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The Impact of BMH-21 on Parasite Load, Biochemical Indices, *ESAG6* and *ITS-I* Genes of *Trypanosoma brucei brucei* Infected Albino Rats

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Abstract

This study investigated the in vivo anti-trypanosomal activity of BMH-21. Wistar albino rats were randomly divided into eight groups, each consisting of five rats: IT20, IT40, and IT60. These groups received treatments with 20, 40, and 60 mg/kg body weight (bw) of BMH-21, respectively, after being infected. Additional groups included IUT (Infected-untreated), UUT (Uninfected-untreated), UT60 (Uninfected but given 60 mg/kg bw of BMH-21), P60 (administered 60 mg/kg bw of BMH-21 once, infected after 72 hours, with treatment continuing daily until Day 4), and ITDA (Infected and treated with 3.5 mg/kg bw of diminazine aceturate). Infection was induced by intraperitoneal injection of 0.5 ml of blood containing approximately 10^3 cells/ml. Standard protocols were followed for parasitological, biochemical, histopathological, and gene expression analyses. Graded doses of BMH-21 significantly reduced ($p<0.05$) parasitemia in the infected-treated rats. Treatment with 60 mg/kg bw of BMH-21 resulted in a significant increase ($p<0.05$) in PCV, RBC, Hb, and WBC counts in infected-treated animals compared to the IUT group. Serum ALT activity significantly decreased ($p<0.05$) in the UT60 and P60 groups compared to IUT. Serum AST levels also showed a significant decrease

in the IT20, UT60, and P60 groups. Additionally, the expression levels of the T. b. brucei ESAG6 and ITSI genes were down-regulated by 40 mg/kg bw of BMH-21, with fold reductions of 5 and 3, respectively, compared to the calibrator. BMH-21 demonstrated anti-trypanosomal potential against T. b. brucei, improved the biochemical parameters of infected animals, and reduced the expression levels of ESAG6 and ITSI genes.

Keywords: BMH-21; *Trypanosoma brucei brucei*; RNA polymerase I (RNAPI); Variant surface glycoprotein (VSG); Internal transcribed spacer-I (ITS-I); Expression site associated gene 6 (ESAG6).

1. Introduction

Trypanosomiasis is a vector-borne parasitic disease caused by protozoa of the genus Trypanosoma, primarily transmitted to humans and animals through the bites of tsetse flies (Glossina genus), which acquire the parasite during a blood meal from infected human or animal hosts [1]. Additionally, transmission can occur from mother to fetus via the placenta, through mechanical means involving other blood-sucking insects, accidental pricks with contaminated needles, and through sexual contact [2].

Human African trypanosomiasis (sleeping sickness) is caused by two subspecies of Trypanosoma brucei: *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* [3]. The chronic form of the disease, caused by *T. b. gambiense*, is endemic in 24 West and Central African countries and accounts for 97% of reported sleeping sickness cases. In contrast, *T. b. rhodesiense* causes the acute form of the disease, occurring in 13 countries in Eastern and Southern Africa, representing about 3% of reported cases [2]. A third subspecies, *Trypanosoma brucei brucei* (*T. b. brucei*), is responsible for the animal form of the disease and is not pathogenic to humans due to its sensitivity to the trypanolytic factor in human serum [3].

Trypanosomiasis progresses through two stages. The first, known as the haemo-lymphatic stage, involves the parasites multiplying in subcutaneous tissues, blood, and lymph, leading to symptoms such as fever, joint pain, itching, headaches, and swollen lymph nodes [2]. The second stage, referred to as the neurological or meningo-encephalitic stage, is marked by behavioral changes, confusion, sensory disturbances, and poor coordination. These symptoms arise when the parasites cross the blood-brain barrier and infect the central nervous system [2].

Trypanosomes evade host immune responses through antigenic variation of their variant surface glycoprotein (VSG) coats. The genes responsible for encoding these surface glycoproteins, such as the procyclin gene (in the insect form of the parasite) and the VSG gene (in the bloodstream form of the parasite), are transcribed by RNA polymerase I (RNAPI). Additionally, the VSG-expression site associated genes (VSG-ESAGs) and ribosomal RNA genes, including their intervening internal transcribed spacer I (ITS-I) genes, also rely on RNAPI for transcription [4-6]. T. brucei VSG-ESAG 6 and 7 encode transferrin receptors that allow the parasite to absorb iron from the host's blood circulation [7, 8]. This iron uptake deprives the host of the iron necessary for erythropoiesis and may contribute to the anemia that is a hallmark of trypanosomiasis.

The management of trypanosomiasis has primarily relied on chemotherapy. For Animal African Trypanosomiasis, treatments include homidium bromide, homidium chloride, diminazine aceturate, quinapyramine sulfate, isometamidium chloride, melarsomine dihydrochloride, and suramin sodium. In the case of Human African Trypanosomiasis, drugs such as pentamidine, suramin, melarsoprol, eflornithine, nifurtimox, the nifurtimox–eflornithine combination therapy (NECT), and fexinidazole are commonly used [9-12].

Various drugs have been tested in experimental treatments of T. b. brucei in rats, including kelamidium [13], levamisole with or without Vitamin C [14], and combinations like diminazine aceturate with arteether [15]. However, the use of chemotherapeutic agents has faced significant challenges due to their high toxicity, complex administration protocols, high costs, specificity to certain subspecies and disease stages, and reduced efficacy caused by the emergence of drug-resistant parasites [16-18]. These issues have prompted the search for new, safer, and more effective therapies with broad-spectrum activity that are not limited by subspecies or disease stages, leading to the consideration of (N-[2-(dimethylamino) ethyl]-12-oxo-12h-benzo [g] pyrido [2, 1-b] quinazoline-4-carboxamide) (BMH-21).

BMH-21 is a small, planar tetracyclic molecule that intercalates into double-stranded DNA, particularly favoring GC-rich sequences. Molecular modeling has shown that BMH-21 aligns flatly between GC base pairs, with its positively charged side chain possibly interacting with the DNA backbone. This novel compound inhibits RNA polymerase I transcription by binding to ribosomal DNA, resulting in a blockade of RNA polymerase I and the degradation of its large catalytic subunit (RPA194).

According to Kerry, *et al.* [19], BMH-21, along with other RNA polymerase I inhibitors like quarfloxin and CX-5461, specifically inhibited RNA polymerase I transcription in vitro in *T. brucei*. This inhibition was marked by the reduction of RNA precursor transcripts from Polymerase I, disruption of the parasites' nucleolus and VSG expression site body, and a halt in cell proliferation. Additionally, there was a decrease in the transcription of ribosomal RNA and Variant Surface Glycoprotein genes. Investigating BMH-21's antitrypanosomal activity in vivo will provide a strong foundation for considering its potential as a treatment for trypanosomiasis. Therefore, this study aimed to assess BMH-21's effectiveness against *T. brucei* in living organisms.

2. Materials and Methods

2.1. Experimental Animals and Parasites

Wistar albino rats were sourced from the Department of Veterinary Pharmacology and Toxicology at the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. The rats were housed in laboratory cages, fed standard rat chow, and provided with water ad libitum. The *T. b. brucei* (Federe strain) was obtained from the

Nigerian Institute for Trypanosomiasis Research (NITR) in Kaduna, Nigeria, and confirmed via nested polymerase chain reaction.

2.2. Test Drugs

The study utilized BMH-21 (N-[2-(dimethylamino)ethyl]-12-oxo-12H-benzo[g] pyrido[2,1-b] quinazoline-4-carboxamide), procured from MedChem Express, Monmouth Junction, NJ, USA, and diminazine aceturate (DA), from Aether Centre, Beijing, Biology Co., Ltd. A 50 mg/ml stock solution of BMH-21 was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) with gentle warming. Diminazine aceturate did not require warming.

2.3. Intra-peritoneal Acute Toxicity Study of BMH-21 on Female Rats

The acute toxicity of BMH-21 was assessed using the OECD guidelines [20].

2.4. In Vivo Anti-*T. b. brucei* Effect of BMH-21

Wistar albino rats were assigned randomly into eight groups, each consisting of five rats. Infection was administered via intraperitoneal injection of 0.5 ml of blood containing approximately 10^3 trypanosome cells/ml. Treatment started 96 hours post-infection (day 4) and continued daily for 3 days (days 4-6), with the exception of the P60 group, which received 60 mg/kg BMH-21 once 72 hours before infection and then continued treatment for 5 days (days 0-4). The groups were as follows: UUT: Uninfected-untreated, IUT: Infected-untreated, ITDA: Infected-treated with standard trypanocide (diminazine aceturate, 3.5 mg/kg) twice (days 4 and 5), IT20: Infected-treated with 20 mg/kg BMH-21, IT40: Infected-treated with 40 mg/kg BMH-21, IT60: Infected-treated with 60 mg/kg BMH-21, UT60: Uninfected but treated with 60 mg/kg BMH-21, P60: Animals received 60 mg/kg BMH-21 once 72 hours before infection, then treated daily for 5 days (days 0-4). Treatment was stopped in P60 on day 4. Parasitemia was monitored daily using the "Rapid Matching" method of Herbert and Lumsden [21]. Blood, kidney, and liver samples were collected for analysis at the end of the 5 days treatment for P60 and after 3 treatment days for other groups for further investigation. Haematological parameters were determined using Mindray Bc-5000 Automatic Hematology Analyzer (Guangzhou Medsinglong Medical Equipment Co., Ltd). The serum levels of biochemical parameters were determined using standard assay kits: Creatinine and urea (Agappe Diagnostics Switzerland GmbH), ALT and AST (Randox Laboratories Ltd. United Kingdom) following manufacturer's instructions. Histological changes in the liver and Kidney tissues were examined according to the method described by Avwioro [22].

2.5. Assay for the Effect of BMH-21 on the Expression of ESAG6 and ITS-I Genes of *T. b brucei* infected Rats

Total RNA extraction was done with Quick-RNA^{TM} MiniPrep (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instruction. Complementary DNA (cDNA) was synthesized using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs Inc) according to the instructions of the manufacturer. RNA and cDNA qualities were assessed using Nanodrop Spectrophotometer (Nanodrop One C, Thermo Fisher Scientific, Madison, USA). Quantitative real-time PCR (qPCR) was performed on a BioRad qPCR System using Lunar Universal qPCR Master Mix according to the manufacturer's instruction. Primers used for the qPCR amplification of *ITS-I gene* were TRYP 1 and TRYP 2 [23] while those of *ESAG6 gene* were designated as ESAG6F and ESAG6R. *α-Tubulin gene* was used as control. Primer sequences are listed in Table 4. The fold changes were calculated according to the $2^{-\Delta CT}$ method of Schmittgen and Livak [24].

2.6. Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (version 20, IBM Corporation, NY, USA). Normality was tested with Shapiro-Wilk's test. Data with normal distribution were analyzed using One-way ANOVA, with differences between means identified using Duncan's post-hoc test. Non-normally distributed data were analyzed with the Kruskal-Wallis test. Sample size was n = 5 unless otherwise specified.

2.7. Ethical Approval

The research received ethical approval from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), with approval number ABUCAUC/2021/069. All animal handling adhered to the committee's guidelines.

3. Results

3.1. Intra-peritoneal Acute Toxicity of BMH-21 on Female Albino Rats

The acute toxicity profile of BMH-21 was evaluated, with the results summarized in Table 1. Rats given 5 mg/kg and 50 mg/kg BMH-21 showed initial weakness but recovered by day 1 with no lasting toxicity. The 300 mg/kg dose caused panting and hiding behavior but resolved after 3 hours with no further toxicity. Rats administered 2000 mg/kg exhibited staggering, loss of appetite, and restlessness, leading to mortality within 24 hours. The intraperitoneal LD_{50} of BMH-21 was estimated to be between 300 and 2000 mg/kg body weight ($300 < LD50 < 2000$) mg/kg body weight.

Table-1. Intra-peritoneal acute toxicity profile of BMH-21 on female wistar albino rats

Doses of BMH-21 (mg/kg body weight)	Observations from the animals	Recovery period	Lethality
5	Weakness	<24 hrs.	Nil
50	Weakness	<24 hrs.	Nil
300	Weakness, panting, animal sought hiding places within the cage, loss of appetite	24 hrs.	
2000	Lethargy, loss of appetite, groaning sounds	Nil	Death in <24 hrs.

The intra-peritoneal LD_{50} of BMH-21 was determined to be between 300 and 2000 mg/kg body weight (300 < LD50 < 2000). The sample size for this study was 4.

3.2. Effect of BMH-21 Treatment on Parasitaemia in *T. b. brucei*-Infected Rats

Parasitaemia was detected in the infected rats 3 days after infection, with treatment beginning on Day 4 (refer to Table 2). The parasite load in the infected-untreated (IUT) group increased progressively throughout the study period. By Days 6 and 7, significant reductions in parasitaemia were observed in the IT20, IT40, IT60, ITDA, and P60 groups compared to the IUT control ($p<0.05$). No significant differences in parasitaemia were noted between the diminazine aceturate-treated group and those receiving 20, 40, or 60 mg/kg BMH-21 on Days 6 and 7 ($p>0.05$). In the P60 group, parasites were not detected by microscopy until Day 6 post-infection (2 days after treatment cessation), but parasitaemia was reestablished by Day 7 (3 days after treatment was stopped).

Table-2. Effect of BMH-21 and DA on the Parasitaemia (x 10^6 Cells/mL) of *T. b. brucei* infected rats

Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
IUT	0.00±0.0	0.00±0.0	38.33±35.5^a	45.08±35.5^a	152.53±65.1^b	237.47±77.3^b	214.96±85.2^b
ITDA	0.00±0.0	0.00±0.0	0.60±0.3^a	37.95±25.1^a	31.62±23.8^{ab}	11.18±5.9^a	0.251±0.0^a
IT20	0.00±0.0	0.00±0.0	19.84±12.3^a	69.32±24.7^{ab}	34.73±11.8^{ab}	44.15±21.3^a	25.27±11.8^a
IT40	0.00±0.0	0.00±0.0	39.52±15.0^a	157.22±31.3^b	43.42±11.8^{ab}	13.85±6.7^a	0.06±0.06^a
IT60	0.00±0.0	0.00±0.0	11.99±7.5^a	78.78±30.1^{ab}	39.49±7.9^{ab}	5.95±3.7^a	0.06±0.06^a
P60	0.00±0.0	0.00±0.0	0.00±0.0^a	0.00±0.0^a	0.00±0.0^a	0.00±0.0^a	34.78±12.6^a

Treatment in all groups, except P60, started on Day 4 post-infection (pi) and concluded on Day 6 pi. The sample size (N) = 5 animals. Data were analyzed using One-way ANOVA with Duncan's post-hoc test for multiple comparisons. A p-value <0.05 indicated significant differences among groups, with values marked by different letters (a-b) signifying significant differences between treatments.

3.3. Effect of Treatment on Animal Survival

As shown in Figure 1, the survival rate was 100% in the UUT, ITDA, UT60, and P60 groups. The infected-untreated control (IUT) had a 20% survival rate, while the IT40 and IT60 groups each had a 40% survival rate. The group treated with the lowest dose of BMH-21 (IT20) had a 60% survival rate.

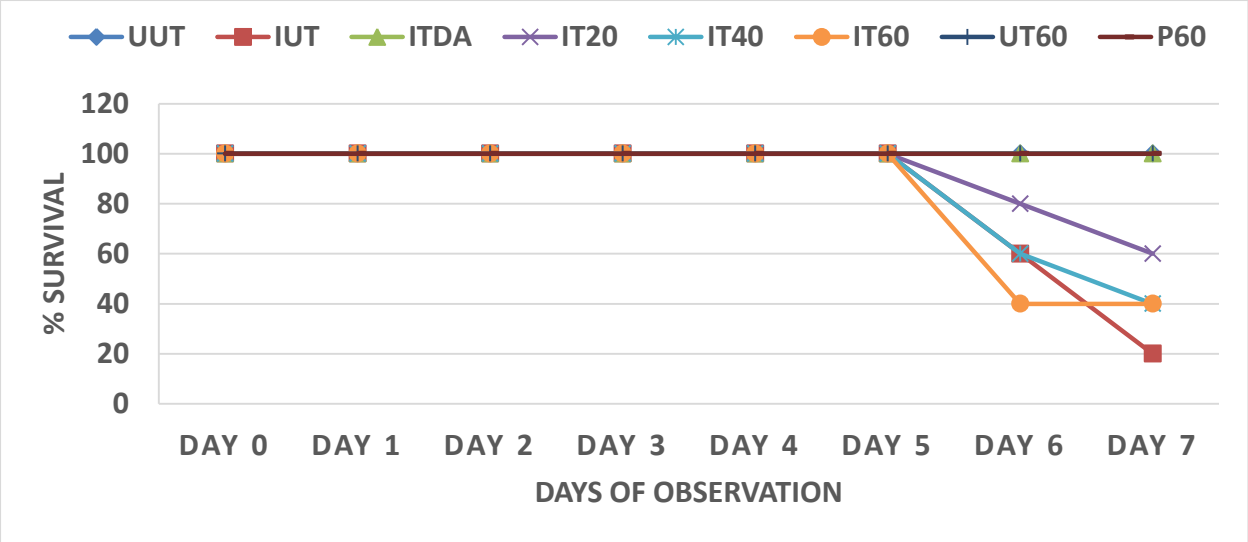


Figure-1. Effect of BMH-21 on the Percentage Survival of *T. b. brucei* Infected

3.4. Effect of BMH-21 on biochemical parameters of *T. b. brucei* infected rats

The biochemical parameters of *T. b. brucei* infected rats treated with BMH-21 shown in Table 3 indicates that the urea level in IT20 and P60 decreased significantly compared to IUT, ITDA and IT40. However, there was no significant change in serum urea levels of UUT compared to IT40, IT60, UT60 and P60. The serum creatinine of ITDA, IT40, UT60 and P60 when compared to each other did not show significant changes ($p>0.05$). However, their levels decreased significantly ($p<0.05$) relative to IUT, IT20, and IT60. The data also showed elevation of creatinine in IT20 and IT60 relative to other BMH-21 treated groups. The serum ALT activities were significantly lower ($p<0.05$) in UUT, ITDA, UT60 and P60 compared to IUT. There were increased ALT activities ($P<0.05$) in IT20, IT40 and IT60 compared to the uninfected-untreated control (UUT). Furthermore, serum AST activity was significantly decreased in the UT60

group relative to all other groups. AST activity in IUT, IT40 and IT60 showed significantly ($p<0.05$) elevation compared to other groups. The AST activities in ITDA, IT20 and P60 did not show significant changes compared to UUT.

The data also reveals that groups IUT, IT20 and P60 recorded significant decrease ($P<0.05$) in their PCV when compared to the uninfected-untreated (UUT) control as well as the group treated with diminazine aceturate. However, there was no significant ($p>0.05$) differences in the PCV of UUT when compared ITDA, IT40, IT60 and UT60. The white blood cell count (WBC) of P60 increased significantly ($p<0.05$) relative to UUT, IUT, ITDA, IT20 and UT60. For the red blood cell (RBC) count, UUT showed RBC level comparable to ITDA, IT40, IT60, UT60 and P60 whereas a significant ($P<0.05$) reduction in RBC count was noticed in IUT and IT20 compared to UUT. The haemoglobin concentrations of UUT, ITDA, IT40, IT60, and UT60 are comparable to each other while the RBC count of IUT depreciated significantly ($p<0.05$) relative to UUT, ITDA, IT40, IT60 and UT60.

3.5. Effect of BMH-21 on Histology of Liver and Kidney of *T. b brucei* Infected Rats

The histological changes in the kidney and liver are shown in Figure 2 and 3 respectively. Figure 2 shows normal histological features of the kidney in the uninfected-untreated group (UUT) and tubular necrosis for the infected control (IUT). The group treated with standard drug displayed hyperplasia of inflammatory cells. More so, treatment with 40 mg/kg (IT40) resulted in tubular distortion whereas IT20, IT60, UT60 and P60 showed tubular necrosis. Normal liver histological features were shown in UUT, ITDA, IT20 and UT60. The groups infected and treated with 40 and 60 mg/kg as well as P60 displayed hepatic necrosis (Figure 3).

Table-3. Effect of BMH-21 on Biochemical parameters of *T. b. brucei* infected rats

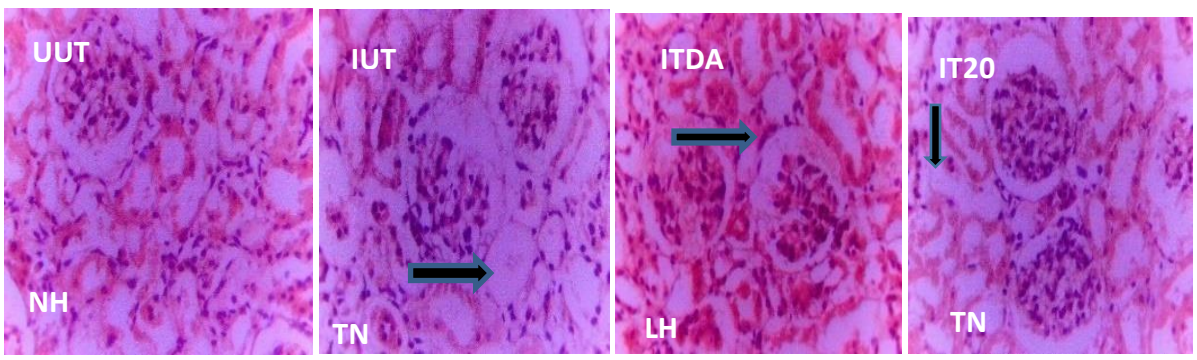
Groups	PCV (%)	WBC (10^3/µL)	RBC (10^6/ µL)	HB (g/dL)	Urea (mg/dL)	Creatinine (mg/dL)	ALT (U/L)	AST (U/L)
UUT	51.07 ± 7.0^{c}	6.64 ± 2.3^{a}	7.88 ± 0.8^{c}	13.17 ± 1.0^{c}	38.95 ± 3.19^{ab}	1.46 ± 0.12^{b}	46.67 ± 17.24^{a}	104.33 ± 2.31^{bc}
IUT	28.15 ± 8.3^{a}	6.75 ± 1.5^{a}	4.97 ± 1.0^{a}	8.47 ± 1.9^{a}	70.79 ± 8.20^{b}	1.63 ± 0.31^{bc}	138.67 ± 3.51^{c}	199.67 ± 4.04^{d}
ITDA	48.83 ± 2.9^{c}	9.69 ± 2.0^{ab}	7.59 ± 0.7^{bc}	12.40 ± 0.7^{bc}	54.39 ± 19.0^{b}	0.45 ± 0.29^{a}	50.00 ± 9.54^{a}	91.00 ± 1.73^{b}
IT20	36.40 ± 9.2^{ab}	10.61 ± 4.8^{ab}	5.90 ± 1.6^{ab}	9.90 ± 2.5^{ab}	35.35 ± 1.97^{a}	1.94 ± 0.15^{c}	91.67 ± 28.87^{bc}	116.67 ± 20.20^{bc}
IT40	43.00 ± 4.1^{bc}	12.75 ± 1.9^{abc}	6.74 ± 0.3^{abc}	11.40 ± 0.7^{bc}	63.77 ± 3.78^{b}	0.89 ± 0.11^{a}	120.33 ± 0.58^{bc}	180.67 ± 0.58^{cd}
IT60	42.97 ± 5.1^{bc}	17.01 ± 11.0^{bc}	7.23 ± 1.1^{bc}	13.10 ± 1.0^{c}	56.84 ± 14.78^{ab}	1.5 ± 0.43^{bc}	112.00 ± 16.46^{bc}	202.33 ± 10.12^{d}
UT60	46.87 ± 4.3^{bc}	7.24 ± 3.1^{a}	7.10 ± 0.9^{bc}	12.07 ± 0.6^{bc}	40.73 ± 1.78^{ab}	0.65 ± 0.14^{a}	49.33 ± 0.57^{a}	62.00 ± 13.86^{a}
P60	37.57 ± 1.9^{ab}	19.97 ± 1.1^{c}	6.07 ± 0.7^{abc}	10.00 ± 1.0^{ab}	30.03 ± 6.19^{a}	0.79 ± 0.20^{a}	77.67 ± 6.35^{ab}	101.91 ± 6.95^{bc}

Results are expressed as mean ± SD. Values with different superscripts down the column are significantly different from one another at $p<0.05$ (One-way ANOVA, Duncan-HSD multiple range *post hoc* test for normally distributed samples). Except where otherwise stated, treatment started on day 4 and ended on day 6. **P60**: Administered 60 mg/kg bw of BMH-21 72 hours before infection day (day 0) and treatment continued immediately after infection once daily until day 4. The experiment was terminated on day 7 and blood samples were collected for assessment. Creatinine was not normally distributed, so Kruskal-Wallis test was used to confirm the differences among the groups while the rest were not normally. Sample size (N) = 3.

Table-4. List of Primers used in gene expression study

Name of Primers	Sequences 5'-3'	Sources
Tryp 1 Tryp 2	AAGCCAAGTCATCCATCG TAGAGGAAGCAAAAG	(Adams *et al*., 2006)
ESAG 6F ESAG 6R	TGCACAGGGCAGAAAGAGGC CCCCGCTACCGTGAAAGGTC	
α-Tubulin F α-Tubulin R	GAGGAGGTGGGAAGGGTATATG GAAGGCGTGTTGATGAGTTGTA	(Kerry *et al*., 2017)

Tryp 1 and 2 were used for the qPCR amplification of the ITS-I region of *T. b. brucei* ribosomal DNA. α-Tubulin F and α-Tubulin R were forward and reverse primers respectively used for the amplification of the house keeping gene (alpha tubulin). ESAG 6F and ESAG 6R were the forward and reverse primers respectively used in qPCR for the amplification of the expression site associated gene 6.



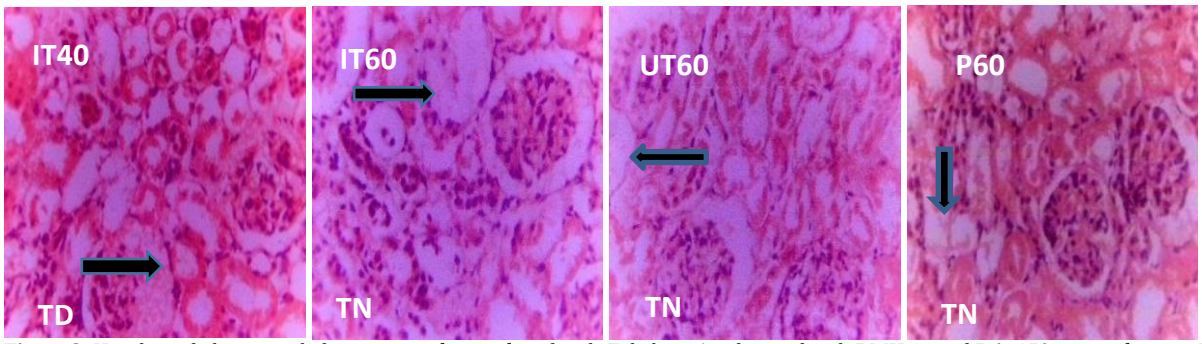


Figure-2. Histological changes in kidney tissues of rats infected with *T. b. brucei* and treated with BMH-21 and DA, 250× magnification, H & E Stain.

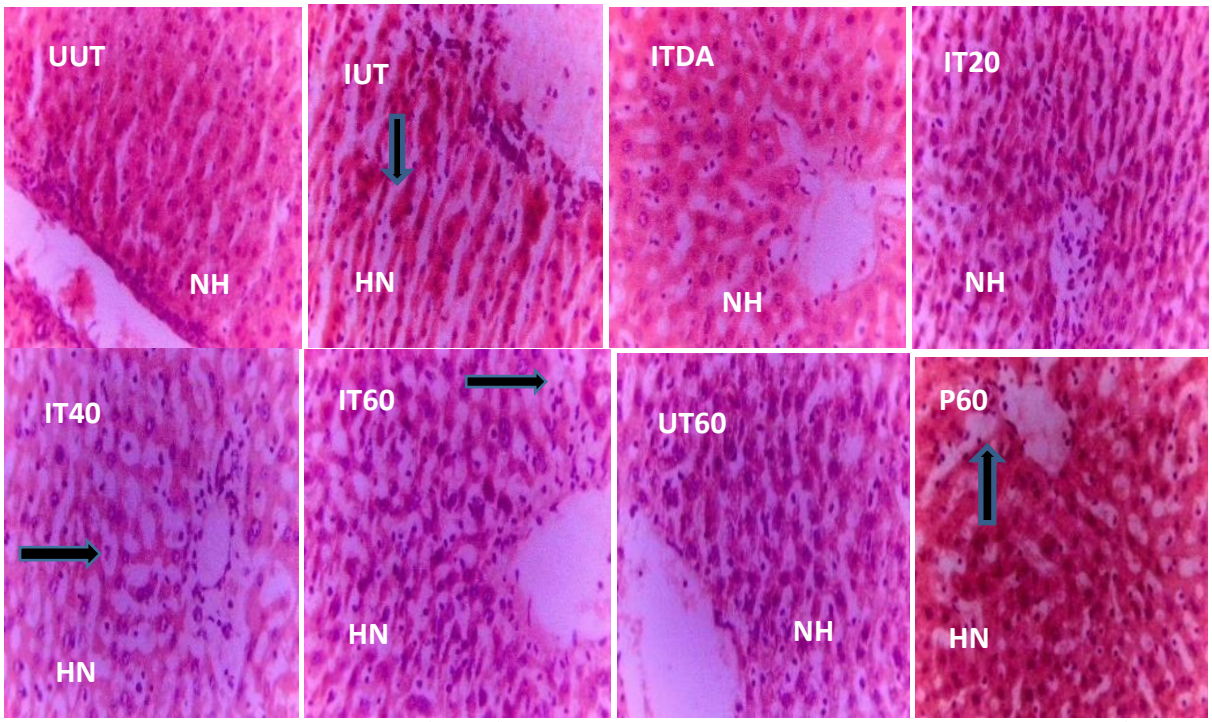
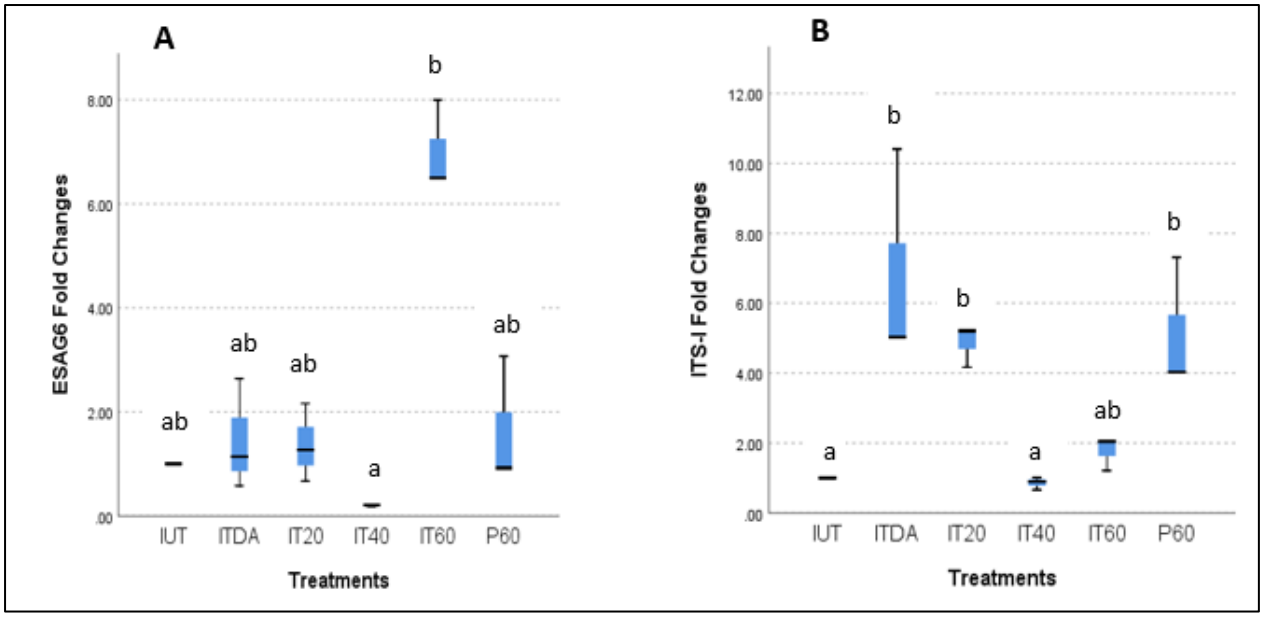


Figure-3. Histological changes in liver tissues of rats infected with *T. b. brucei* and treated with BMH-21 and DA, 250× magnification, H & E Stain.

Arrows point at the histological changes observed in the tissues which are named in white ink below the arrows in each of the plate. **TN**: Tubular necrosis, **LH**: Lymphocyte hyperplasia, **TD**: Tubular distortion, **NH**: Normal hepatocytes, **HN**: Hepatic necrosis, **TD**: Tubular distortion. Except otherwise stated treatment started on day 4 post-infection and ended on day 6 post-infection.

3.6. Effect of BMH-21 on the Expression Levels of *ESAG6* and *ITS-I* Genes

The effect of treatment on the expression levels of *ESAG6* and *ITS-I* genes are shown in **Figures 4 (A and B)**. Treatment with 40 mg/kg body weight of BMH-21 (IT40) resulted in a decrease in the expression level of *ESAG6* with a reduction fold change of 5 relative to the calibrator and normalized against *α-tubulin* gene Figures 4 (A) while other treatments gave rise to upregulation of *ESAG6* expression. The data also showed that treatment with the 3.5 mg/kg of DA (ITDA) resulted in an increase in the expression level of *ESAG6* though the observed increase was not significant compared to other treatments. IT60 also generated an increased expression level of ESAG6 which is significantly higher than IT40. Figures 4 **B** shows that treatment with 40 mg/kg (IT40) of BMH-21 resulted in decreased expression of *ITS-I* gene with reduction fold changes of 3 compared to the infected-untreated control. The relative abundance of *ITS-I* gene was significantly increased in ITDA, IT20, and P60 compared to IUT and IT40.



Figures-4.

Figures 4: Effect of BMH-21 treatment on the gene expression of (A) T. b. brucei VSG expression site associated gene 6 (ESAG6) and (B) internal transcribed spacer I (ITS-I) gene of T. b. brucei ribosomal DNA (rDNA). Sample size (N) = 3. Boxplots with different alphabets (a-b) are significantly different from each other. (Kruskal-Wallis test was used to determine the statistical differences in the groups and values with $p<0.05$ were taken to be significantly different from others). Except otherwise stated, treatment started on day 4 and ended on day 6. P60: Administered 60 mg/kg bw of BMH-21 72 hours before infection day (day 0) and treatment continued immediately after infection once daily until day 4. The experiment was terminated on day 7 and blood samples were collected for gene expression studies.

4. Discussion

This study evaluated the *in vivo* antitrypanosomal potential of BMH-21, a small molecule inhibitor of RNA polymerase I (RNAP I) enzyme. Rapidly proliferating eukaryotic cells typically rely on RNAP1 enzyme for the transcription of their ribosomal RNA gene unit [4]. In trypanosomes however, RNAP I is responsible for the transcription of variant surface glycoprotein (VSG) genes, VSG expression site associated genes as well as the ribosomal RNA gene unit which contains the internal transcribed space-I (ITS-I) region.

The major and overriding criterion in the selection of medicines for use in health services is safety [25]. The foregoing informed the acute toxicity study of BMH-21. There were no serious toxicity signs/symptoms in rats given 5-300 mg/ kg bw of BMH-21 but at 2000 mg/kg bw mortality was recorded. Therefore, the mean lethal dose (LD_{50}) was taken to be greater than 300 mg/kg but less than 2000 mg/kg bw (300 mg/kg < LD_{50} <2000 mg/kg bw) in line with the Globally Harmonized Classification System of chemical compounds [20].

In view of the above, the compound was examined for its in *vivo* antitrypanosomal potential using albino rats experimentally infected with *T. b. brucei* (Federe strain). Amongst the three different doses (20, 40 and 60 mg/kg body weight) used in the study, 40 mg/kg could be taken as the minimum effective dose as it reduced the parasitaemia of the infected rats to a level not detected by microscopic examination of tail vein blood. This finding is in tandem with the report of Jutamaad, *et al.* [26], which stated that if the mean lethal dose of a substance is three times more than the minimum effective dose, such substance should be given further consideration as a safe drug candidate.

The study showed that the parasitaemia of the infected-untreated animals progressively increased which could be due to proliferation of the parasites within the host as there was no interference but for the host's immune system which trypanosomes notoriously evade through their mechanism of antigenic variation. At days 6 and 7, the parasitaemia of groups infected and treated with 20, 40 and 60 mg/kg bw of BMH-21 as well as diminazine aceturate 3.5 mg/kg (D A) showed significant decrease ($p<0.05$) relative to the infected-untreated control. In fact, 40, 60 mg/kg bw and DA at day 7 reduced the parasite load to a level that could not be detected by microscopy. This clear suppression of parasitemia by BMH-21 in rat hosts lends credence to the findings of Kerry, *et al.* [19] which reported *in vitro* anti-*T. brucei* activity of BMH-21.

However, delay in parasite establishment was recorded in P60 which was administered 60 mg/kg bw of BMH-21 once within 72 hours before inoculation and subsequently treated once daily for 5 days. Parasite was first detected in P60 after 7 days infection/treatment as against the other treatment groups where the parasite showed a pre-patent period of 72 hours (3 days). The establishment of parasite in this group three days after treatment was stopped suggests that BMH-21 has trypanostatic effect against *T. b. brucei in vivo.*

Survival of the experimental animals was also monitored, with 100 % survival recorded in UUT, ITDA, UT60 and P60 at the final day of the experiment (Day 7). Expectedly, the infected-untreated (IUT) group had the least percentage survival may be due to the progressive proliferation of the parasite which might have caused pathological

changes in the host hence the increased mortality. Animals in the P60 group completely survived the parasite challenge (for 7 days before the experiment was terminated) possibly because of the trypanostatic ability displayed by BMH-21. The groups infected and treated with 40 and 60 mg/kg bw recorded 40% survival each while 60% survival was seen in the group infected and treated with 20 mg/kg bw. A possible reason for the mortality here could be the inability of BMH-21 to remedy the pathological changes caused by the parasites before administration of the compound judging from the 100% survival recorded in UT60 which was given highest dose of BMH-21 without infection. The survivability of the experimental animals defied dose dependent pattern and could be due to idiosyncratic interaction of the animals with the pharmacologic agent. The infected-untreated control (IUT) had a survival of 20% (80% mortality) which supports the deadly nature of trypanosomes especially when untreated. No record of mortality occurred in the control group (UT60) which was not infected but administered highest dose (60 mg/kg bw) of the test compound, which suggests that the tested doses of BMH-21 may not be responsible for the cases of mortality recorded in the infected and treated groups.

According to John, *et al.* [27], alteration in haematological parameters is characteristic of African trypanosomiasis which greatly influences the disease's pathogenesis. In the light of the above, we investigated the effect of BMH-21 on some haematological parameters of the infected-treated rats and also in the control groups. The PCV, RBC and Hb concentration of the infected-untreated group in this experiment were decreased when compared to other groups. This may be due to haemolysis and consequent anaemic condition which usually sets in during trypanosome infection. Our finding is consistent with that of Fidelis, *et al.* [28] who reported a decrease in the RBC count, hemoglobin and PCV, although they remained within the reference values. Decreases in erythrocytes have been associated with erythrocytes desialylation in the acute phase of disease, stage of high parasitemia [29]. Treatment with BMH-21 sustained these haematological parameters within physiological levels in a manner comparable to that of the standard drug (diminazine aceturate) except for IT20 whose PCV, RBC and Hb were significantly reduced ($p<0.05$) when compared to the uninfected-untreated control. A possible explanation to this could be that the 20 mg/kg was not good enough to elicit the required anti-trypanosomal effect within the period of observation hence unable to restore the haematological parameters altered by the parasite in the host.

The WBC count showed a dose-dependent increase in the BMH-21 treated groups which was higher though not significantly ($P>0.05$) than that of the group treated standard drug. This rise in WBC count following treatment may be an indication that BMH-21 has an immune stimulatory function or that its suppression of parasitaemia relieved the animals of the biological assault hence the increase in leucocyte level. The defense of the body system from invasion of infectious agents/foreign bodies are vested on the white blood cells which play important roles in the establishment of phagocytes/antibodies against recognizable antigens of invading parasites [30, 31]. However, the P60 group showed a significantly increased WBC count ($p<0.05$) relative to UUT, IUT, ITDA IT20 and UT60 which could be due to the immune system responding to the rebounding parasitaemia in the host when effect of BMH-21 diminished following withdrawal of drug administration. Hence the host's system recruiting its immune arsenals to combat the assault of the invading haemoflagellates by encouraging the production of more WBCs [32].

The infected-untreated control group (IUT) displayed the least level of WBC count when compared to the treated groups. It has been suggested that early leukopenia during trypanosomiasis may be influenced by the bulk trypanosome antigen [33]. Support for this comes from the fact that after the decline in parasitemia, the leukocyte values tend to return to the pre-infection phase and sometimes even exceed these values [28].

The onset of renal pathology in infected hosts could be implicated by the elevation of serum creatinine and urea levels [34]. The liver and kidney play important roles in the biosynthesis of erythropoietin responsible for regulation of erythropoiesis and ultimately ability to respond to anaemic conditions [34]. Expectedly, the serum urea and creatinine levels were high in the infected-untreated group (IUT) may be due to the infection which may have caused alteration in the kidney architecture, hampering the clearance of these biomarkers hence their pronounced presence in the serum. The serum urea concentration in the infected-untreated control was higher than most of the other groups which could be associated with the presence of the parasite causing distortions in the kidneys' architecture, hence encouraging leakage of urea into the serum or hampering its clearance. Similarly, high urea concentrations were noticed in ITDA, IT40 and IT60. Damage to the kidney histology by the parasites could be responsible since lower dose of the compound produced urea level comparable to that of the uninfected-untreated control. The increase in the urea levels of the treated groups which did not differ significantly from the infected-untreated control may be an indication of damage to the kidney cells. However, the least serum urea level was recorded in P60 which was administered 60 mg/kg bw of BMH-21 once 72 hours before infection and treatment continued thereafter for 5 days. If BMH-21 was that toxic to the kidney the reverse of the serum urea level in P60 could have been the case.

The serum creatinine concentration in this study did not follow a particular pattern, such that UUT, IUT, and IT60 had comparable higher levels which were all significantly ($p>0.05$) higher than those of ITDA, IT40, UT60 and P60. These differences could be as a result of differences in the absorption, distribution, metabolism and excretion of BMH-21 by the individual animals. However, it can be adduced that BMH-21 was not toxic since the animals treated with the highest dose of the test compound presented a low serum creatinine concentration. Under physiological conditions, creatinine is formed as a result of muscle metabolism and its excretion is assumed to be at a relatively constant rate notwithstanding age, diet, however, creatinine elevation in the serum implicates conditions affecting glomerular filtration [35].

Liver damage too could be signaled by alteration in the serum levels of biomarker enzymes; therefore, assessment of serum activity of such enzymes could give insight into the integrity of liver tissues [36]. AST and ALT activities in the serum were increased in IT40 and IT60 groups. However, this change was very minimal in UT60, suggesting that the elevation in these parameters might have been caused by the parasite not necessarily the test

compound. According to Bashir, *et al.* [37], AST and ALT are indicators of hepatic integrity and can be relied on at certain levels to assess the extent of hepatocellular damage. The ALT activities provide more reliable information for the assessment of hepatocyte integrity than AST. Under normal physiological conditions, the plasma contains a basal level of the enzymes; but in the event of cellular damage, the enzymes leak into the extracellular fluid giving rise to their elevated plasma concentrations [38].

To further confirm the changes in biochemical parameters, histological changes in kidney and liver tissue architecture were investigated. Overall, BMH-21 treatment showed moderate histopathological changes in kidney evident as tubular distortion and necrosis. Hepatic necrosis was also noticed in the liver tissues.

The study sought to confirm the possible mode of action of BMH-21. The expressions of *ITS-I* and *ESAG6* genes which depend on RNAP I for their transcription were assayed. The study revealed a down regulation in the expression levels of *ESAG6* by the administration of 40 mg/kg body weight of BMH-21 with a 5-fold change reduction which may be likely due to BMH-21 inhibition of RNA Pol I enzyme transcription of the gene. Similarly, there was a decrease in the expression of *ITS-I* gene in IT40 with a fold reduction of 3 relative to the calibrator whereas all other treatments brought about up-regulation in the expression level of *ESAG6* and *ITS-I* gene. Our finding agrees partly with that of Kerry, *et al.* [19] who reported that BMH-21 decreased transcription of *T. brucei* rRNA and VSG genes *in vitro*. The standard drug diminazine aceturate showed up regulation of the genes investigated which implied that it has a different mechanism of action other than RNA polymerase I enzyme inhibition.

The increase in the expression levels of *ESAG6* and *ITS-I* genes in the P60 group may be due to the parasitaemia surge after treatment was withdrawn. Parasitaemia was noticed to be high at the point of termination of this experiment in this group hence the activity of RNA polymerase I enzyme was expected to be high thus manifesting in high level of expression of the RNAP I-dependent genes. The reason the expression level study produced a mixed picture is probably due to the fact that it was carried out in live animals. It is likely that different animals would have metabolized the drug at different rates so that the parasites in the different animals were exposed to different concentrations of the drugs over time.

5. Conclusion

This study established that BMH-21 possesses *in vivo* anti-trypanosomal effect on *T. b. brucei* in a manner similar to diminazine aceturate. BMH-21 displayed *in vivo* efficacy against *T. b brucei* as parasites were reduced from the blood of infected rats. Generally, it can be said that treatment with BMH-21 reversed some of the biochemical pathologies caused by the infection. It also resulted in a decrease in the expression levels of *ESAG6* and *ITS-I* genes suggesting inhibition of RNA polymerase I enzyme which is responsible for transcription of the above genes.

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