisher Scientific) were prepared with parasite (50 μ L/well of either *T. congolense* IL3000 (1×10⁵ cells/mL), *T. b. brucei* GUTat3.1, *T. b. gambiense* IL1922, *T. b. rhodesiense* IL1501 (2.5×10³ cells/mL), *T. evansi* Tansui, or *T. equiperdum* IVM-t1 (1×10⁴ cells/mL)) and 50 μ L of the test compound and were incubated at 37 °C and 5% CO₂ (33 °C for *T. congolense* IL3000) for 72 h. Phase-contrast microscopy was then used to examine the plates for trypanosomes, and Cell-TiterGlo solution (25 μ L, Promega) was added. The GloMax-Multi+ Detection System plate reader was then used to determine bioluminescence at 450 nm.^[36a]

All compounds with more than 50% trypomastigote growth inhibition at 25 μ g/mL qualified for IC₅₀ determination. Prepared Nunc MicroWell 96-well optical bottom plates containing qualifying compounds (50 μ L/well) at seven concentrations of a two-fold serial dilution (highest: 25 μ g/mL; lowest: 0.390625 ng/mL) and the abovementioned parasite concentrations (50 μ L/well) were incubated at 37 °C and 5% CO₂ (and 33 °C) for *T. congolense* IL3000) for 72 h. Eflornithine, nifurtimox, pentamidine, suramin, and diminazene aceturate served as standard drugs, and Cell-TiterGlo was then used to evaluate trypanocidal activity as specified in the initial assay above.

4.3. Preliminary Treatment Efficacy Evaluation

The experiment was approved by the Animal Ethics Committee of the Obihiro University of Agriculture and Veterinary Medicine (Approval No. 22–15). Healthy female 6-week-old BALB/c mice (CLEA Japan, Inc., Tokyo, Japan) were used in this study. All mice had *ad libitum* access to normal chow and water. The virulent *T. congolense* strain IL3000 was propagated in a mouse and used for infection (these parasites were passaged once in the mouse before the experiment). The experimental mice were intraperitoneally injected with 100 μ L of *T. congolense* strain IL3000 in phosphate-buffered saline containing 10% glucose (PSG) at 1×10³ cells/mouse. Mice were randomly allocated to two groups of two mice each as follows: group I served as positive control (since both mice were infected but not treated). In contrast, group II mice were infected and treated with either 100 mg/kg of test compound **4b** orally or 10 mg/kg of test compound **4b** intraperitoneally. Treatment was initiated six d.p.i. upon confirmation of parasitemia using the wet smear technique with fresh blood collected from the tail vein. Treatments were newly prepared every day at a concentration of 100 mg/kg using 10% DMSO in corn oil for oral administration and 10 mg/kg using 1% DMSO in PBS for intraperitoneal

administration and continued for seven consecutive days. The number of trypanosomes in the peripheral blood (diluted to a suitable concentration with PSG) was assessed using a cell counting chamber. The Kaplan-Meier method was used to generate survival curves, and the differences between groups were analyzed using the log-rank test. All analyses were performed using GraphPad Prism version 8 software (GraphPad, Inc., San Diego, CA, USA).

4.4. Intramacrophage Antileishmanial Assay

The resazurin assay and two *Leishmania* strains assessed the efficacy of synthesized isatinylhydantoin derivatives against intramacrophage amastigotes. A literature modified technique^[51] was applied as detailed in Zuma et al.^[45] All synthesized derivatives were first tested for growth inhibition, and those with growth inhibition of more than 60% were considered for further IC₅₀ determination.^[41]

Briefly, 96 well plates of adherent THP1 macrophage cells were infected with *Leishmania donovani* (strain 9515 (MHOM/IN/95/9515)) or *L. major* (strain NIH S (MHOM/SN/74/Seidman)) stationary phase promastigotes with a MOI of 30:1 and then treated with either 10 μ M test compound for activity screening or seven three-fold dilution concentrations of compounds for IC₅₀ determination for 72 h. Upon the completion of the treatment, the host cells were lysed with sodium dodecyl sulphate for 30 sec and promastigote growth medium containing 10% FBS was added to terminate the lysis. To determine the antileishmanial activity, resazurin was added and the plates incubated in the dark for 24 and 72 h for the IC₅₀ determination to assess anti-amastigote activity and potential recovery to proliferative promastigotes, respectively. The absorbances, data analysis, and IC₅₀ determinations were carried out as described in the in Zuma et al.^[45]

4.5. Data Analysis

The in vitro experiments were carried out in triplicate. GraphPad Prism software (GraphPad, Inc., San Diego, CA, USA) was used to compute the reported half maximum cytotoxic (CC₅₀, μ M) or inhibitory concentration (IC₅₀, μ M) of each test compound by non-linear regression (curve fit) and represented as the mean $\pm$ standard deviation (SD). Each compound's selectivity index (SI) was determined using the following formula: CC₅₀ or IC₅₀ of Vero, MDBK or THP1 cells/IC₅₀ of parasite species.

Ethics

Ethics approval for this study was received from the Human Research Ethics Committee of the North-West University (NWU-00093-22-A1). The animal study was approved by the Animal Ethics Committee of the Obihiro University of Agriculture and Veterinary Medicine (Approval No. 22–15).

Disclaimer

Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

Author Contributions

Conceptualization: [David D. N'Da]; Methodology: [Keamogetswe Sechoaro, David D. N'Da, Janine Aucamp, Helena D. Janse van Rensburg]; Formal analysis and investigation: [David D. N'Da, Keamogetswe Sechoaro, Christina Kannigadu, Helena D. Janse van Rensburg and Janine Aucamp]; Writing-original draft preparation: [Keamogetswe Sechoaro; Helena D. Janse van Rensburg]; Writing-review and editing: [David D. N'Da]; Funding acquisition: [David D. N'Da]; Resources: [David D. N'Da]; Supervision: [David D. N'Da].

Acknowledgements

This work was based upon research financially supported by the National Research Foundation (NRF) (UID 140476) and the North-West University, Potchefstroom Campus. All *In vitro* evaluation of cytotoxicity and trypanocidal activity was supported by the Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number 16 K18793 [Grants-in-Aid for Young Scientists (B)], Grant Number 148781 [NRF-JSPS Bilateral Joint Research Program] and Ohyama Health Foundation, Inc. The authors thank Dr. Johan Jordaan for the HRMS analysis and Dr. Daniel Otto for the NMR analysis.

The following reagents were obtained through BEI Resources, NIAID, NIH: *Leishmania donovani*, strain 9515 (MHOM/IN/95/9515), NR-48822; *Leishmania major*, strain NIH S (MHOM/SN/74/Seidman), NR-48819; *Trypanosoma cruzi*, strain Tulahuen, Clone C4 (+ *lacZ*), NR-18959

The following reagents were provided by Dr. Hirumi: *T. b. gambiense*, strain IL1922; *T. b. rhodesiense*, strain IL1501; *T. b. brucei*, strain GUTat3.1; *T. congolense*, strain IL3000; *T. evansi*, strain Tansui.

Conflict of Interests

The authors have no conflict of interest to declare.

Data Availability Statement

The data supporting this study's findings are provided in the supplementary information.

Keywords: Trypanosoma • Leishmania • Isatin • Hydantoin • Hybrid • *In vitro* • *In vivo*

- [1] WHO, Neglected tropical diseases <https://www.who.int/news-room/questions-and-answers/item/neglected-tropical-diseases> [Date of access: 24 May 2024], **2024 a**.
- [2] B. P. Kamdem, F. F. Boyom, *Drugs Drug Candidates* **2023**, 2(3), 624–652.
- [3] M. P. Barrett, S. L. Croft, *Br. Med. Bull.* **2012**, 104(1), 175–196.
- [4] A. Seetsi, D. D. N'da, N. Molefe-Nyembe, K. Sukanuma, T. Ramatla, O. Thekisoe, *Fundam. Clin. Pharmacol.* **2024**, 38(1), 72–83.
- [5] H. D. Janse van Rensburg, D. D. N'Da, K. Sukanuma, *ACS Omega* **2023**, 8(45), 43088–43098.
- [6] WHO, Trypanosomiasis, human African (sleeping sickness; <https://www.who.int/news-room/fact-sheets/detail/trypanosomiasis-human-african-sleeping-sickness>) [Date of access: 22 May 2024] **2023 a**.
- [7] a) DNDI, US FDA approves fexinidazole as the first all-oral treatment for sleeping sickness. <https://dndi.org/press-releases/2021/us-fda-approves-fexinidazole-as-first-all-oral-treatment-sleeping-sickness/> [Date of access: 10 July 2024], **2021 a**; b) DNDI, Fexinidazole for T.b. gambiense. <https://dndi.org/research-development/portfolio/fexinidazole/> [Date of access: 10 July 2024], **2021 b**; c) S. Bernhard, M. Kaiser, C. Burri, P. Mäser, *Diseases* **2022**, 10(4), 90.
- [8] WHO, Chagas disease (American trypanosomiasis); <https://www.who.int/news-room/questions-and-answers/item/chagas-disease> [Date of access: 22 May 2024], **2023 b**.
- [9] M.-J. Pinazo, C. Forsyth, I. Losada, E. T. Esteban, M. García-Rodríguez, M. L. Villegas, I. Molina, C. Crespo-Andújar, M. Gállego, C. Ballart, *Lancet Infect. Dis.* **2024**, 24 (4), 395–403.
- [10] WHO, Number of new reported cases of human African trypanosomiasis (T.b. rhodesiense); <https://www.who.int/data/gho/data/indicators/indicator-details/GHO/number-of-new-reported-cases-of-human-african-trypanosomiasis-t-b-rhodesiense> [Date of access: 22 May 2024], **2023 c**.
- [11] L. A. B. Freitas, A. C. da Silva Santos, G. de Cássia Silva, F. N. do Nascimento Albuquerque, E. D. Silva, C. A. de Simone, V. R. A. Pereira, L. C. Alves, F. A. Brayner, A. C. L. Leite, *Chem. Biol. Interact.* **2021**, 345, 109561.
- [12] H. D. Janse van Rensburg, K. Sukanuma, D. D. N'Da, *Mol. Divers.* **2023**, 28 (4), 2667–2680.
- [13] F. Giordani, L. J. Morrison, T. G. Rowan, H. P. De Koning, M. P. Barrett, *Parasitology* **2016**, 143(14), 1862–1889.
- [14] M. T. Seck, A. G. Fall, M. Ciss, M. T. Bakhoun, B. Sall, A. M. Gaye, G. Gimonneau, M. D. Bassène, R. Lancelot, M. J. Vreysen, *Parasite* **2024**, 31, 11.
- [15] M. Desquesnes, P. Holzmüller, D. H. Lai, A. Dargantes, Z. R. Lun, S. Jittaplapong, *Biomed. Res. Int.* **2013**, 2013, 194176.
- [16] N. I. Toaleb, R. M. Shaapan, *Egyptian J. Vet. Sci.* **2024**, 55(4), 1131–1142.
- [17] Y. Gizaw, M. Megersa, T. Fayera, *Trop. Anim. Health. Prod.* **2017**, 49(1), 887–897.
- [18] Abera, *Int. J. Pure Med. Res.* **2023**, 8(4), 1–7.
- [19] a) H. Berhe, M. Kumar Cinthakunta Sridhar, M. Zerihun, N. Qvit, *Pharmaceutics* **2024**, 16(2), 227; b) WHO, **2023 d**. Leishmaniasis. <https://www.who.int/news-room/fact-sheets/detail/leishmaniasis>, [Date of access: 22 May 2024].
- [20] N. H. Zuma, J. Aucamp, M. Viljoen, D. D. N'Da, *ChemMedChem* **2022**, 17(10), e202200023.
- [21] I. Ramírez-Macías, C. Marín, H. Es-Samti, A. Fernández, J. J. Guardia, H. Zentar, A. Agil, R. Chahboun, E. Alvarez-Manzaneda, M. Sánchez-Moreno, *Parasitol. Int.* **2012**, 61(3), 405–413.
- [22] R. Ettari, L. Tamborini, I. C. Angelo, N. Micale, A. Pinto, C. De Micheli, P. Conti, *J. Med. Chem.* **2013**, 56(14), 5637–5658.
- [23] J. L. Siqueira-Neto, A. Debnath, L. I. McCall, J. A. Bernatchez, M. Ndao, S. L. Reed, P. J. Rosenthal, *PLoS Negl. Trop. Dis.* **2018**, 12(8), e0006512.
- [24] H. Mahmoudzadeh-Niknam, J. H. McKerrow, *Exp. Parasitol.* **2004**, 106(3–4), 158–163.
- [25] V. C. Santos, A. E. R. Oliveira, A. C. B. Campos, J. L. Reis-Cunha, D. C. Bartholomeu, S. M. R. Teixeira, A. P. C. A. Lima, R. S. Ferreira, *Sci. Rep.* **2021**, 11(1), 18231.
- [26] I. Chianzu, E. Hansell, J. Gut, P. J. Rosenthal, J. H. McKerrow, K. Chibale, *Bioorg. Med. Chem. Lett.* **2003**, 13(20), 3527–3530.
- [27] A. Sabt, W. M. Eldehna, T. M. Ibrahim, A. A. Bekhit, R. Z. Batran, *Eur. J. Med. Chem.* **2023**, 246, 114959.
- [28] S. Cho, S.-H. Kim, D. Shin, *Eur. J. Med. Chem.* **2019**, 164, 517–545.
- [29] C. Wang, Q. Zhao, M. Vargas, J. O. Jones, K. L. White, D. M. Shackelford, G. Chen, J. Saunders, A. C. F. Ng, F. C. K. Chiu, Y. Dong, S. A. Charman, J. Keiser, J. L. Vennerstrom, *J. Med. Chem.* **2016**, 59(23), 10705–10718.
- [30] A. Buchynskyy, J. R. Gillespie, Z. M. Herbst, R. M. Ranade, F. S. Buckner, M. H. Gelb, *ACS Med. Chem. Lett.* **2017**, 8(8), 886–891.
- [31] D. Dalvie, S. Nair, P. Kang, C.-M. Loi, *Metabolism, Pharmacokinetics and Toxicity of Functional Groups*, Royal Society of Chemistry Cambridge, UK **2010**, 275–327.
- [32] E. Lanzarotti, L. A. Defelipe, M. A. Marti, A. G. Turjanski, *J. Cheminform.* **2020**, 12(1), 30.
- [33] C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Adv. Drug Deliv. Rev.* **1997**, 23(1), 3–25.
- [34] a) A. Daina, O. Michielin, V. Zoete, *Sci. Rep.* **2017**, 7(1), 42717; b) A. Daina, V. Zoete, *ChemMedChem* **2016**, 11(11), 1117–1121.
- [35] J. L. Siqueira-Neto, O.-R. Song, H. Oh, J.-H. Sohn, G. Yang, J. Nam, J. Jang, J. Cechetto, C. B. Lee, S. Moon, *PLoS Neg. Trop. Dis.* **2010**, 4(5), e675.
- [36] a) K. Sukanuma, P. Allamanda, H. Hakimi, M. Zhou, J. M. Angeles, S.-i. Kawazu, N. Inoue, *J. Vet. Med. Sci.* **2014**, 76(11), 1437–1441; b) K. Sukanuma, S. Yamasaki, N. I. Molefe, P. S. Musinguzi, B. Davaasuren, E. Mossaad, S. Narantsatsral, B. Battur, B. Battsetseg, N. Inoue, *Int. J. Parasitol-Drug* **2017**, 7(2), 200–205; c) L. Munsimbwe, A. Seetsi, B. Namangala, D. D. N'Da, N. Inoue, K. Sukanuma, *Molecules* **2021**, 26(11), 3372.
- [37] a) K. Katsuno, J. N. Burrows, K. Duncan, R. H. Van Huijsduijnen, T. Kaneko, K. Kita, C. E. Mowbray, D. Schmatz, P. Warner, B. Slingsby, *Nat. Rev. Drug Discov.* **2015**, 14(11), 751–758; b) S. Nwaka, A. Hudson, *Nat. Rev. Drug Discov.* **2006**, 5(11), 941–955.
- [38] a) E. A. Adewusi, P. Steenkamp, G. Fouché, V. Steenkamp, *Nat. Prod. Commun.* **2013**, 8(9), 1213–1216; b) J. Fu, H. Chen, D. N. Soroka, R. F. Warin, S. Sang, *J. Agric. Food Chem.* **2014**, 62(20), 4632–4642.
- [39] S. Liu, M. Su, S. J. Song, J. H. Jung, *Mar. Drugs* **2017**, 15(10), 329.
- [40] N. S. Finiuk, V. P. Hreniuh, Y. V. Ostapiuk, V. S. Matyichuk, D. A. Frolov, M. D. Obushak, R. S. Stoika, A. M. Babsky, *Biopolym. Cell* **2017**, 33(2), 135–146.
- [41] G. De Muylder, K. K. Ang, S. Chen, M. R. Arkin, J. C. Engel, J. H. McKerrow, *PLoS Negl. Trop. Dis.* **2011**, 5(7), e1253.
- [42] M. De Rycker, S. O'Neill, D. Joshi, L. Campbell, D. W. Gray, A. H. Fairlamb, *PLoS Negl. Trop. Dis.* **2012**, 6(11), e1932.
- [43] a) M. P. Pai, Cottrell, M. L. Kashuba, A. D. M. & Bertino, J. S., in *Principles and Practice Of Infectious Diseases* (Ed: J. E. Bennett, R. Dolin, M. J. Blaser), Elsevier, W. B. Saunders **2015**, 252–262; b) N. Wald-Dickler, P. Holtom, B. Spellberg, *Clin. Inf. Dis.* **2017**, 66(9), 1470–1474.
- [44] E. M. Czekanska, *Mammalian Cell Viability: Methods and Protocols* (Ed: M. J. Stoddart), Humana Press, Totowa, NJ **2011**, 27–32.
- [45] N. H. Zuma, J. Aucamp, H. D. Janse van Rensburg, D. D. N'Da, *Eur. J. Med. Chem.* **2023**, 246, 115012.
- [46] a) N. I. Molefe, S. Yamasaki, A. M. C. Macalanda, K. Sukanuma, K. Watanabe, X. Xuan, N. Inoue, *Parasitol. Res.* **2017**, 116, 2407–2415; b) R. Nath, S. Pathania, G. Grover, M. J. Akhtar, *J. Mol. Struct.* **2020**, 1222, 128900.
- [47] A. Kulshrestha, V. Bhandari, R. Mukhopadhyay, V. Ramesh, S. Sundar, L. Maes, J. C. Dujardin, S. Roy, P. Salotra, *Parasitol. Res.* **2013**, 112, 825–828.
- [48] N. Martínez-Peinado, C. Martori, N. Cortes-Serra, J. Sherman, A. Rodríguez, J. Gascon, J. Alberola, M. J. Pinazo, A. Rodríguez-Cortes, J. Alonso-Padilla, *Int. J. Mol. Sci.* **2021**, 22(2), 688.
- [49] K. Sukanuma, S. Narantsatsral, B. Battur, S. Yamasaki, D. Otgonsuren, S. P. Musinguzi, B. Davaasuren, B. Battsetseg, N. Inoue, *Parasit. Vectors* **2016**, 9, 1–9.
- [50] H. Hirumi, K. Hirumi, *Parasitology* **1991**, 102(2), 225–236.
- [51] a) S. K. Jain, R. Sahu, L. A. Walker, B. L. Tekwani, *J. Vis. Exp.* **2012** (70), e4054; b) C. A. N. Njanpa, S. C. N. Wouamba, L. R. T. Yamthe, D. Dize, B. M. T. Tchata, P. V. F. Tsouh, M. N. Pouofo, J. B. Jouda, B. L. Ndjakou, N. Sewald, *BMC Complement Med. Ther.* **2021**, 21, 1–12.

Manuscript received: July 15, 2024

Revised manuscript received: September 25, 2024

Accepted manuscript online: September 30, 2024

Version of record online: November 8, 2024